Size Exclusion PEGylation Reaction Chromatography Modelling

A thesis submitted in total fulfillment
of the requirements for the degree of
Masters of Engineering
in Materials and Process Engineering
by

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Abstract

Size exclusion PEGylation reaction chromatography was investigated using a model developed by Fee (2005). Column dispersion was neglected and the PEGylation reaction was modelled as second order. The model allowed up to four PEG groups to be attached to a protein and accounted for succinic acid hydrolysis from activated PEG. The model was adapted to simulate α-lactalbumin PEGylation and succinic acid hydrolysis from activated PEG in a batch stirred tank so rate parameters from stirred tank kinetic experiments could be obtained and the model verified. The model was solved using finite differences and simulations run in Matlab. The effect of reaction parameters such as timing, length and concentration of PEG and protein injection, reaction rates, and model resolution on model simulation results was explored.

In the size exclusion PEGylation simulations it was found that increasing protein concentration increased MonoPEG concentrations and increased the ratio of MonoPEG to starting protein feed concentration. Increasing PEG pulse length and starting PEG concentration initially increased MonoPEG concentration and product ratio until all protein had been PEGylated at which point MonoPEG concentration the product ratio levelled out. Increasing PEG hydrolysis rates did not affect the amount of MonoPEG produced but reduced the activated PEG concentration and increased succinic acid concentration. Optimal conditions for producing MonoPEG were found to be equal concentrations of PEG and protein, with the PEG injection length twice as long as the protein injection, and the PEG injection done immediately after the protein injection.

Acknowledgements

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Contents

List of Figures

List of Tables

Nomenclature

A_c	Cross sectional area (cm^2)
C_A	Solution concentration of solute A in solution (mg/ml)
C_{AO}	Initial concentration of solute A in solution (mg/ml)
C_{APEG}	Solution concentration of active PEG (mg/ml)
C_B	Solution concentration of solute B in solution (mg/ml)
C_{BO}	Initial concentration of solute B in solution (mg/ml)
C_i	Solution concentration of i^{th} component (mg/ml)
C_{IPEG}	Solution concentration of inactivated PEG (mg/ml)
C_M	Solution concentration of solute M in solution (mg/ml)
C_{MO}	Initial concentration of solute M in solution (mg/ml)
$C_{protein}$	Solution concentration of protein (mg/ml)
C_{PEG}	Solution concentration of PEG (mg/ml)
$C_{PEGfeed}$	Initial feed concentration of PEG (mg/ml)
$C_{PEGstart}$	Starting concentration of PEG (mg/ml)
C_{Pfeed}	Initial feed concentration of protein (mg/ml)
C_{Pstart}	Starting concentration of protein (mg/ml)
$C_{P(n)PEG}$	Solution concentration of protein with n number of PEG attached (mg/ml)
C_{SA}	Solution concentration of succinic acid (mg/ml)
d_p	Resin particle diameter (cm)
J	Time divider (dimensionless)
J_{steps}	Total number of steps taken in a run (dimensionless)
k_1	PEGylation reaction rate for protein (ml/mg.s)
k_2	PEGylation reaction rate for monoPEG (ml/mg.s)
k_{hydr}	Hydrolysis rate for activated PEG (1/s)
$k_{(n)}$	PEGylation reaction rate (ml/mg.s)
K_{avi}	Available pore and interstitial volume fraction (cm/s)

L	Axial bed length (cm)
n	Stage number (dimensionless)
N	Total number of stages (dimensionless)
Q	Volumetric flow rate of solution through the column (ml/s)
r_c	Column radius (cm)
$r_{monoPEG}$	Rate of reaction of monoPEG (mg/mls)
$r_{protein}$	Rate of reaction of protein (mg/ml.s)
t	Time (s), and in time steps
$t_{PEGstart}$	Time at which PEG is introduced to column (s) (or in time steps)
$t_{PEGfinish}$	Time at which the PEG pulse is stopped (s)
t_{Pstart}	Time at which protein is introduced to column (s) (or in time steps)
$t_{Pfinish}$	Time at which the protein pulse is stopped (s)
t_{run}	Simulations run time (s)
T	Total time steps (s)
U	Superficial velocity (cm/min)
V_e	Volume of each stage (ml)
V_i	Volume applied for j^{th} step (ml)
x	Distance through column *(cm or steps)*
X	Number of stages (dimensionless)

Greek

ε	Packed bed interstitial void fraction (dimensionless)
π	Constant (3.142)

Chapter 1

Introduction

1.1 Background

Advances in biochemistry, genetics and biotechnology has led to the proliferation of biologically derived pharmaceuticals and therapeutics. These are routinely injected, or taken orally into the human body for treating diseases. However, when a medicine is injected into a human body, it can be rapidly degraded or metabolized, requiring repeated injections to maintain the medicine at a therapeutic blood concentration. Much research has focused on increasing the medicine's halflife in the bloodstream. One approach has been to shield the medicinal compound by attaching polyethylene glycol (PEG) to its surface, a process called PEGylation.

PEG is non toxic, highly soluble in aqueous solutions, readily excretable and has extremely low immunogencity and antigencity (Fee 2003; Chowdhury and Wu 2005). PEGylation reactions involve the covalent attachment of PEG to an amino acid residue on the protein surface. However PEGylation reactions are notoriously difficult to control in terms of where the PEG group is attached on the protein surface and how many PEGs may be attached to one protein. A PEGylation reaction may yield many different types of PEGylated protein in terms of attachment site and number of PEGs attached, making PEGylated proteins difficult to characterize. An additional problem is isolating a particular type of PEGylated product.

One approach to reduce the diversity of PEGylated protein products from a reaction is to perform the reaction inside an axial flow size exclusion column. Size exclusion chromatography separates compounds from a mixture on the basis of size. Size exclusion columns are typically packed with resin particles that have a porous

structure. Molecules too large to enter the resin pores remain in the particle interstices and elute from the column faster than smaller molecules that readily diffuse into the resin particle pores. In size exclusion PEGylation reaction protein is applied to the top of the column over a set time period, followed by buffer and then by PEG. The protein and PEG move down the column as discrete bands at different migration rates. PEG being larger than the protein, moves through the column faster, eventually overtaking the protein. As the PEG overtakes the protein, the two species react, producing PEGylated protein which has one or more PEG groups attached. The PEGylation reaction and unreacted products then separate out into distinct bands as they continue to migrate through the column. Extent of PEGylation reaction can be controlled by tailoring the reaction conditions such as pulse length of PEG or protein and the time in between each pulse.

To obtain optimal conditions for PEGylation reactions within size exclusion column requires extensive experimentation, which is expensive for therapeutic proteins. Simulating a size exclusion PEGylation reaction using computer models has the advantage of reducing the number of experiments required to obtain optimal conditions, hence reducing cost. However little or no research has been presented on modeling PEGylation reactions in size exlusion column.

1.2 Research objective

A size exclusion PEGylation reaction chromatography model was developed by Fee (2005). The objective of this thesis was to maximise the yield of mono-PEGylated protein in a simulated size exclusion column and to study the effect of parameters

such as reactant concentration, reactant injection length, time between injections, reaction rates and volumetric flow rate on mono-PEGylated protein.

1.3 Organization of this thesis

An overview of PEGylation is presented in Chapter Two, its role in modern drug delivery, PEGylation chemistry and examples of PEGylated molecules along with their benefits and downstream processing, and size exclusion chromatography is introduced. The mathematical model and its solution used for simulating size exclusion PEGylation reaction chromatography is detailed in Chapter Three. Methods used to obtain model parameters using batch PEGylation experiments are described in Chapter Four. Experimental and model results are presented and discussed in Chapter Five and conclusions drawn and recommendations for future work are outlined in Chapter Six.

Chapter 2

Literature Review

2.1 Introduction

Advances in biochemistry, genetics and biotechnology has led to the proliferation of biologically derived pharmaceuticals and therapeutics. These are routinely injected, or taken orally into the human body for treating diseases. However, when a medicine is injected into a human body, it can be rapidly degraded or metabolized, requiring repeated injections to maintain the medicine at a therapeutic blood concentration. Much research has focused on increasing the medicine's halflife in the bloodstream. One approach has been to shield the medicinal compound by attaching polyethylene glycol (PEG) to its surface. PEG attachment to a protein increases the protein molecule size preventing it from being excreted and prolonging its circulation time in the blood (Figure 2-1). Davies and Abuchowsky (1977) produced the first PEGylated protein albumin in the 1970s. Since then, PEGylation has become a popular method in medical science and biopharmaceuticals and has been developed to include various PEGylation chemistries and conjugation methods (Veronese and Pasut 2005). PEG is non toxic, highly soluble in aqueous solutions, readily excretable and has extremely low immunogencity and antigencity (Fee 2003; Chowdhury and Wu 2005). These properties can be imparted to the compound PEG is conjugated with (Fee and Van Alstine 2006). Examples of PEGylated drugs include taxol, camptothecin, cisplatinum and doxorubicin (Fee and Van Alstine 2006). PEGylated antibodies have a longer halflife and reduced toxicity and immunogencity than their native counterparts with no change in antigen binding ability (Chowdhury and Wu 2005).

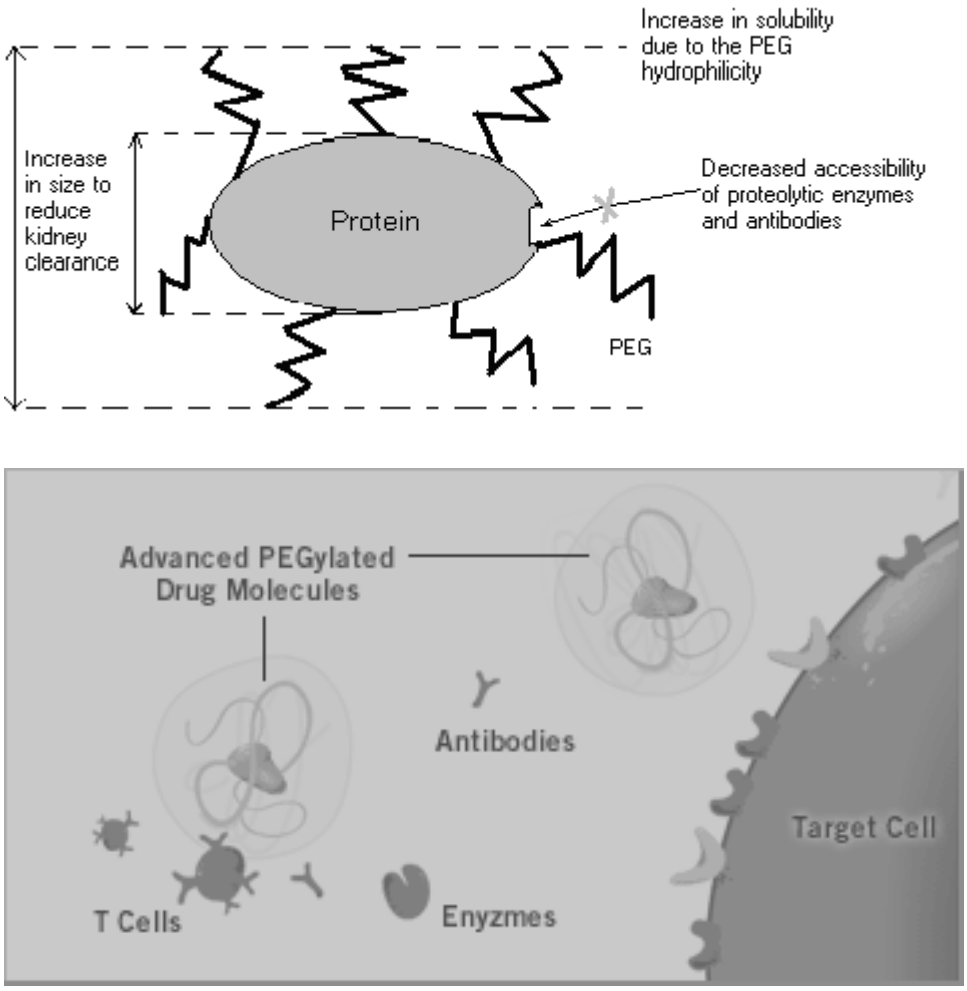


Figure 2-1: PEGylated protein

2.2 Properties of PEG

Polyethylene glycol polymers are neutral, hydrophilic and soluble in various aqueous solutions and organic solvents such as acetone. PEGs are normally soluble in aqueous solution up to 100^0C and are reactive at high temperatures. PEG solubility in aqueous solution decreases with increasing temperature and increasing salt concentration (Fee and Van Alstine 2006).

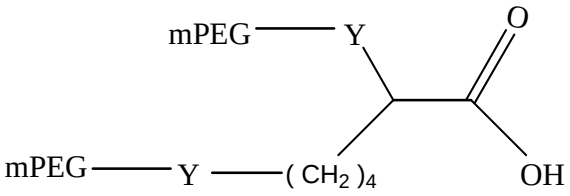
Polyethylene glycol molecules consist of *n* number of repeating ethylene oxide subunits, each 44 Da in size. The average molecular weight of PEG chain is

n x 44 Da. PEG is inert with two terminal hydroxyl groups available for activation or conjugation. PEG can be activated by converting one of the hydroxyl groups to a methoxy or alkoxy group. PEG is available in linear and branched forms. Two or more PEG chains can be joined together with linkers such as lysine or triazine to form branched PEG. Some examples of PEG molecules are shown in Figure 2-2 (Bailon and Berthold 1998).

Linear PEG-OH $H\text{-}(OCH_2CH_2)_n\text{-}OH$

Linear mPEG-OH $Ch_3\text{-}(OCh_2CH_2)_n\text{-}OH$

Branched mPEG2



or

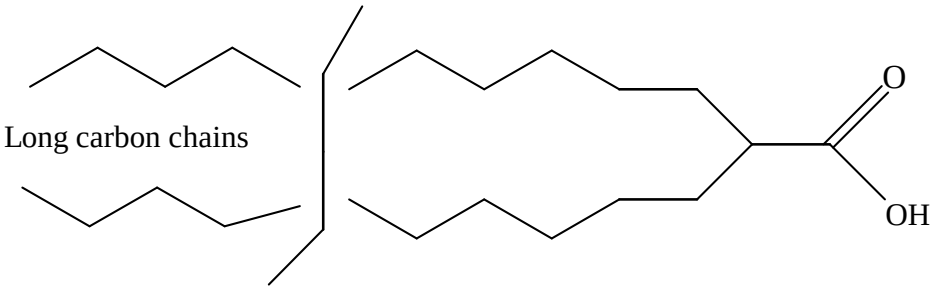


Figure 2-2: Chemical structures of some representative polyethylene glycol (PEG) molecules (Bailon and Berthold 1998).

As PEGs are high molecular weight polymer chains they tend to increase the viscosity of aqueous solutions. Therefore PEGylated molecules can cause solution foaming and can precipitate leading to fouling if passed through a chromatography column. Polyethylene glycol is transparent and non-fluorescent (Veronese 2001).

2.3 Improved protein drugs by PEGylation

Polyethylene glycol (PEG)-conjugated proteins belong to a new class of biomolecules that are neither proteins nor polymers. In 1977, Abuchowski and colleagues demonstrated that Polyethylene glycol conjugated proteins are more effective than their corresponding native form (Bailon and Berthold 1998). Now, PEGylation is used in several classes of drugs such as in enzymes, cytokines, antibodies and oligomers. The main advantages are improved serum half-life reduced immunogencity, low toxicity and increased stability towards metabolic enzymes (Veronese and Pasut 2005).

PEGylation improves the pharmacokinetics properties of the conjugates. A typical example is PEGylated α-interferon Pegasys®, retains only 7% of the antiviral activity of the native protein and still shows a great improvement in pharmacokinetics than its native form (Veronese and Pasut 2005). PEGylated drugs exhibited excellent pharmacokinetics properties than the unmodified parent drug by increasing the serum half life and residence time of the conjugate by many folds higher than those of unmodified drugs (Bailon and Berthold 1998).

Some of the polymers that are used as a shield include, polysaccharides, polyacrylamide, polyvinyl alcohol, poly(N-vinyl-2-pyrrolidone), polyethylene glycol and PEG containing copolymers such as poloxamers, poloxamines and polysorbates. PEG and PEG-containing copolymers are the most effective and the most commonly used polymers tested to date (Kim, Jeong et al. 2004; Mishra, Webster et al. 2004; Owens Iii and Peppas 2006).

In terms of immunogencity and toxicity, PEGylated molecules have shown a reduced antibody response and low toxicities in comparison with the unmodified molecule. The low molecular weight conjugates enters the extravascular tissues and diffuse back in to circulation. The larger the size of the molecule, the slower is the renal clearance (Bailon and Berthold 1998).

2.4 PEGylation chemistry

There are several different methods in which a PEG group can be attached to a protein. A common attachment site is the amino group of lysine using acylation or alkylation reactions (Zalipsky 1995; Veronese 2001; Veronese and Pasut 2005; Fee and Van Alstine 2006). Different conjugation methods can also be used to target thiol, hydroxyl or amide groups of the amino acids on the protein surface using several specific linkers or enzymes (Veronese and Pasut 2005).

2.4.1 Amino group modification

The amino groups are the first targets in PEGylation since they are the most common and exposed groups in proteins. They can be modified using a wide range of reactions. (Veronese and Pasut 2005). Most proteins and antibodies have lysine residues with ε-amino groups present on the surface making PEG conjugation straightforward (Fee and Van Alstine 2006). Common reactions used are alkylation and acylation.

Alkylating PEGs does not modify the charge of amino residues. In the case of PEG-aldehyde, a permanent linkage is obtained by forming a shif-base which is reduced by carborohydride reduction (Figure 2-3). Shif-base formation rate is relatively low and a PEGylation reaction can take up to a day for the completion of conjugation under controlled pH. In this case the α-amino terminal modification is achieved around pH 5 (Veronese 2001).

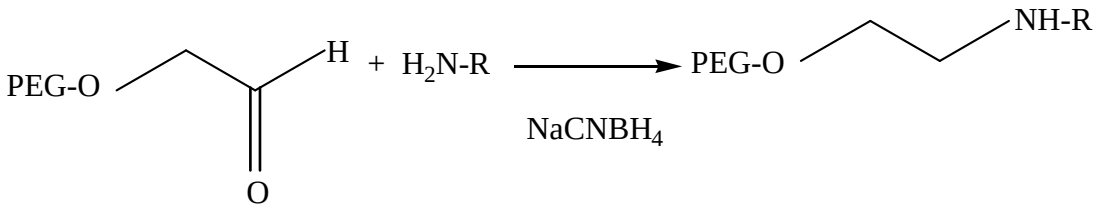
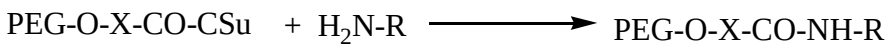


Figure 2-3: Alkylation of PEG-aldehyde (Veronese 2001)

The majority of acylating PEGs are hydroxylsuccinimilidly esters (-OSu) of carboxylated PEGs (Figure 2-4). The distance between the active ester (-COOSu) and the first PEG subunit varies by up to four methylene units. The distance influences its

reactivity towards amino groups and water. For example, the halflife of PEG-O-CH_2–COOSu is 0.75 h and 23 h for PEG-O-CH_2-CH_2-CH_2-COOSu (Veronese 2001).



X= (-CH_2-)n

Figure 2-4: Activated acyl acids (Veronese 2001)

PEG hydroxyl group activated by chloroformates or carbonylimidazole have different acylating chemistries and exhibit lower reaction rates than the –OSu activated PEGs. In case of PEG-p-nitrophenlcarbonate or PEG-trichlorophenylcarbonate selectivity of conjugation is achieved based on the availability of amino groups (Figure 2-5) (Veronese 2001).

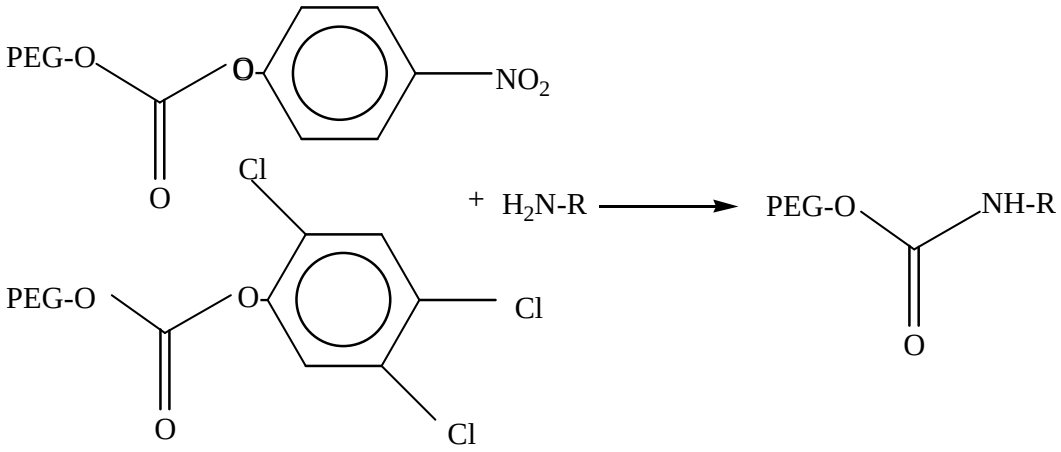


Figure 2-5: PEG-p-nitrophenlcarbonate and PEG-trichlorophenylcarbonate (Veronese 2001)

Kinster et al. (2002) used pH to control amino modification so that the conjugation takes place at the α-amino of the N-terminal instead of α-amino of a lysine residue. This was successfully tested on granulocyte colony stimulating factor, available in the market as Pegfilgrastim used in the treatment of granulocyte depletion during chemotherapy.

2.4.2 Thiol modification

PEGylation by attaching PEG to the cysteine thiol group residue in protein can be highly specific because cysteine is rarely present in protein. Also cysteine is hydrophobic it is buried deep inside the protein structure restricting its availability for PEGylation. Hence, thiol modification of proteins for PEG attachment is rapidly expanding. Genetic manipulation allows a cysteine residue to be inserted at specific location within the protein's amino acid sequence. Another strategy involves reducing protein disulphide bridges to expose new thiol groups for PEG attachment (Veronese and Pasut 2005; Fee and Van Alstine 2006). An example of a thiol reaction is shown in Figure 2-6 where PEGorthopyridyl-disulphide, an activated disulphide, reacts with thiol to yield a stable symmetric disulphide (Veronese 2001; Veronese and Pasut 2005).

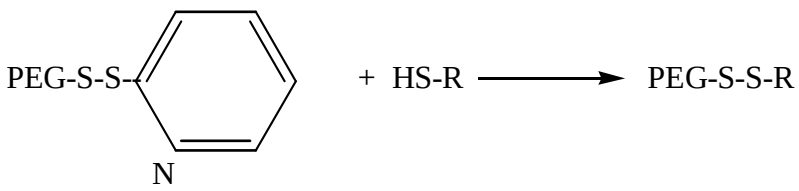
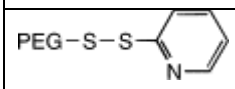
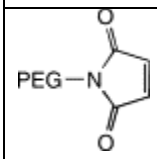
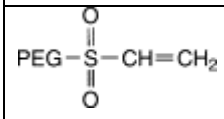
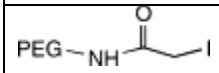


Figure 2-6: Thiol reactive PEG-orthopyridyl-disulphide (Veronese 2001)

One example of where thoil modification has been used is human growth factor (G-CSF). The non-accessible cysteine 17 was modified by first attaching a low molecular weight PEG bearing a thiol reactive group at one end and an azide group at the other that could access cysteine because of its low steric hindrance. A high molecular weight PEG was then attached to the azide end of the low molecular weight PEG (Veronese and Pasut 2005; Fee and Van Alstine 2006).

Some examples of thiol modified PEG include PEG-Vinyl sulphone (via free cysteine), PEG-Iodoacetamide (via free cysteine), PEG-Maleimide (via free cysteine), PEG-Orthopyridyl disulphide (via free cysteine), PEG-Hydrazide (via oligosaccharides) and PEG-Isocyanate (via alcohol or amino group) (Bailon and Berthold 1998). Examples of PEGs reactive with thiol groups are shown in Table 2-1.

Table 2-1: PEGs reactive towards thiol group (Veronese and Pasut 2005)

Structure	Thioreactive PEGs	Properties
	PEG–pyridildisulphide	The most specific towards thiol but yields a cleavable linkage by a reducing agent also *in vivo.*
	PEG–maleimide	Gives stable linkage by double bond addition but can also react with amines at pH >8.
	PEG–vinylsulfone	
	PEG–iodo acetamide	Less reactive, not much used.

2.4.3 Site specific modification

A common problem with amino acid PEG attachment is that it is not site specific. For example, for reactions involving attaching PEG to lysine residues, PEG will be attached anywhere on the protein surface where a lysine residue is. Therefore a PEGylation reaction may produce a mixture of PEGylated products with one or more PEG groups attached and with different attachment sites. In addition, the attachment site may be in the active site of a protein reducing or eliminating its activity. Isolating one particular type of PEGylated product from a mixture becomes formidable task. This can be overcome using site specific amino modification, of which an example was given in the section on thiol modification. Site specific amino modification helps in the purification and characterization of the PEGylated product because only one type is produced. It also preserves protein activity in the conjugate (Veronese and Pasut 2005).

One method of site specific modification involves amino acid mutagenesis, where amino acids with specific functional groups are genetically incorporated in proteins. Deiters et al. (2004) produced a selectively PEGylated protein by attaching an alkyne derivatized PEG to the azido group of an incorporated para-azidophenylalanine using a mild cycloaddition reaction. They showed that amino acids with alkenyl, iodo and keto groups could be incorporated to produce site specific PEGylated proteins with good efficiencies and high selectivity. Other examples of site specific PEGylation used N-Hydroxysuccinimide-activated esters (amide bond), PEG-Epoxide (alkyl

bond), PEG-Carbonyl imidazole (urethane bond), PEG-Tresylate (alkyl bond) and PEG-Aldehyde (N-terminus, Shiff's base) (Bailon and Berthold 1998).

2.4.4 Reversible PEGylation

Unlike larger proteins, PEGylation often inactivates smaller peptides because PEG attachment is more likely to occur within the peptide's active site. Reversible PEGylation can be used to preserve protein activity by performing the reaction with an inhibitor, a substrate, or ligand specific for the macromolecule present, which protects the active site from PEGylation. In Figure 2-7, the protein's (E) active site is protected from PEGylation by attaching it to a ligand such as a substrate or an inhibitor (L) which is linked to an insoluble matrix (Veronese 2001; Veronese and Pasut 2005).

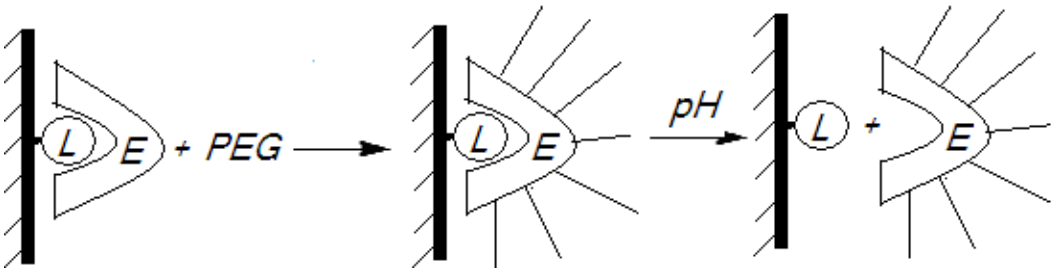


Figure 2-7: Reversible PEGylation.

Salmaso et al (2005) found that small molecular weight PEGs were able to reach the active site despite using a protective agent, while more efficient protection was achieved when large PEG molecules were used for conjugation.

Shechter et al. (2005) developed a reversibly PEGylated peptide, active PEG40-FMS-PYY_{3-36}, derived by coupling PYY_{3-36} with 40 kDa PEG through a spontaneously cleavable linker. PEG40-FMS-PYY_{3-36} half-life was 24 hours, 8 times larger than unmodified PYY_{3-36}. Reversible PEGylation was the only approach successful in case of PYY_{3-36}, whereas acetylation and irreversible PEGylation rendered it inactive.

Salmaso et al. (2005) demonstrated that the reversible PEGylation of biotinylated antibodies prevented the binding in the vicinity of active site and maintained high biological activity.

2.5 Factors affecting PEGylated conjugate behaviour

2.5.1 Site of conjugation and number of polymers attached

The site at which conjugation takes place and the number of polymer chains attached plays an important role in determining the characteristics of PEGylated proteins (Fee 2003; Fee and Van Alstine 2006). In some cases the desired pharmaceutical properties can be achieved by addition of only one polymer molecule, for example, monoPEGylated interferon, but in other cases extensive PEGylation is required, up to 2-3 (20-60 kDa) polymer molecules (Bailon and Berthold 1998; Veronese 2001).

The attachment of PEG to proteins can be done in three different ways

- A single large PEG at a single site
- A branched PEG at a single site
- Several small chains at multiple site (monoPEG- one PEG chain, diPEG- two PEG chains, triPEG- three PEG chains.. etc)

The uni-site PEGylated proteins usually prove to have higher activity since the chance of binding at the receptor domains is low, multiple attachment of PEGs may result in partial or complete loss of bioactivity (Bailon and Berthold 1998).

The advantage of having a mixture of PEGylated conjugates leads to an expected overlapping bioactivity due to various absorption rates is shown in the Figure 2-8. The rate of absorption and receptor saturation increases with the degree of PEGylation (Bailon and Berthold 1998).

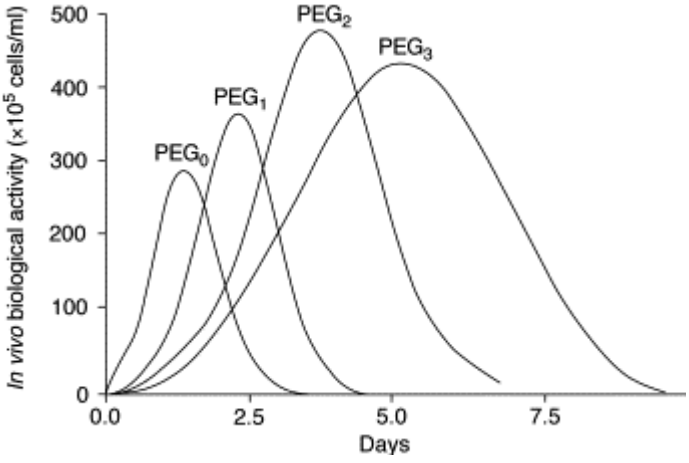


Figure 2-8: Overlapping bioactivities of components of a polyethylene glycol (PEG) conjugate mixture (Bailon and Berthold 1998).

The growth hormone-releasing factor (GRF) (1-29) has three primary amino groups, N-terminal, Lys^{19} and Lys^{21} to which a PEG group can be attached. The PEGylated GRF (1-29) showed different biological activity depending on the amino group where the PEG is attached. Youn et al. (2004) separated seven PEG conjugated GRF (1-29) (three mono-, three di- and tri-PEGylated GRF (1-29)) by RP-HPLC in a single run, with high resolution. The conjugates were then analyzed using MALSI-TOF-MS followed by proteolytic digestion.

Ton et al. (2005) used dialysis and FPLC to obtain pure mPEG-CPA conjugates with specific number of mPEG chains. The dialysis step removed all low molecular impurities, including free PEG, making it easy for fractionation.

Tattini et al. (2005) observed that the modification of BSA-PEG in a ratio of (4:1) showed a lower degree of structural alterations and lower variation on the physiochemical characteristics in comparison with BSA-PEG (2:1).

2.5.2 Size of PEG

Fee and Alstine (2004) suggested that the viscosity radius of a tetra-PEGylated molecule with 5 kDa PEG is equivalent to the one which results from mono-PEGylation with a 20 kDa PEG as measured by (SEC) chromatography. The latter is the more effective that the first, since the activity of native protein decreases with increased number of attachments. The direct relationship between PEG mass and the in-vitro activity are illustrated in the Figure 2-9 (Bailon and Berthold 1998).

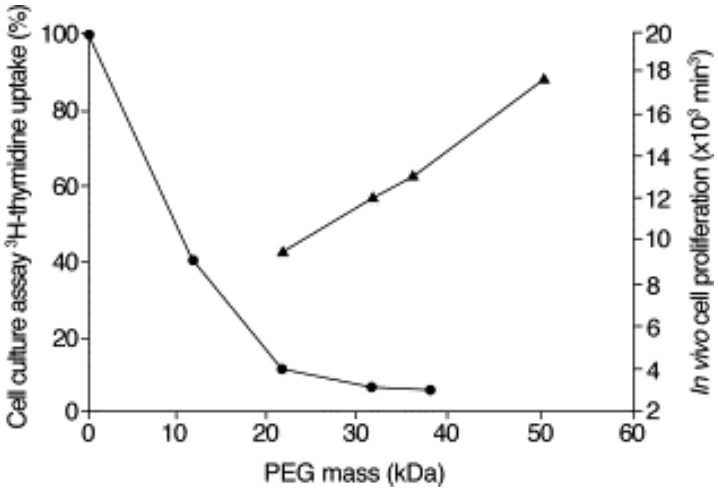


Figure 2-9: In vitro and in vivo biological activities as a function of polyethylene glycol (PEG) mass (Bailon and Berthold 1998). Circles are the cell culture assay uptake and triangles are the in-vivo cell proliferation.

As the PEG size increases of a molecule, its distribution through the animal or human body decreases. One example is PEGylation of avidin, a toxic protein found in egg white. Avidin is a xenoprotein processing poor pharmacokinetics and immunological properties. Salmaso et al (2005) found that conjugating avidin with 5 kDa PEG reduced the avidin distribution and localized it to the liver and kidney, whereas, the 20kDa conjugate was confined to the blood stream. They showed that the conjugation of high polymer mass on protein surface suppressed avidin immunogencity and antigencity regardless of the polymer size and shape used.

2.5.3 Stabilizing agents

Hinrichs et al (2006), illustrates that various PEGylated nanoparticles can be stabilized by oligosaccharides, which are compatible with PEG. For example, inulin was able to prevent full aggregation of DOTA/DOPE liposomes, EPC/Chol liposomes, DOTAP/DOPE lioplexes and PEI polyplexes during freezing thawing or freeze drying, irrespective of the degree of PEGylation of these nanoparticles.

2.5.4 Molecular flexibility

The polymer should have molecular flexibility, since rigid molecules lack in significant radius of gyration (Rg) resulting in poor camouflaging of membrane antigens. Hence, Rg depends on the intra-chain mobility and lies close to its linear length (L) of the rigid polymer. The flexible linear PEG molecules have high Rg and provide more protection than a rigid linear PEG (Figure 2-10) (Scott and Chen 2004).

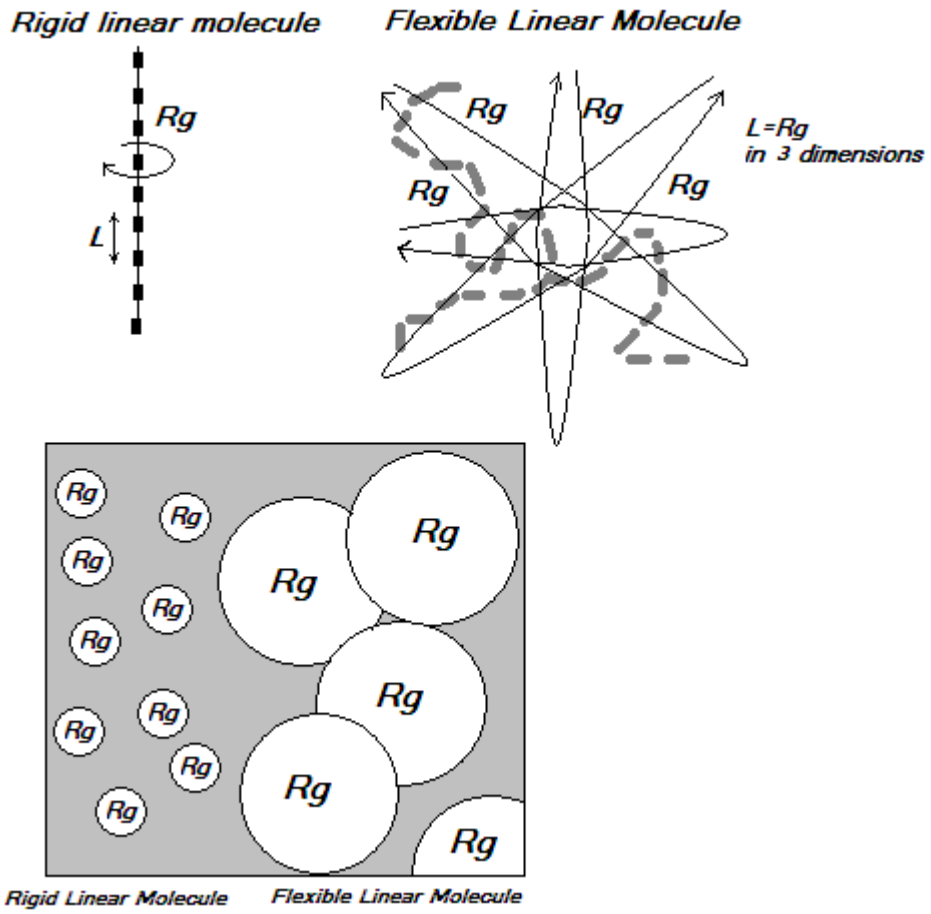


Figure 2-10: Rigid Linear Molecule and Flexible Linear Molecule (Scott and Chen 2004).

2.5.5 Conditions affecting PEGylation reactions

Usually, the cost of native pure protein used in PEGylation is very high. Hence, reaction extent and specificity are critical objectives (Fee and Van Alstine 2006). The degree of PEGylation is strongly influenced by coupling conditions such as buffer pH, protein to PEG molar ratio, reaction duration, temperature and reaction stoicheometry (Bailon and Berthold 1998).

pH can change in the PEGylation reaction mixture due to PEG reaction with amino groups of proteins and fast aqueous hydrolysis of PEG, altering reaction kinetics. Yun, Yang et al. (2005) were able to control pH and reduce PEG hydrolysis by mixing SCM-PEG (succinimidyl carboxymethyl-mPEG5000) with sodium tetraborate to make a small dense PEG-pellet. They found that the aqueous hydrolysis amount of SCM-PEG was decreased and the fluctuation of pH was controlled by simultaneous release of sodium tetraborate from the PEG-pellet, and that a greater degree of modification compared to the traditional PEGylation technique.

PEGylation conditions can be controlled using reaction engineering, increasing the yield and minimizing cost. Batch processing is the most common and the easiest method for PEGylation, the other being unidirectional and single-pass processes such as in chromatography column (Bailon and Berthold 1998).

2.6 Limitations and drawbacks in the use of PEG

Polyethylene glycol is polydisperse, i.e. it a batch of PEG consists of a mixture of molecules whose chain lengths form a Gaussian distribution. This can affect the conjugate biological properties, mainly immunogencity and circulation half-life, for example in low molecular weight drugs such as peptides, oligomers and antibodies. Improvements in PEG production has resulted in PEGs that have less variation in chain length (Veronese and Pasut 2005).

Another concern of using PEG is excretion from the human body. Large PEG molecules tend to accumulate in the liver and spleen leading to macromolecular

syndrome and other toxic effects (Owens Iii and Peppas 2006). Also the high water co-ordination of the PEG molecule increases its hydrodynamic volume up to 3-5 times, which may prevent it from being excreted by the kidneys. The kidney retains molecules greater than 30 kDa in size. However, PEG chain length may be reduced for example by enzymes such as cytochrome (Veronese and Pasut 2005).

The loss of biological activity is one of the main drawbacks. For example, PEGylated α-interferon Pegasys® retains only 7% of the antiviral activity (Veronese and Pasut 2005). In addition PEG is non-detectable due to its transparent and non-fluorescent properties and moreover does not release products easy to quantify upon hydrolysis (Veronese 2001).

2.7 Batch PEGylation

In case of batch wise PEGylation, a heterogeneous product mixture of unreacted fuctionalized PEG, unreacted native protein, some by-products due to hydrolysis and PEGylated proteins (species) with a range of PEGylation sites and extent of conjugation is obtained depending on the reaction conditions. For example, succinimidyl-propionic-acid-PEG (PEG-SPA) is readily hydrolysed in water to yield N-hydroxysuccinimide and some unreactive PEG residues affecting the reaction kinetics adding to the complexity for processing and purification. In case of amino-activated PEGs, hydrolysis is accompanied by aminolysis, hence a molar excess of PEG to protein ratio is very important, and 50:1 PEG to protein ratio may be used (Fee and Van Alstine 2006).

The other by-products of PEGylation may also consist of low quantities of cross-linked conjugates, considered significant in products intended for therapy. Hence mono-methoxy PEG (mPEG-x) reagents are used to avoid cross-linking (Fee and Van Alstine 2006).

The main disadvantage of the batch process is that the reactants, products and the by-products are mixed and need to be separated after the completion of the reaction. In the unpublished work by Fee, mono-PEGylated proteins are yielded using a fed-batch process; were small quantities of PEG is added to a large excess of proteins. In this case, a partition is required to constantly remove PEGylated proteins from the reactor to avoid multiple PEGylation, once an appreciable amount of PEGylated protein is accumulated. This method can only control the extent of PEGylation at the cost of low protein conversion (Fee and Van Alstine 2006).

This is of the great concern for proteins PEGylated for the pharmaceutical use, but is of less concern for other types of PEGylated molecules used for miscellaneous purposes, example, catalysis, pesticides, detergents etc (Fee and Van Alstine 2006).

2.8 Packed bed process

Thus, the bio-separation or purification based on the size of molecules, surface charge and hydrophobicity may be successful. Hence, size exclusion reaction chromatography, ion-exchange chromatography and reverse phase chromatography seems to be the best suited or commonly used methods (Fee and Van Alstine 2006; Owens Iii and Peppas 2006).

The “on column” or packed bed process is being used in attempt to influence both the site and extent of conjugation, example, oligopeptide PEGylation. This method is found to be quite useful in case of smaller molecular weight substances. This is done by; immobilizing the proteins to the solid phase in the column; the reactants and by-products are washed in the mobile phase giving partial separation followed by elution of products. This technique was used by Monkarsh et al. 1997 to PEGylate α-interferon. The native protein was bound to the ion exchanger and PEG was then passed through the column. Some attempts have been made to achieve more activated conjugates using the same process, where the active site of protein is held towards the solid-phase interface preventing conjugation occurring at the active site (Fee and Van Alstine 2006).

In 2003, Fee used size exclusion reaction chromatography (SERC) to exploit the differing linear velocities of the species of differing sizes to control the reaction extent. This was done by injecting a single pulse of protein and PEG, lowest molecular size first since the larger reactants with higher linear velocity catches up with the smaller ones forming a moving reaction zone and at the same time separation of the species in a single unit operation aiming towards low cost of production. This approach is useful for therapeutic proteins less than 20 kDa (Fee and Van Alstine 2006).

2.9 Purification engineering

The general purification or bio-separation strategy is based on few important factors such as, molecular size for membrane separations or size exclusion reaction

chromatography, its iso- electric point and surface charge distribution for ion-exchange chromatography and hydrophobicity for hydrophobic interaction chromatography (Fee and Van Alstine 2006).

PEGylation of any bio-molecule gives rise to two basic types of purification challenge, the first being separation of the target PEG-biomolecules from other reaction products and the second is the sub-fractionation on the basis of positional isomerism (Fee and Van Alstine 2006).

Fee and Alstine (2006) designed a table showing PEGylated protein processing consisting of proteins with different molecular weights with the conjugation methods used for PEGylation and purification steps. The summary of the common conjugation methods are listed below

- Succinimidyl propionate (18%)
- N-hydroxy succinimide (18%)
- Succinimide carbonate (16%)
- Aldehyde (12%)
- Maleimide (8%)
- Dithiopyridyl (8%)

Common purification methods used are:

- Size exclusion (33.75%)
- Ion exchange (27.5%)
- Dialysis and Lyophilisation (11.25%)
- Ultrafiltration (10%)
- Reverse phase (8.75%)

This shows that size exclusion and ion exchange are the most common methods used for purification of PEGylated species.

2.9.1 Size-based separations

Size exclusion reaction chromatography can be used to separate PEGylated species from other components, given that the SEC column is calibrated in terms of molecular size rather than molecular weight, which has shown consistent results for protein and PEG standards (Fee and Van Alstine 2006). In 2004, using this technique, Fee and Alstine found that the size of PEGylated proteins can be accurately predicted from the radius of a hypothetical PEG molecule having the same molecular weight as the total conjugated PEG and the native protein radius. The resolution of SEC decreases proportionally with the increase in the extent of PEGylation.

Epoxy group of each PEG is said to affect approximately 16 water molecules as suggested by various physical studies and proteins on the other hand interacts via covalent, hydrogen bond, van der waals and other interactions in pure water, significantly affects the structure of the conjugates (Fee and Van Alstine 2006).

The ability to separate PEGylated species from one another, increases with molecular weight, but becomes less effective as the extent of PEGylation increases. Fee and Alstine (2006), has shown that, only mono- and di-PEGylated species can be effectively separated by SEC when a 5 kDa PEG molecule is used and the separation becomes more difficult as the ratio of protein to PEG molecular weight increases.

The positional isomers of the same PEGylation extent has different biological activities, but identical molecular weights and are much more difficult to separate, since they have similar physiochemical properties. The studies by Fee and Alstine (2006) suggest that the existing SEC media is suitable for separation of PEG-proteins from naïve proteins and moderate separation of PEG proteins of differing N.

2.9.2 Charged-based separations

Ion-exchange chromatography is the most important technique used in PEGylation. PEG is a neutral polymer, but has a tendency to affect the charge properties of proteins in three different ways as noted in the work published by Fee and Alstine (2006). In first case, the presence of the PEG conjugate may shield the surface charge of a protein, thereby weakening the binding to ion-exchange resins. Secondly, by conjugation to amine acid residues that alter their charge nature at certain pH values and thirdly, surface localized PEG may hydrogen bond with acid or other groups. Fee and Alstine (2006) have given a good explanation on charged-based separation and hydrophobicity based separation in their published work.

Monkarsh et al. (1997), where able to separate 11 positional isomers of mono-PEGylated α-interferon using cat ion exchange chromatography in analytical scale.

Wu et al. (2005), PEGylated human basic fibroblast growth factor (fb-FGF) with 5 kDa PEG via the cysteine residue to obtain a conjugate with 80% modification and retaining 60% of the mitogenic activity which was successfully purified using ion-exchange chromatography.

Yun et al. (2004) used two consecutive ion exchange chromatography steps to successfully separate and purify the PEGylated rhg-CSF. The cation-exchange chromatography was first used to separate the PEGylated species from the un-PEGylated rhg-CSF, followed by anion-exchange chromatography to separate the PEGylated species (mono-, di- and tri-PEGylated rhg-CSF) and the excess free PEG.

2.10 FDA approved PEGylated drugs

There are already a few drugs approved by FDA and are available in the market for therapeutical applications. Some of the successfully approved PEGylated conjugates include PEGylated α-interferons, for use in the treatment of hepatitis C (PEGasys® from Hoffman- LaRoche and PEG intron® from Schering-Plough/Enzon), PEGylated growth hormone receptor antagonist (PEG Somavert® from Pfizer), PEG-asparaginase (Oncospar® from Enzon), adenosine deaminase (ADAGEN® from Enzon), and granulocyte colony stimulating factor (Neulasta® from Amgen) (Fee and Van Alstine 2006). PEGylated α-interferons used for the treatment of hepatitis C, PEGasys® from Hoffman- LaRoche and PEG intron® from Schering-Plough/Enzon have sales in excess of $ 1 billion per year (Harris and Veronese 2003).

FDA has approved PEGylated aptamers, 28mer oligomer aptanib for the treatment of age-related macular degeneration of retina (Veronese and Pasut 2005).

The requirements for the approval of new PEG modified conjugates are more stringent and the characterization of each polymer is compulsory, example are α-interferon conjugates from PEGasys and PEG-Intron, for which all the binding sites in

primary sequence were established (Veronese and Pasut 2005). Due to its non-toxicity and non-immunogencity, FDA has approved PEG for using as a base in foods, cosmetics and pharmaceuticals (Wu, Li et al. 2006).

2.11 Other applications

The three main areas of application of PEG conjugates are

- PEG ligands in aqueous two phase partitioning
- Bioreactor use of PEG co factors and PEG catalysts
- PEG-drug conjugates for controlled delivery of biologically active substance (Zalipsky 1995).

The chemotherapy agent L-asparaginase used in the treatment of acute lymphoblastic leukemia therapy has two main disadvantages, the need for frequent intramuscular injection and a very high rate of allergic reactions. These problems were overcome by conjugation with polyethylene glycol. The conjugate was less likely to cause hypersensitivity and provided a long duration form of the drug. Thus the initial purpose of PEGylation is achieved (Graham 2003).

Scott and Chen (2003), suggested that the covalent modification of cells and tissues with methoxypoly(ethylene glycol) (mPEG) can significantly diminish immunologic recognition of other allogenic tissues and enhance the induction of tolerance, tested for a specialized form of cellular transplantation. They used a chemical linker to substitute the terminal hydroxyl group of mPEG polymer for conjugation. Saline

injected animals injected with cyclosporine methoxyPEG showed a mean survival time of 11 days while the animals injected with unmodified spenocytes was 8 days.

Some of the theoretical and commercial results of PEGylation are shown in Table 2-2.

Table 2-2: Approved PEG conjugates (Veronese and Pasut 2005)

PEG conjugates	Type of PEGylation	Year to market	Disease
With proteins			
PEG–asparaginase (Oncaspar®)	Random, linear PEG	1994	Acute lymphoblastic leukemia
PEG–adenosine deaminase (Adagen®)	Random, linear PEG	1990	Severe combined immunodeficiency disease (SCID)
PEG–interferon α2a (Pegasys®)	Random, branched PEG 40 kDa	2002	Hepatitis C
PEG–interferon α2b (PEG–Intron®)	Random, linear PEG 12 kDa	2000	Hepatitis C and clinical trials for cancer, multiple sclerosis, HIV/AIDS
PEG–G-CSF (pegfilgrastim, Neulasta®)	Selective, linear PEG 20 kDa	2002	Treating of neutropenia during chemotherapy
PEG–growth hormone receptor antagonist (Pegvisomant, Somavert®)	Random, linear PEG 5 kDa (genetic modified protein)	2002	Acromegaly
With oligonucleotides			
Branched PEG–anti-VEGF aptamer (Pegaptanib, Macugen™)	Selective, branched PEG 40 kDa	2004	Macular degeneration (age-related)

Lee et al. (2005), studied that, PEGylation of islets have a dose reducing effect on the immunosuppressive medication and synergistically improved the survival time of islets in the transplantation site without causing the infiltration of immune cells.

Pancreatic islet transplantation is a method of cell therapy used to treat diabetes mellitus. The SPA group of mono-methoxy PEG-SPA was bound with the amine groups of collagen matrix of the isolated islets. They found that most of the transplanted islets were generally rejected within 2 weeks and PEGylated islets survived for 100 days in three out of seven rats; the remaining four rats survived 30 days on the average (Figure 2-11).

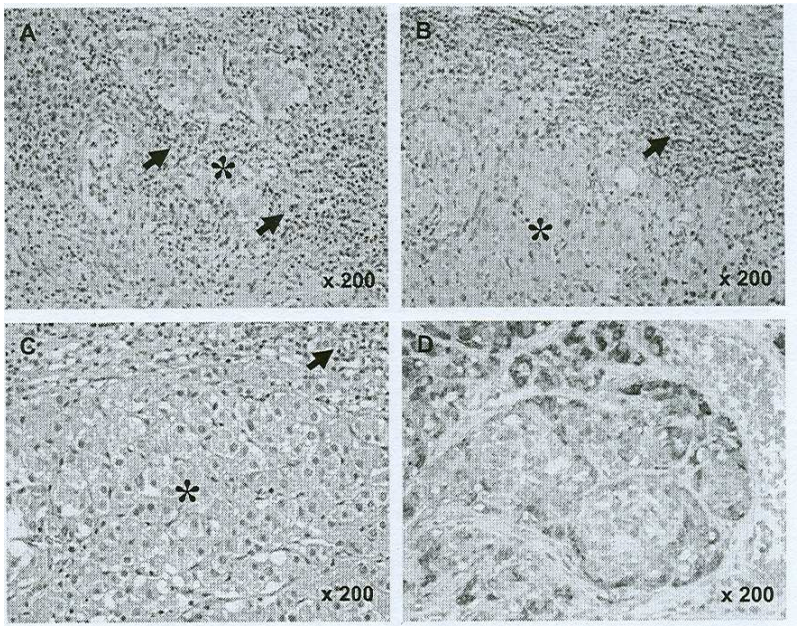


Figure 2-11: Histological analysis of transplanted islets (A) unmodified islets rejected within 2 weeks, (B) PEGylated islets rejected at day 30, (C, D) PEGylated islets survived for 100 days (Lee et al 2006)

Sainathan et al. (2005) showed that the PEGylated Granulocyte-macrophage colony-stimulating factor (GM-CSF) has a half-life of 48h compared to 6h of un-PEGylated GM-CSF. The PEGylation of GM-CSF was carried out at lysine residue.

Acar et al. (2005) developed a novel superparamagnetic iron oxide nanoparticles coated with polymerized PEGylated bilayers, were successful in resisting the

aggregation after γ-irradiation. The superparamagnetic iron oxide nanoparticles are clinically used for magnetic resonance imaging and many other applications such as magnetic drug delivery, cell tracking and hyperthermia.

Wang et al (2004) tested the anti-HIV activity and cytotoxicity of the PEGylated TCS derivatives to find that, there was a substantial drop in HIV activity with a increased half-life up to 17-22 fold but only a small decrease in cytotoxicity.

Antibodies are a fast growing area in pharmaceutical science because of their ability to target specific compounds. The first monoclonal antibody (mAb) tested as therapeutic in humans was OKT3 in 1986. There are 18 FDA approved monoclonal antibodies available in the market and 150 in clinical developments (Chowdhury and Wu 2005). Engineering specific domains of an antibody can alter its properties to a great extent. Also, the additional domains can be used to link them with other molecules to achieve desired properties providing scope for PEGylation. PEG chains are attached to the antibodies mainly to alter the pharmacokinetics and reducing its toxicity by altering PI (Chowdhury and Wu 2005).

Chowdhury and Wu (2005) successfully linked PEG to antibodies while completely preserving the antigen binding ability. This was achieved by site-specific PEGylation using maleimide chemistry. In the case of scFv-immunotoxin, they introduced a free cysteine as a linker for PEG conjugation. PEGylation of this antibody showed an increase in the plasma half-life with reducing toxicity and immunogencity with no change in antigen binding ability of the antibodies (data unpublished) (Chowdhury and Wu 2005).

2.12 Conclusion

In this chapter, the chemistry of PEGylation, properties of PEG, factors affecting the behavior of PEGylated conjugate, limitations and problems related to PEGylation, processes used for PEGylation, purification engineering and other applications of PEGylation was reviewed. Size exclusion reaction chromatography and ion exchange chromatography are two promising purification processes used so far in isolating, purifying and producing PEGylated compounds. Size exclusion reaction chromatography separates the PEGylated species on the basis of their molecular weight and ion exchange chromatography is used for charge based separations. The properties of PEG along with the reaction conditions play an important role in determining the reaction kinetics, the site and the extent of PEGylation.

Chapter 3

Modelling

3.1 Introduction

In a PEGylation reaction performed in an axial flow size exclusion column, protein is applied to the top of the column over a set time period, followed by buffer and then by activated PEG. The protein and PEG move down the column as discrete bands at different migration rates depending on the species molecular weight. PEG being larger than the protein, moves through the column faster, eventually overtaking the protein. As the PEG overtakes the protein, the two species react, producing PEGylated protein which has one or more PEG groups attached. The PEGylation reaction and unreacted products then separate out into distinct bands as they continue to migrate through the column. Continuity equations used to model these reactions and product separation in an axial flow size exclusion column are presented in this chapter. The continuity equations were developed by Fee (2005) and solved using the finite difference method (Lay 2005). Column dispersion is neglected in the continuity equation but is approximated when the model is solved by treating the column as a series of well mixed tanks. PEGylation reaction is modelled as second order. The model allows up to four PEG groups to be attached to a protein and accounts for succinic acid hydrolysis from activated PEG. The model was adapted to simulate PEGylation and PEG hydrolysis in a batch stirred tank so rate parameters from stirred tank kinetic experiments could be obtained.

3.2 PEGylation reaction

In a typical PEGylation reaction, one or more activated PEG groups are attached to an amino acid on the target protein surface. The reaction yields a PEGylated protein with *n* PEG groups and succinic acid. In modeling this reaction it is assumed that

- one PEG group is attached at a time at a reaction rate *k*,
- reaction rate *k* is dependent on the number of PEG groups already attached, and
- the reaction is irreversible.

$$\text{PEG.SA + (n)PEG.Protein} \xrightarrow{k_{(n)}} \text{(n+1)PEG.Protein +SA} \qquad (1)$$

The change in molar concentration of PEGylated protein with time is given by

$$\frac{\partial C_{P(n+1)PEG}}{\partial t} = k_{(n)} C_{P(n)PEG} C_{APEG} - k_{(n+1)} C_{P(n+1)PEG} C_{APEG} \qquad (2)$$

Where $C_{P(n)PEG}$ is the molar concentration of protein with *n* PEG groups attached ((n)PEG.Protein in equation 1), *t* is time (s) and $k_{(n)}$ is a second order PEGylation rate constant (L/M.s) and C_{APEG} is the molar concentration of activated PEG (PEG.SA in equation 1).

The change in unPEGylated protein molar concentration with time is given by

$$\frac{\partial C_{P(n=0)PEG}}{\partial t} = -k_{(n=0)} C_{P(n=0)PEG} C_{APEG} \tag{3}$$

A mole of PEG attached to protein yields one mole of succinic acid, therefore change in succinic acid concentration in a PEGylation reaction is given by

$$\frac{\partial C_{SA}}{\partial t} = \sum_{n=0}^{N-1} k_{(n)} C_{P(n)PEG} C_{APEG} \tag{4}$$

Where C_{SA} is succinic acid molar concentration and N is the maximum number of PEG groups attached to a protein.

In addition succinic acid will irreversibly dissociate from PEG in the absence of protein at a rate k_{hydr}

$$\text{PEG.SA} \xrightarrow{k_{hydr}} \text{PEG} + \text{SA} \tag{5}$$

Therefore total change in succinic acid molar concentration is given by

$$\frac{\partial C_{SA}}{\partial t} = k_{hydr} C_{APEG} + \sum_{n=1}^{N} k_n C_{P(n)PEG} C_{APEG} \tag{6}$$

Where k_{hydr} is a first order reaction rate (1/s).

The total change in activated PEG and inactivated PEG molar concentration is

$$\frac{\partial C_{APEG}}{\partial t} = -k_{hydr} C_{APEG} - \sum_{n=0}^{N-1} k_{(n)} C_{P(n)PEG} C_{APEG} \tag{7}$$

$$\frac{\partial C_{IPEG}}{\partial t} = k_{hydr} C_{APEG} \tag{8}$$

where C_{IPEG} is inactivated PEG molar concentration.

3.3 PEGylation reaction in an axial flow size exclusion column

Modeling a PEGylation reaction in an axial flow size exclusion column is complex because the model needs to track the concentration of activated PEG (C_{APEG}), inactivated PEG (C_{IPEG}), succinic acid (C_{SA}) and protein ($C_{P(n)PEG}$) with 0 to N PEG groups attached, with time and distance through the column. For simplicity it is assumed that:

- Extra-column and intra-column dispersion is negligible
- Concentration is uniform with radius
- Flow velocity is uniform with radius and distance through the column
- The solid and liquid phases are incompressible

- The PEGylation reaction is isothermal

- There is no adsorption of protein, PEG and succinic acid to the solid phase

- Retention time in the column is a function of available pore and interstitial volume which is dependent on the molecular weight of the protein, PEG, PEGylated protein and succinic acid.

The change in concentration of the i^{th} component due to convection is given by performing a mass balance on a thin section of height Δx of an axial flow column of radius r_c.

$$C_i\big|_{t+\Delta t}\pi {r_c}^2\Delta x K_{avi} = C_i\big|_t \pi {r_c}^2 \Delta x K_{avi} + C_i\big|_x Q\Delta t - C_i\big|_{x+\Delta X} Q\Delta t \tag{9}$$

Where C_i is the ith component concentration, r_c is column radius, K_{avi} is available pore and interstitial volume fraction, and Q is volumetric flowrate of solution through the column.

Simplifying equation 9 gives

$$\frac{\left(C_i\big|_{t+\Delta t} - C_i\big|_t\right)}{\Delta t} = \frac{\left(C_i\big|_x - C_i\big|_{x+\Delta x}\right)}{\Delta x}\frac{Q}{\pi {r_c}^2 K_{avi}} \tag{10}$$

Letting Δt and Δx approach zero gives

$$\frac{\partial C_i}{\partial t} = \frac{\partial C_i}{\partial x}\frac{Q}{\pi r_c{}^2 K_{avi}} \tag{11}$$

To account for change in concentration of the i^{th} component due to reaction a reaction term is included

$$\frac{\partial C_i}{\partial t} = \frac{\partial C_i}{\partial x}\frac{Q}{\pi r_c{}^2 K_{avi}} + \frac{\partial C_i}{\partial t}(Rxn) \tag{12}$$

Where $\frac{\partial C_i}{\partial t}(Rxn)$ is given by equations 2, 3, 6, 7 or 8. For example, the change in concentration of a PEGylated protein with *n* number of PEG groups is

$$\frac{\partial C_{P(n+1)PEG}}{\partial t} = \frac{\partial C_{P(n+1)PEG}}{\partial x}\frac{Q}{\pi r_c{}^2 K_{avi}} + k_{(n)} C_{P(n)PEG} C_{APEG}$$

$$- k_{(n+1)} C_{P(n+1)PEG} C_{APEG} \tag{13}$$

3.3.1 Finite difference solution for axial flow size exclusion column chromatography

An axial flow column bed is divided into *X* stages along the axis. Each stage is modeled as a well-mixed tank (Figure 3-1).

Under ideal conditions, the maximum number of stages required can be calculated by

$$X_{\max} = \frac{L}{16 \cdot d_p} \tag{14}$$

where L is the axial bed length and d_p is the diameter of the resin particles (Levenspiel 1993).

The height (Δx), cross sectional area (A_c), volume of each stage (V_e) and change in time (Δt) are given by

$$\Delta x = \frac{L}{X} \tag{15}$$

$$A_c = \pi r_c^2 \tag{16}$$

$$V_e = A_c \cdot \Delta x \tag{17}$$

$$\Delta t = \frac{V_e \cdot \varepsilon}{Q \cdot J} \tag{18}$$

where ε is the packed bed interstitial void fraction. Normally J, a dimensionless parameter for reducing Δt for each time step, is set to 3, but can be increased if the model becomes unstable when change in concentration due to reaction is high giving negative concentration values for the time step.

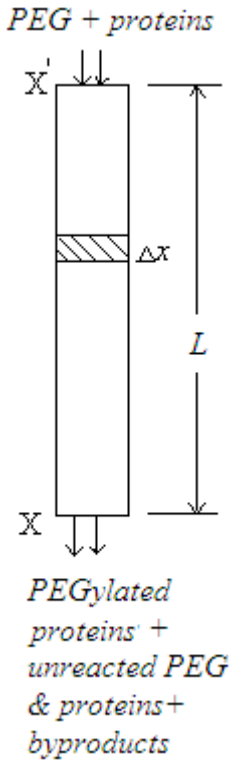


Figure 3-1: Axial flow model

Total time steps T is given by,

$$T = \frac{t_{run}}{\Delta t} \tag{19}$$

Where

$$t_{run} = \sum_{j=1}^{J_{steps}} \frac{V_j}{Q} \tag{20}$$

J_{steps} is the total number of steps taken in a run and V_i is the volume applied for j^{th} step.

In finite difference form equation 9 becomes

$$C_i\big|_{x,t}\pi r_c{}^2\Delta x K_{avi} = C_i\big|_{x,t-1}\pi r_c{}^2\Delta x K_{avi} + C_i\big|_{x-1,t-1}Q\Delta t - C_i\big|_{x,t-1}Q\Delta t \qquad (21)$$

Simplifying and substituting in equations 16 and 17, equation 21 becomes

$$C_i\big|_{x,t} = C_i\big|_{x,t-1} + \frac{\left(C_i\big|_{x-1,t-1} - C_i\big|_{x,t-1}\right)Q\Delta t}{V_e K_{avi}} \qquad (22)$$

Including change in concentration due to reaction in equation 22, the finite difference equations for C_{APEG}, C_{IPEG}, C_{SA}, $C_{P(n)PEG}$ with 0 to 4 PEG groups attached, assuming PEGylation of a protein stops once 4 PEGs are attached, are:

Where $1 < x \le X + 1$ and $1 < t \le T + 1$

UnPEGylated protein (n=0)

$$C_{P(n=0)PEG}\big|_{x,t} = C_{P(n=0)PEG}\big|_{x,t-1} + \frac{\left(C_{P(n=0)PEG}\big|_{x-1,t-1} - C_{P(n=0)PEG}\big|_{x,t-1}\right)Q\Delta t}{V_e K_{avP(n=0)PEG}}$$

$$- k_{(n=0)}C_{P(n=0)PEG}\big|_{x,t-1}C_{APEG}\big|_{x,t-1}\Delta t \qquad (23)$$

MonoPEG (n=1)

$$C_{P(n=1)PEG}\big|_{x,t} = C_{P(n=1)PEG}\big|_{x,t-1} + \frac{\left(C_{P(n=1)PEG}\big|_{x-1,t-1} - C_{P(n=1)PEG}\big|_{x,t-1}\right)Q\Delta t}{V_e K_{avP(n=1)PEG}}$$

$$+\left(k_{(n=0)}C_{P(n=0)PEG}\big|_{x,t-1} - k_{(n=1)}C_{P(n=1)PEG}\big|_{x,t-1}\right)C_{APEG}\big|_{x,t-1}\Delta t \qquad (24)$$

DiPEG (n=2)

$$C_{P(n=2)PEG}\big|_{x,t} = C_{P(n=2)PEG}\big|_{x,t-1} + \frac{\left(C_{P(n=2)PEG}\big|_{x-1,t-1} - C_{P(n=2)PEG}\big|_{x,t-1}\right)Q\Delta t}{V_e K_{avP(n=2)PEG}}$$

$$+\left(k_{(n=1)}C_{P(n=1)PEG}\big|_{x,t-1} - k_{(n=2)}C_{P(n=2)PEG}\big|_{x,t-1}\right)C_{APEG}\big|_{x,t-1}\Delta t \qquad (25)$$

TriPEG (n=3)

$$C_{P(n=3)PEG}\big|_{x,t} = C_{P(n=3)PEG}\big|_{x,t-1} + \frac{\left(C_{P(n=3)PEG}\big|_{x-1,t-1} - C_{P(n=3)PEG}\big|_{x,t-1}\right)Q\Delta t}{V_e K_{avP(n=3)PEG}}$$

$$+\left(k_{(n=2)}C_{P(n=2)PEG}\big|_{x,t-1} - k_{(n=3)}C_{P(n=3)PEG}\big|_{x,t-1}\right)C_{APEG}\big|_{x,t-1}\Delta t \qquad (26)$$

TetPEG (n=4)

$$C_{P(n=4)PEG}\big|_{x,t} = C_{P(n=4)PEG}\big|_{x,t-1} + \frac{\left(C_{P(n=4)PEG}\big|_{x-1,t-1} - C_{P(n=4)PEG}\big|_{x,t-1}\right)Q\Delta t}{V_e K_{avP(n=4)PEG}}$$

$$+\ k_{(n=3)}C_{P(n=3)PEG}\big|_{x,t-1}C_{APEG}\Big|_{x,t-1}\Delta t \qquad (27)$$

Activated PEG

$$C_{APEG}\big|_{x,t} = C_{APEG}\big|_{x,t-1} + \frac{\left(C_{APEG}\big|_{x-1,t-1} - C_{APEG}\big|_{x,t-1}\right)Q\Delta t}{V_e K_{avAPEG}} - \left(k_{hydr} + \sum_{n=0}^{n=3} k_{(n)} C_{P(n)PEG}\big|_{x,t-1}\right) C_{APEG}\big|_{x,t-1}\Delta t \quad (28)$$

Inactivated PEG

$$C_{IPEG}\big|_{x,t} = C_{IPEG}\big|_{x,t-1} + \frac{\left(C_{IPEG}\big|_{x-1,t-1} - C_{IPEG}\big|_{x,t-1}\right)Q\Delta t}{V_e K_{avIPEG}} + k_{hydr} C_{APEG}\big|_{x,t-1}\Delta t \quad (29)$$

Succinic acid

$$C_{SA}\big|_{x,t} = C_{SA}\big|_{x,t-1} + \frac{\left(C_{SA}\big|_{x-1,t-1} - C_{SA}\big|_{x,t-1}\right)Q\Delta t}{V_e K_{avSA}} + \left(k_{hydr} + \sum_{n=0}^{n=3} k_{(n)} C_{P(n)PEG}\big|_{x,t-1}\right) C_{APEG}\big|_{x,t-1}\Delta t \quad (30)$$

For boundary conditions

$$1 \le x \le X+1 \text{ and } t = 1$$

$$C_{P(n=0:N)PEG}\big|_{t,x} = 0,\ C_{SA}\big|_{t,x} = 0,\ C_{APEG}\big|_{t,x} = 0,\ C_{IPEG}\big|_{t,x} = 0 \quad (31)$$

$$t_{Pstart} \le t \le t_{Pfinish} \text{ and } X = 1$$

$$C_{P(n=0)PEG}\big|_{t,x} = C_{Pfeed} \quad (32)$$

$$t_{PEGstart} \le t \le t_{PEGfinish} \quad \text{and} \quad X = 1$$

$$C_{APEG}\big|_{t,x} = C_{PEGfeed} \tag{33}$$

C_{Pfeed} and $C_{PEGfeed}$ are the initial feed concentrations of protein and PEG respectively.

3.3.2 Stirred tank rate kinetic experiments

In modeling PEGylation reactions in stirred tanks, the entire vessel is treated as a well mixed tank, i.e. there is no change in concentration with distance (x), and there is no net fluid flow in or out of the tank (Q=0). Therefore equations 23-30 reduce to change in concentration over time due to reaction only. For example, equation 30 for succinic acid becomes

$$\text{At } 1 < t \le T+1$$

$$C_{SA}\big|_t = C_{SA}\big|_{t-1} + \left(k_{hydr} + \sum_{n=0}^{n=3} k_{(n)} C_{P(n)PEG}\big|_{t-1} \right) C_{APEG}\big|_{t-1} \Delta t \tag{34}$$

The boundary conditions are

$$\text{At } t = 1$$

$$C_{P(n=1:N)PEG}\big|_t = 0\,,\; C_{SA}\big|_t = 0\,,\; C_{IPEG}\big|_t = 0$$

$$C_{P(n=0)PEG}\big|_t = C_{Pstart}$$

$$C_{APEG}\big|_t = C_{PEGstart} \tag{35}$$

Where C_{Pstart} and $C_{PEGstart}$ are the starting protein and PEG concentrations respectively. Total time steps T is obtained from equation (19).

3.4 Conclusion

Finite difference models were developed to simulate stirred tank and size exclusion chromatography PEGylation of proteins. Model parameters can be obtained from stirred tank rate kinetic experimental data by fitting model curves to experimental results. These parameters can then be used in SEC PEGylation simulations.

Chapter 4

Methodology

4.1 Introduction

This chapter presents the methods used for the experiments repeated in this thesis. There are two main areas of experimental work:

- Determining the hydrolysis rate of the PEG-SPA 2kDa and 20kDa in a batch process. The rate kinetic experiment was carried out in a stirred beaker and the samples were tested using size exclusion chromatography.
- Determining the rate of PEGylation. The reaction was carried out at room temperature in a continuously stirred beaker and the analysis was done using size exclusion chromatography (AKTAexplor 10 liquid chromatography at UV Abs 280nm, Amersham Biosciences, Sweden).

4.2 Reagents

The following reagents where used:

- Feed proteins: α- Lactalbumin from bovine milk (85% pure by polyacrylamide gel electrophoresis (PAGE)), M_r =14.2 kDa, Sigma (St.Louis, MO), Bovine serum albumin (BSA) from bovine plasma (98% pure by PAGE), M_r =66.7 kDa, Sigma (St.Louis, MO) and β- Lactoglobulin from bovine milk (90% pure by PAGE), M_r =36.0 kDa, Sigma (St.Louis, MO).
- Linear PEG (mPEG-SPA) reagent of M_r 2 kDa and branched PEG reagent of M_r 20kDa (Nektar Therapeutics, Huntsville, AL)

- Elution buffer: PBS (phosphate buffered saline) made of 7.4 pH, Sigma (St.Louis, MO)
- Polyethylene glycol (PEG) 3120-71,000 M_r size exclusion calibration kits (Phenomenex US)
- Blue Dextran 2000, M_r =2000 kDa, Sigma (St.Louis, MO)
- 0.1 M HCl (University of Waikato)
- 0.1% trifluroacetic Acid (TFA)

4.3 Equipment

The equipment used is:

- AKTAexplorer 10 liquid chromatography system (UV Abs at 280nm), Amersham Biosciences, Sweden
- Superdex 200 HR 10/30 column, Amersham Biosciences, Sweden

4.4 Batch kinetics

4.4.1 Succinic acid hydrolysis from activated PEG

Batch kinetic experiments of PEG-SPA 2000 were carried out in a continuously stirred beaker. The setup consisted of a magnetic stirrer, 25ml beaker and 1ml sampling bottles. 10 mg of PEG-SPA 2000 was added to 10 ml PBS buffer (7.4 pH) in a constantly stirred beaker and 1 ml of the sample is collected from the beaker at desired time intervals and acidified with two drops of 0.1 M HCl to stop the hydrolysis. The same procedure was repeated for the 20 kDa PEG. The samples for linear and branched PEG were analyzed using an AKTA10 30cm long size exclusion

column (Superdex 200 HR 10/30 column, Amersham Biosciences, Sweden), and succinic acid peaks were measured using an inline spectrophotometer set at an absorbance of 280nm. The peaks were analyzed using AKTA software to obtain peak area, peak height and retention volume. The peak area was then used to calculate the mass and concentration of succinic acid. The change in concentration of hydrolysed PEG's was plotted against time to determine the rate of hydrolysis.

4.4.2 Batch PEGylation

In experiments done by Li (2004) 10mg α-lactalbumin was PEGylated with 40mg 20kDa linear polyethylene glycol in a stirred beaker containing 10ml PBS buffer (pH 7.4). Samples were taken at desired time intervals and the reaction stopped in the sample by adding 2 drops of 1M HCl. The samples were analyzed using an AKTA10 30cm long size exclusion column (Superdex 200 HR 10/30 column, Amersham Biosciences, Sweden), and protein peaks were measured using an inline spectrophotometer set at an absorbance of 280nm. The peaks were integrated using AKTA software to obtain peak area, which was then used to calculate mass by comparing data to a calibration curve. The change in mass over time of the different protein peaks was used to determine reaction rates. The rate parameters from this analysis are used in the axial flow size exclusion reaction model simulations. (see chapter 3 modeling).

4.5 Column calibration

The Superdex2000 size exclusion column was calibrated using 40 μL of 20 mg/mL proteins from the calibration kit, 1mg/mL blue detran, 1.0 mg/mL ferritin, or 10mg/mL of each mPEG. All solutions were made in PBS buffer at pH 7.4 and run at 0.5 mL/min. samples were automatically injected in to the size exclusion column. The standards were run after every 3-4 months.

Chapter 5

Results and Discussion

5.1 Introduction

Batch PEG succinic acid hydrolysis results and analysis of batch PEGylation of α-lactalbumin data and size exclusion PEGylation reaction chromatography model simulations are presented in this chapter. Models presented in Chapter 3 are used to fit experimental data to obtain model parameters. The effect of reaction parameters such as timing, length and concentration of PEG and protein injections, reaction rates, and model resolution are explored.

5.2 Batch PEG hydrolysis

Results of batch PEG succinic acid hydrolysis experiments are shown in Figures 5-1 and 5-2 and Tables 5-1 to 5-4. Free succinic acid concentration increased with time until after 100 minutes when all of the succinic acid had been hydrolysed from the PEG. A high degree of succinic acid hydrolysis was observed early in both experiments, which suggested either a systematic experimental error because the results for both 2kDa PEG and 20 kDa PEG were consistent, or that succinic acid had already hydrolyzed from the PEG prior to the start of both experiments. A systematic experimental error due to chromatographic analysis could not be confirmed because a calibration curve of succinic acid concentration versus peak height was not done. Succinic acid hydrolysis prior to the start of the experiments could possibly have been identified by introducing the activated PEG to an acidified solution buffer and immediately analyzing the solution for free succinic acid. The results for the linear and branched PEG were almost identical, demonstrating that the method was

repeatable despite using two different types and sized PEG. Therefore the mechanism for hydrolysis of succinic acid from PEG is unaffected by the PEG size.

A good model fit was obtained when succinic acid hydrolysis that occurred prior to the experiment had been taken into account, demonstrating that a first order model was suitable for simulation succinic acid hydrolysis from activated PEG.

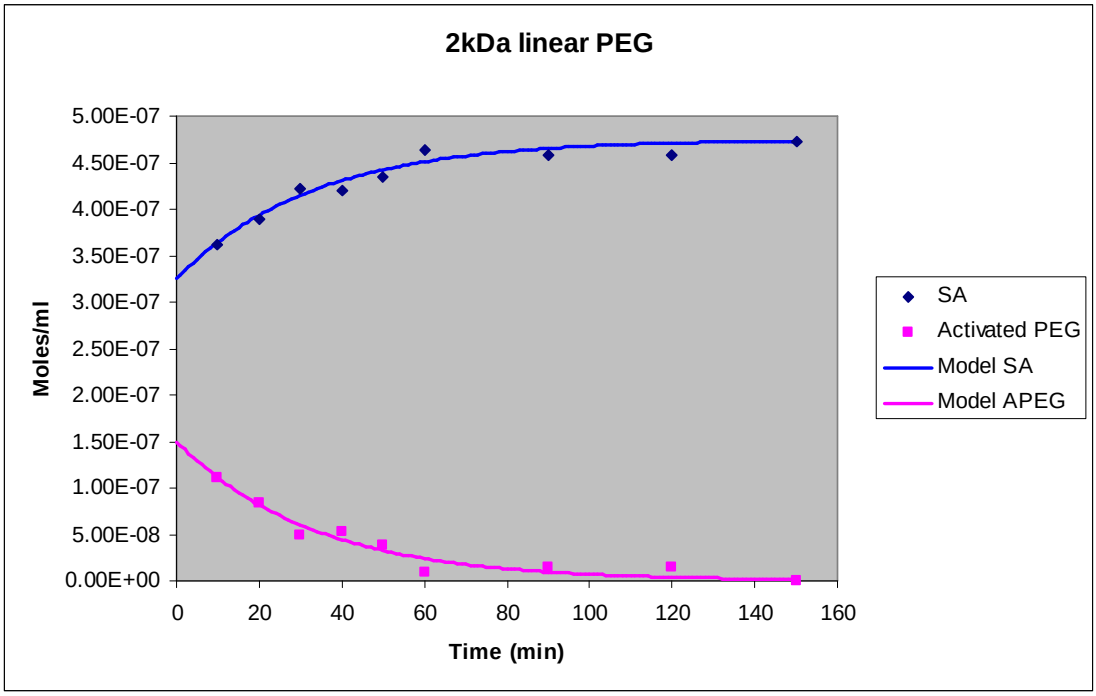


Figure 5-1: hydrolysis of 2kDa linear PEG

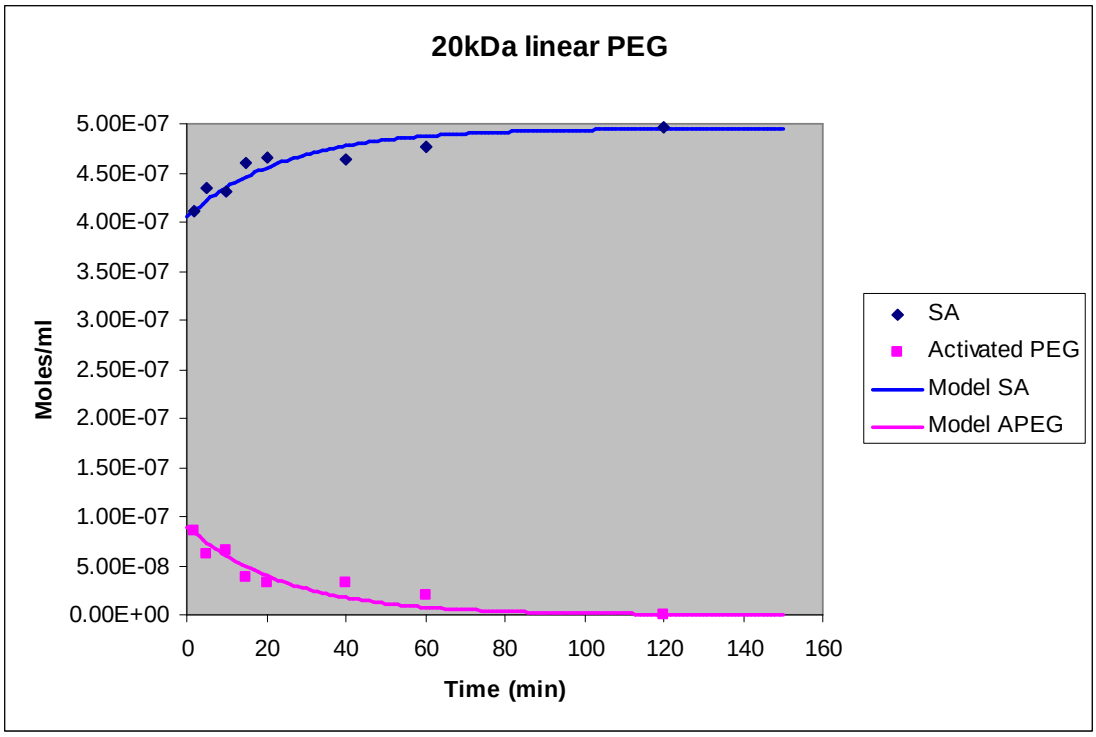


Figure 5-2: hydrolysis of 20kDa branched PEG

Table 5-1: Results for the hydrolysis of 2kDa linear PEG

Time (min)	Retention volume (ml)	Peak height (mAU)	Peak area (mAU*ml)	Mass of succinic acid (mg)
10	20.44	4.831	3.539	0.00045
20	20.46	5.821	3.802	0.00048
30	20.46	6.126	4.133	0.00053
40	20.46	5.705	4.098	0.00052
50	20.46	6.368	4.250	0.00054
60	20.44	6.665	4.534	0.00058
90	20.44	6.499	4.480	0.00057
120	20.44	6.578	4.483	0.00057
150	20.45	6.498	4.616	0.00059

Table 5-2: Results for the hydrolysis of 20kDa branched PEG

Time (min)	Retention volume (ml)	Peak height (mAU)	Peak area (mAU*ml)	Mass of succinic acid (mg)
2	20.42	53.521	37.749	0.00049
5	20.42	56.311	39.924	0.00051
10	20.42	55.412	39.558	0.00051
15	20.42	59.389	42.148	0.00054
20	20.4	59.901	42.641	0.00055
40	20.41	59.433	42.540	0.00055
60	20.41	61.339	43.691	0.00056
120	20.4	63.7	45.587	0.00059

Table 5-3: Calculated moles of succinic acid and activated PEG remaining for 2kDa PEG.

Time (min)	Moles SA in 1 ml sample	Moles activated PEG remaining
10	3.62E-07	1.10E-07
20	3.89E-07	8.32E-08
30	4.23E-07	4.94E-08
40	4.19E-07	5.30E-08
50	4.35E-07	3.75E-08
60	4.64E-07	8.45E-09
90	4.58E-07	1.39E-08
120	4.58E-07	1.36E-08
150	4.72E-07	0.00E+00

Table 5-4: Calculated moles of succinic acid and activated PEG remaining for 20kDa PEG.

Time (min)	Moles SA in 1 ml sample	Moles activated PEG remaining
2	4.12E-07	8.55E-08
5	4.35E-07	6.18E-08
10	4.31E-07	6.57E-08
15	4.60E-07	3.75E-08
20	4.65E-07	3.21E-08
40	4.64E-07	3.32E-08
60	4.76E-07	2.07E-08
120	4.97E-07	0.00E+00

5.2.1 Calculations for the mass of succinic acid

The following calculations were done to obtain the mass and molar concentration of succinic acid and PEG.

For 2kDa linear PEG:

Molecular weight of succinic acid = 118.09 g/mol

Molecular weight of 2kDa PEG = 2000 g/mol

Molecular weight of activated PEG = 2118.09 g/mol

Mass of 2kDa PEG used for hydrolysis in 10ml PBS buffer = 10mg

Concentration of PEG = 1mg/ml = 0.001g/ml

Moles of activated PEG per ml = Concentration of PEG / Molecular weight of activated PEG = 0.001/ 2118.09 = 4.721x 10^{-7} moles/ml

Mass of succinic acid = moles of activated PEG per ml x molecular weight of succinic acid = 4.721×10^{-7} x 118.09 = 5.575×10^{-5} gm/ml

Therefore total mass of succinic acid in 10 ml PBS buffer = 0.0005575 g

Maximum peak area = 4.616

Total mass of succinic acid = 0.0005575 g

Mass of succinic acid per unit area = total mass of succinic acid / maximum peak area = 0.0005575 / 4.616 = 0.000127 mg

Therefore mass of succinic acid is given by,

Mass of succinic acid = mass of succinic acid per unit area x peak area of the sample

For 20kDa branched PEG:

Molecular weight of succinic acid = 118.09 g/mol

Molecular weight of 20kDa PEG = 20000 g/mol

Molecular weight of activated PEG = 20118.09 g/mol

Mass of 20kDa PEG used for hydrolysis in 10ml PBS buffer = 100.1mg

Concentration of PEG = 10mg/ml = 0.01g/ml

Moles of activated PEG per ml = Concentration of PEG / Molecular weight of activated PEG = 0.01/ 20118.09 = 4.971×10^{-7} moles/ml

Mass of succinic acid = moles of activated PEG per ml x molecular weight of succinic acid = 4.971×10^{-7} x 118.09 = 5.87×10^{-5} gm/ml

Therefore total mass of succinic acid in 10 ml PBS buffer = 0.000587 gm

Maximum peak area = 45.5871

Total mass of succinic acid = 0.000587 gm

Mass of succinic acid per unit area = total mass of succinic acid / maximum peak area

= 0.000587 / 45.5871 = 1.287 x 10^{-5} mg

Therefore mass of succinic acid is given by,

Mass of succinic acid = mass of succinic acid per unit area x peak area of the sample

5.2.2 Model parameters for succinic acid hydrolysis from PEG

The following parameters were used to obtain a good model fit with experimental data in Figures 5-1 and 5-2.

For hydrolysis of 2kDa linear PEG:

Starting activated linear PEG = 1.5 x 10^{-7} mol/ml

Starting succinic acid = 3.25 x 10^{-7} mol/ml

Rate of hydrolysis of 2kDa PEG $k_{hydrolysis}$ = 0.03 1/min

$\Delta t = 1\text{min}$

For hydrolysis of 20kDa branched PEG:

Starting activated branched PEG = 9.00 x 10^{-8} mol/ml

Starting succinic acid = 4.05 x 10^{-7} mol/ml

Rate of hydrolysis of 2kDa PEG $k_{hydrolysis}$ = 0.04 1/min

$\Delta t = 1\text{min}$

5.3 Rate kinetics for PEGylation reaction

The results for the batch PEGylation of a-lactalbumin with 20kDa PEG is shown in Figure 5-3, and Table 5-5. Native protein concentration drops rapidly in the first 5 to 10 minutes after which the rate of decrease slows. MonoPEG increases rapidly in the first 5 minutes after which it decreases due to additional PEG groups being attached in the PEGylation reaction. DiPEG peaks around 30 minutes whereas TriPEG continues to increase over time. These results show that one or two PEG groups can be relatively attached easily as is demonstrated by the MonoPEG and DiPEG peaks in the first five minutes. TriPEG formation occurs at a much slower rate either because all available surface lysine has been used for PEG attachment, in which case, the activated PEG must somehow attach to lysine residues within the protein, or because the PEG groups form a shield around the protein restricting further PEG attachment. Values listed in Table 5-5 are used to determine the rate of formation of monoPEG.

Table 5-5: The values obtained from the unpublished work by Li (2004)

Reaction time (min)	Protein concentration (mg/ml)	MonoPEG concentration (mg/ml)	DiPEG concentration (mg/ml)	TriPEG concentration (mg/ml)
5	0.17	0.49	0.31	0.011
10	0.061	0.33	0.30	0.094
30	0.007	0.15	0.37	0.380
60	0	0.03	0.18	0.497

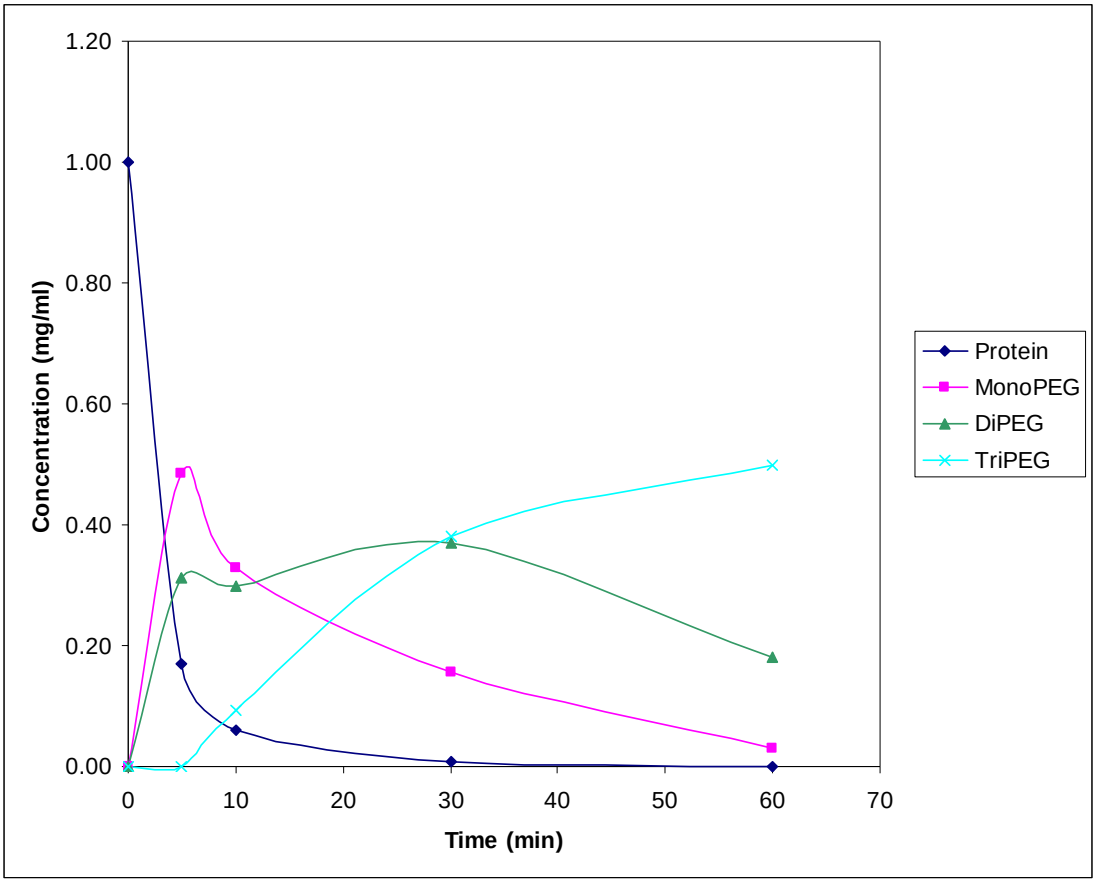


Figure 5-3: PEGylated and native a-lactalbumin concentrations with time in batch PEGylation reaction (Li 2004).

The initial reaction between the PEG moles and the number of lysine moles is assumed to be irreversible because the PEGylation reaction involves a covalent attachment of activated PEG to a lysine residue. The reaction is assumed to be second order due to involvement of two reacting species, PEG and protein. Therefore from Chapter 3 the reaction is,

$$\text{PEG.SPA} + (n)\text{PEG.Protein} \xrightarrow{k_{(n)}} (n+1)\text{PEG.Protein} + \text{SA}$$

The rate of reaction is given by,

$$-r_A = k \cdot C_A \cdot C_B \tag{36}$$

where A = PEG.SPA and B=proteins.

Table 5-6: The experimental data and the model values

t (min)	Concentration of protein (mg/ml)	X_A	$\ln[M(1-X_A)/M(1-X_A)]$
5	0.17	0.83	1.426
10	0.061	0.94	2.42
30	0.007	0.993	4.53
60	0	1.000	6.47

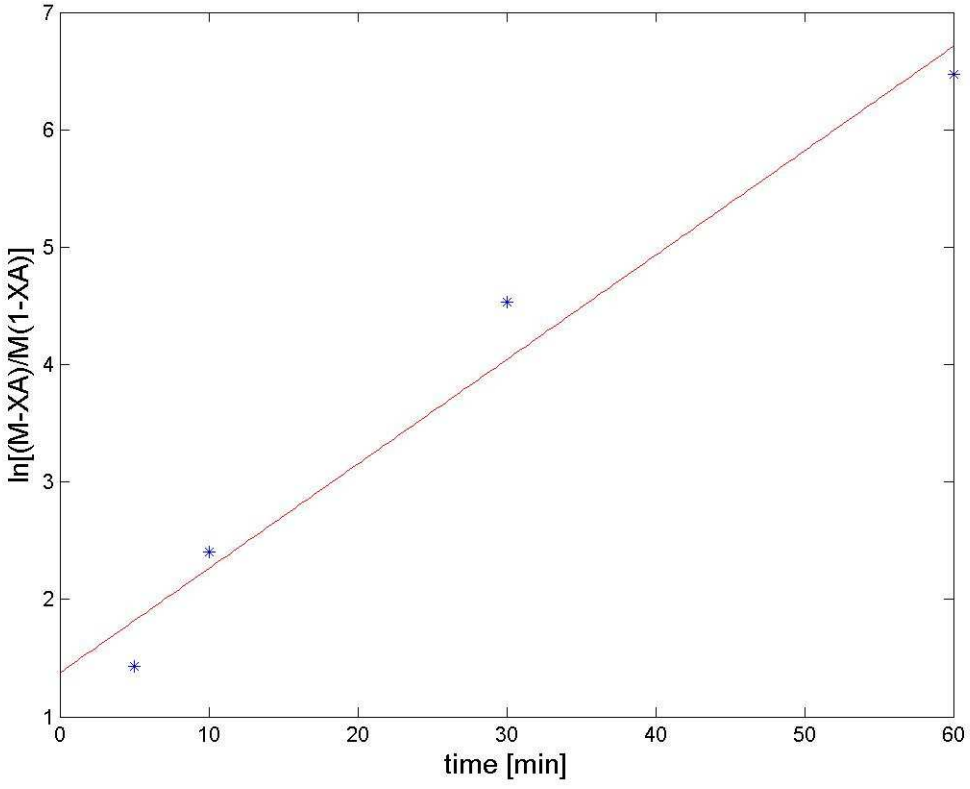


Figure 5-4: MonoPEG formation against time. Where, M= molar ratio and XA = molar concentration. Test for second order kinetics

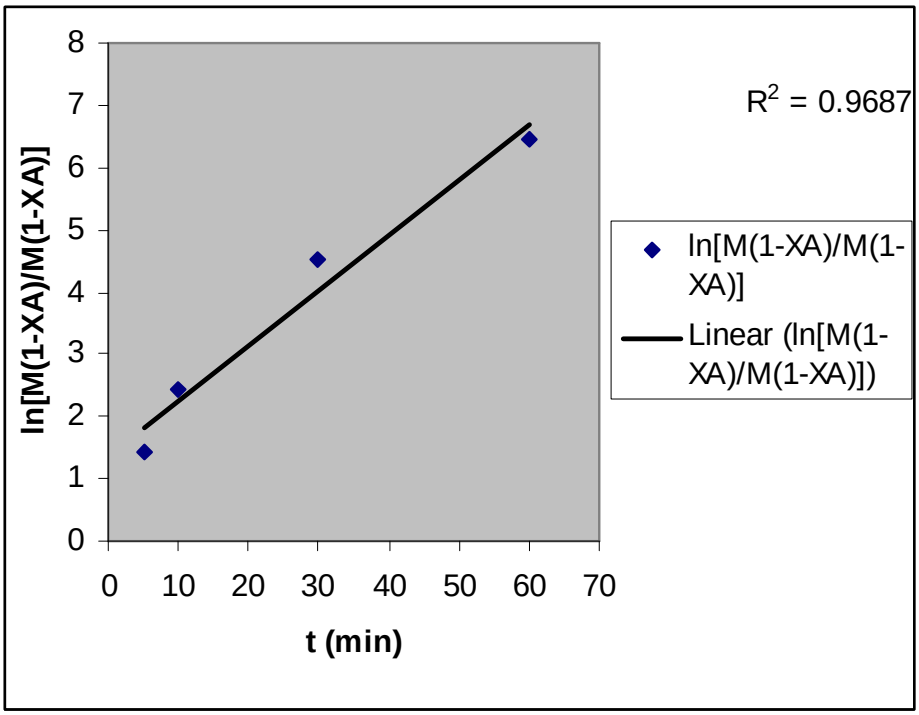


Figure 5-5: Ditto Test for second order kinetics, fit is satisfactory

From Figure 5-4, slope is given by,

$$(M-1)\cdot k_1 = 0.09 \tag{37}$$

k_1 is given by,

$$k_1 = {}^{0.09}\!/\!_{(M-1)} = {}^{0.09}\!/\!_{(2.84-1)} = 0.0489 \tag{38}$$

Where molar ratio M is given by,

$$M = \frac{C_{AO}}{C_{BO}} \tag{39}$$

Molecular weight of protein= 14.2kDa and PEG= 20 kDa, hence molar concentration of protein is given by,

$$C_{AO} = \frac{1}{14.2 * 1000} \cdot gmol \tag{40}$$

Molar concentration of PEG is given by,

$$C_{AO} = \frac{4}{20 * 1000} \cdot gmol \tag{41}$$

Therefore molar ratio M is given by,

$$M = \frac{C_{AO}}{C_{BO}} = \frac{4 * 14.2}{20} = 2.48 \tag{42}$$

The overall rate equation is given by,

$$-r_{protein} = 0.0489 \cdot C_A \cdot C_B \tag{43}$$

The first reaction where MonoPEG is formed by attaching PEG to a single protein lysine residue is a very fast reaction; therefore Mono PEG concentration peaks around 5 minutes. Then it decomposes into Di and Tri PEG by subsequent attachment of more PEG molecules to the PEGylated protein. The formation of Di and TriPEG is dependent on the rate of MonoPEG formation assuming that PEG attachment is sequential. This problem was solved by using MATLAB to fit model data to native protein and PEGylated protein concentrations. The order of the Di and TriPEG formation was found to be 1.5.

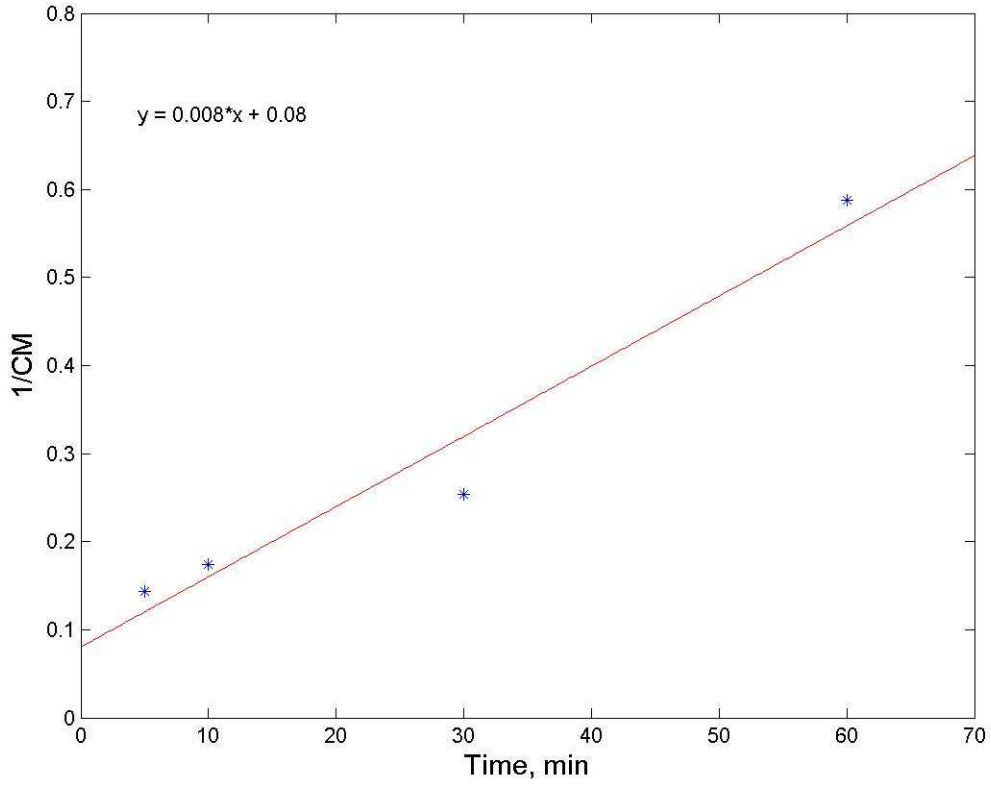


Figure 5-6: Reaction rate of MonoPEG Solid line represents model where as points represent experimental data. Where, CM= concentration of monoPEG

Given that the order of the reaction is 1.5,

$$\frac{-dC_M}{dt} = k_2 \cdot C_M^{1.5} \tag{44}$$

Integrating equation (44) gives,

$$\frac{1}{C_M^{0.5}} - \frac{1}{C_{M0}^{0.5}} = k_2 \cdot t \tag{45}$$

The slope from Figure 5-5 is,

$$k_2 = 0.016 \tag{46}$$

By substituting (46) in equation (44), the rate equation for DiPEG formation is

$$rate = -\frac{dC_M}{dt} = 0.016 \cdot C_M^{1.5} \tag{47}$$

The overall rate equation is given by,

$$-r_{monoPEG} = k_1 C_{Protein} C_{PEG} - k_2 C_{monoPEG} C_{PEG} \tag{48}$$

Substituting the values of k_1 and k_2 in equation (48) gives,

$$-r_{monoPEG} = 0.0489 \cdot C_{Protein} \cdot C_{PEG} - 0.0016 \cdot C_{monoPEG} \cdot C_{PEG} \tag{49}$$

A reasonable model fit in Figure 5-3 was achieved with the data given, which indicates the model is suitable. However goodness of fit could be further verified if more experimental data had been obtained.

5.4 Evaluation of results obtained from MatLab simulations

5.4.1 Batch model

The following assumptions were made in case of batch model Matlab simulations

- The PEGylation reactions were assumed to be first order
- Arbitrary values were used for component properties and retarding factors

The batch model integrity was tested by doing a mass balances on protein and PEG in and out of the simulated column is as shown in the Table 5-7 and Table 5-8.

Constant variables:

Mass of protein= 20 mg

Time t=180min

Volume of the beaker V= 25ml

Reaction rate of protein k= 0.003 ml/mg.s

Rate of hydrolysis of PEG $k_{hydrolysis}$ = 0.001 1/s

The Figure 5-7 shown below is an example of the batch model indicating the changes in mass of the reaction components at different feed mass of PEG and also indicates the percentage of protein converted in each case.

The mass of mono PEG formed increases in the beginning but starts to decrease as the reaction proceeds leading to an increase in higher degree of PEGylated products. A similar trend is obtained in case of di PEG and tri PEG but a constant increase can be seen in the case of tri PEG. The conversion of protein increases proportionally with the mass of the active PEG.

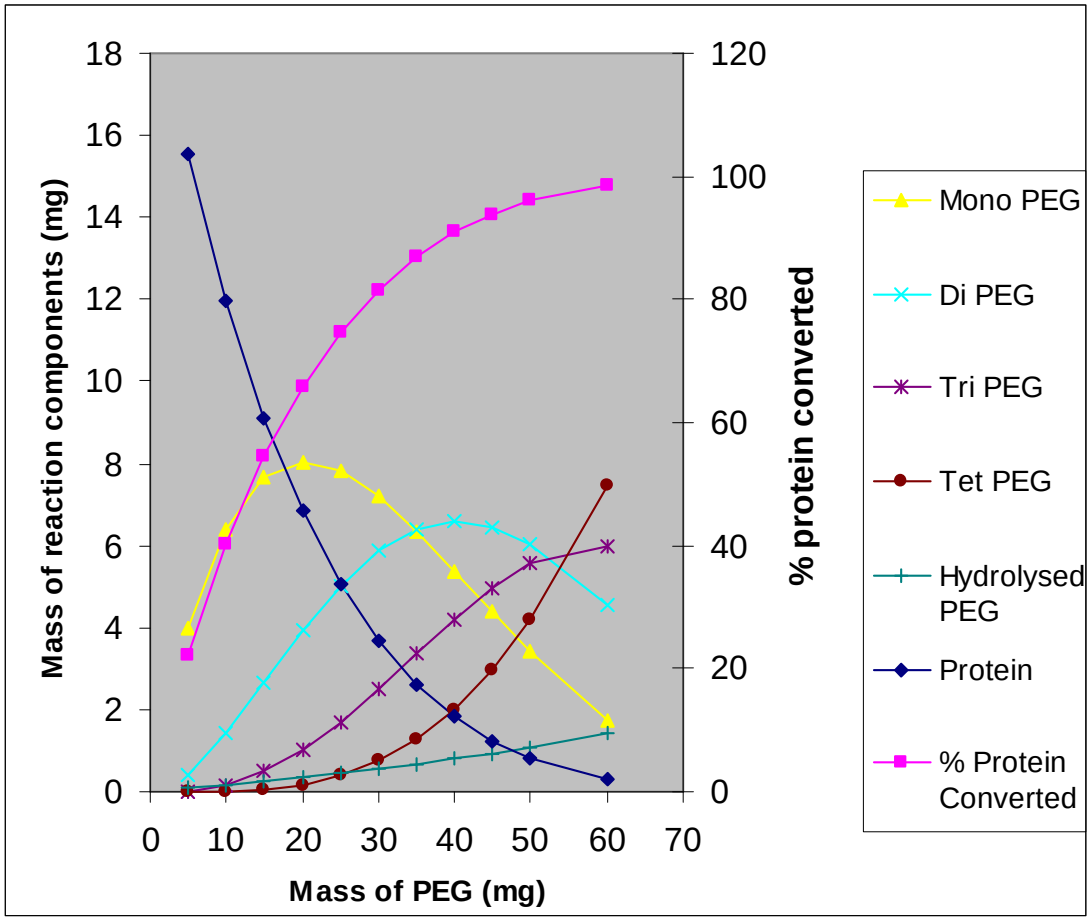


Figure 5-7: mass of the reaction components and percentage of protein converted at differing mass of PEG

5.4.2 Axial flow size exclusion reaction model

The MatLab model for the axial flow size exclusion reaction is based on two assumptions

- The PEGylation reactions were assumed to be first order
- Arbitrary values were used for component properties and retarding factors

The axial flow model integrity was tested by doing a mass balances on protein and PEG in and out of the simulated column. This check was carried out by setting the

PEGylation reaction rate *k* at 0mL/mg.s for unPEGylated protein. The model was also rechecked by passing PEG only through the simulated column. The results of the test are shown in Table 5-7 and Table 5-8.

1. Assuming k= 0 mL/mg.s

C_{PEG}=40mg/mL $C_{protein}$=40mg/mL t=300min U=0.15 cm/min

Table 5-7: Mass balance at k= 0 mL/mg.s

			Mass (mg)	Ratio
Mass of protein (mg)	47.124	Protein	46.878	0.995
Mass of PEG (mg)	94.248	PEG	77.314	0.820
Mass in (mg)	141.37	MonoPEG	0	0
Mass out (mg)	141.37	DiPEG	0	0
Concentration of protein (mg/mL)	40	TriPEG	0	0
Length of Protein pulse (min)	10	TetPEG	0	0
		Hydrolysed		
Concentration of PEG (mg/mL)	40	PEG	17.112	0.180
Length of PEG pulse (min)	20	Succinic acid	0.134	0.001

2. At zero protein or $C_{protein}$ = 0 mg/mL

C_{PEG}=40mg/mL t=300min U=0.15 cm.min

Table 5-8: Mass balance at $C_{protein}$ = 0 mg/mL

			Mass (mg)	Ratio
Mass of protein (mg)	0	Protein	0	NaN
Mass of PEG (mg)	94.248	PEG	77.004	0.820
Mass in (mg)	94.248	MonoPEG	0	NaN
Mass out (mg)	94.248	DiPEG	0	NaN
Concentration of protein (mg/mL)	0	TriPEG	0	NaN
Length of Protein pulse (min)	0	TetPEG	0	NaN
Concentration of PEG (mg/mL)	40	Hydrolysed PEG	17.112	0.180
Length of PEG pulse (min)	20	Succinic acid	0.134	0.001

At k=0 ml/mg.s and $C_{protein}$= 0 mg/ml the amount of mass fed in is equal to the amount of mass out indicating that the model is working properly. All the values generated are shown in Table 5-7 and Table 5-8.

Varied protein concentration

The simulations for MatLab model axial flow size exclusion column were carried out at different input concentration of proteins. The concentration of PEG.SPA was kept constant at 40mg/mL. All the other parameters were kept constant. The results of the

studies by varying the concentration are shown in the Figure 5-8 (product mass vs. concentration of protein) and Figure 5-9 (product ratio vs. concentration of protein).

Constant variables:

C_{PEG} =40mg/ml

Mass of PEG= 47.124 mg

Superficial velocity U=0.15cm/min

Time t=300min

Length of column= 60 cm

Reaction rate of protein k= 0.003 ml/mg.s

Protein start and finish = 5 to 15 minutes

PEG start and finish = 16 to 26 minutes

Table 5-9: The concentration and the mass of protein used for simulations

Concentration of protein (mg/mL)	Mass of protein (mg)
5	5.8905
10	11.781
15	17.671
20	23.562
25	29.452
30	35.343
35	41.233
40	47.124

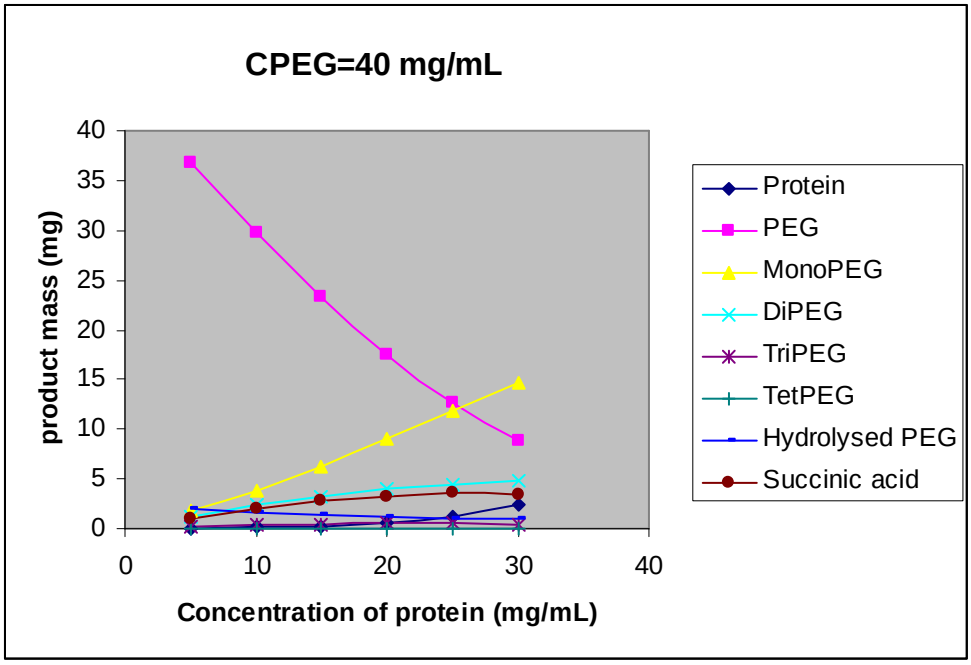


Figure 5-8: product mass vs protein concentration (mg/ml)

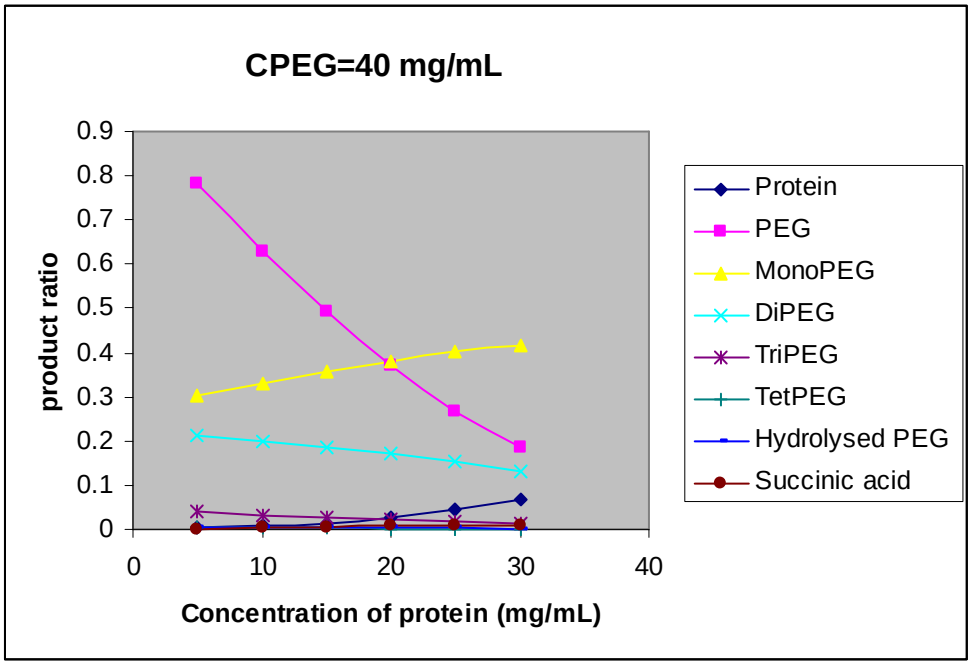


Figure 5-9: product ratio vs. protein concentration (mg/ml)

From the results of the simulation (Figure 5-8 and Figure 5-9), the concentration of PEG decreases with increasing concentration of proteins. As the concentration of protein is gradually increased, the amount of protein converted gets lower with an increase in monoPEG to diPEG ratio. When concentration of protein equals the concentration of PEG, a substantial amount of protein is wasted which is undesirable since the aim is to achieve cost effectiveness and to increase the yield of mono PEGylated protein.

Increasing PEG pulse

The simulations for axial flow size exclusion column was carried out at varied pulse length for different concentrations of PEG (C_{PEG}=10mg/mL and C_{PEG}=20mg/mL). The concentration of protein is set constant at $C_{Protein}$= 40mg/mL. The results are shown in Figure 5-10 and Figure 5-11.

Constant variables:

$C_{protein}$ =40mg/ml

Mass of protein= 47.124 mg

Superficial velocity U=0.15cm/min

Time t=300min

Length of column= 60 cm

Reaction rate of protein k= 0.003 ml/mg.s

Protein start and finish = 5 to 15 minutes

PEG start = 16 minutes

Table 5-10: The pulse length and the mass of PEG used for simulations

Pulse length of PEG (min)	Mass of PEG (C_{PEG}=10mg/mL) (mg)	Mass of PEG (C_{PEG}=20mg/mL) (mg)
10	11.78	23.56
20	23.56	----------
30	35.34	70.69
40	47.12	----------
50	58.91	117.81
60	70.69	----------
70	82.47	164.93
90	---------	212.06

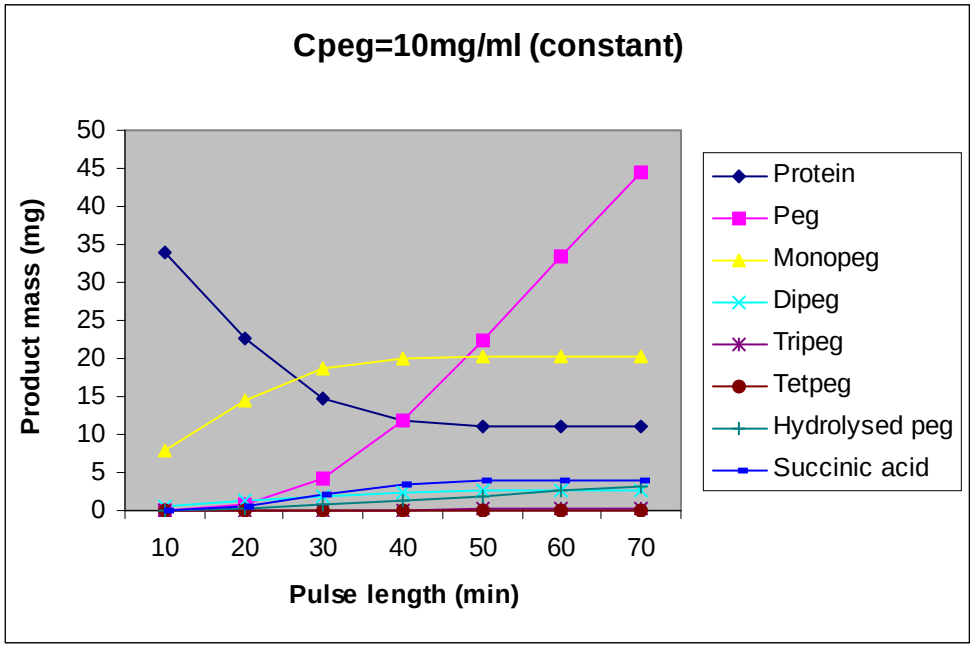


Figure 5-10: product mass vs pulse length of PEG (C_{PEG}= 10mg/mL)

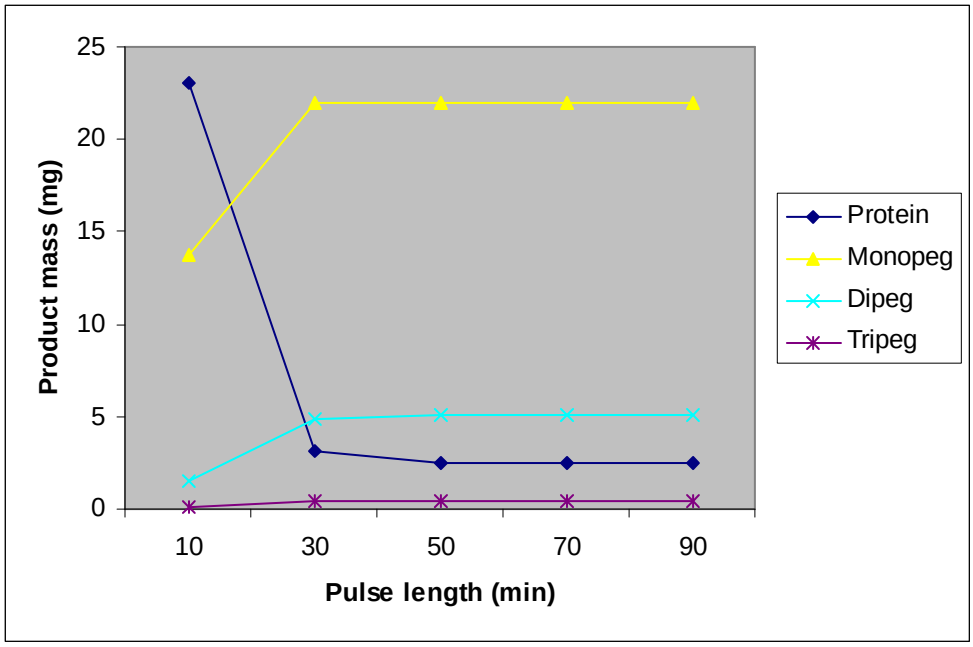


Figure 5-11: product mass vs pulse length of PEG (C_{PEG}= 20mg/mL)

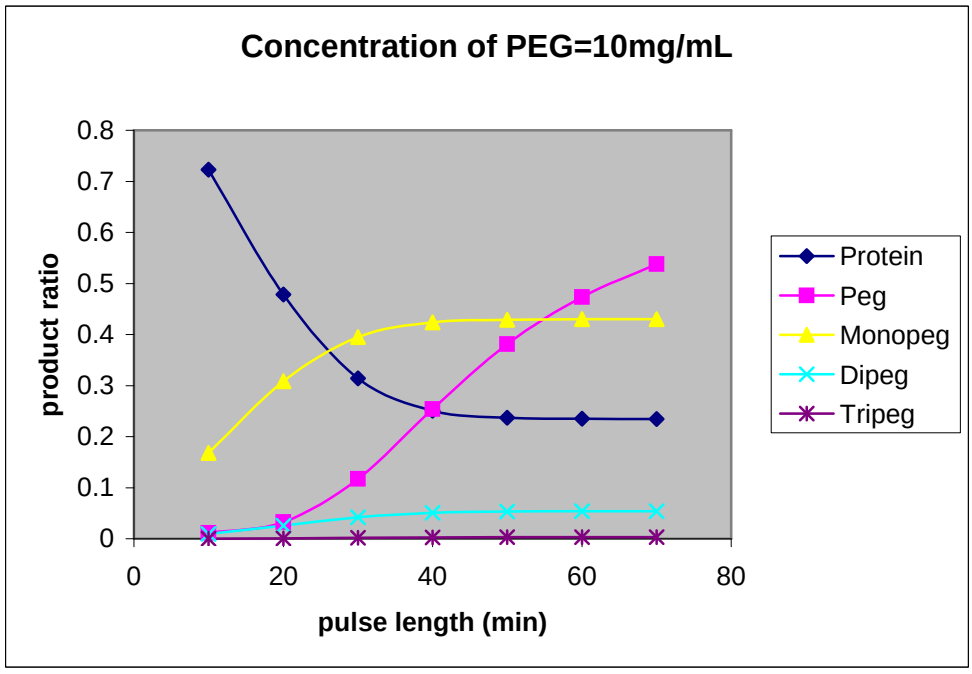


Figure 5-12: product ratio vs PEG pulse length (C_{PEG}= 10mg/mL)

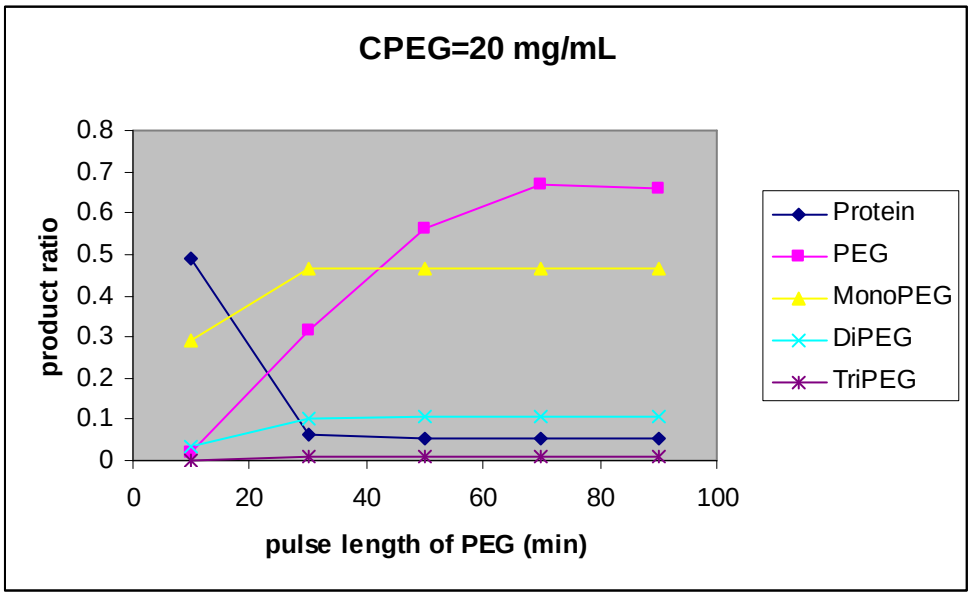


Figure 5-13: product ratio vs pulse length of PEG (C_{PEG}= 20mg/mL)

The addition of PEG after 30 minutes does not make any difference to the mass of the other species because all the products and the unreacted protein have already moved out of the column (Figure 5-12 and Figure 5-13). Hence to increase the time of contact between reactants it is suggested to have a longer column or the feed injection flow needs to be minimized. The length of the column used to run the model simulations was 60cm and the flow rate was set at 0.118 cm^3/min.

Table 5-11: The pulse length and the mass of PEG used for simulations

Pulse length of PEG (min)	Mass of PEG (C_{PEG}=40mg/mL) (mg)
10	47.124
11	51.836
12	56.549
13	61.261
14	65.973
20	94.248

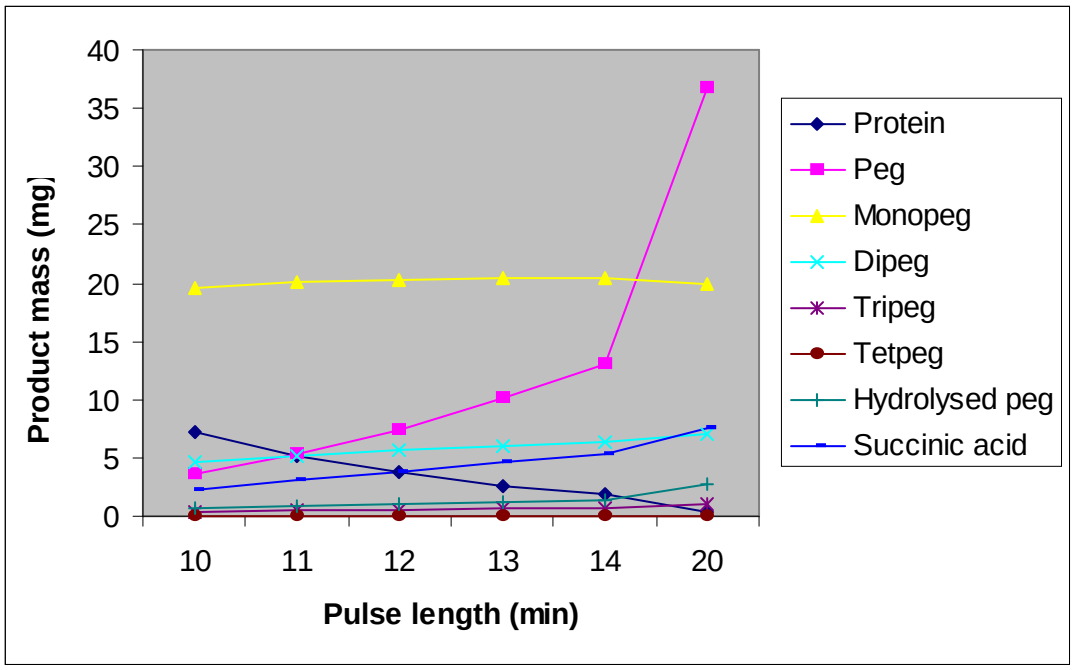


Figure 5-14: product mass vs pulse length of PEG (C_{PEG}= 40mg/mL)

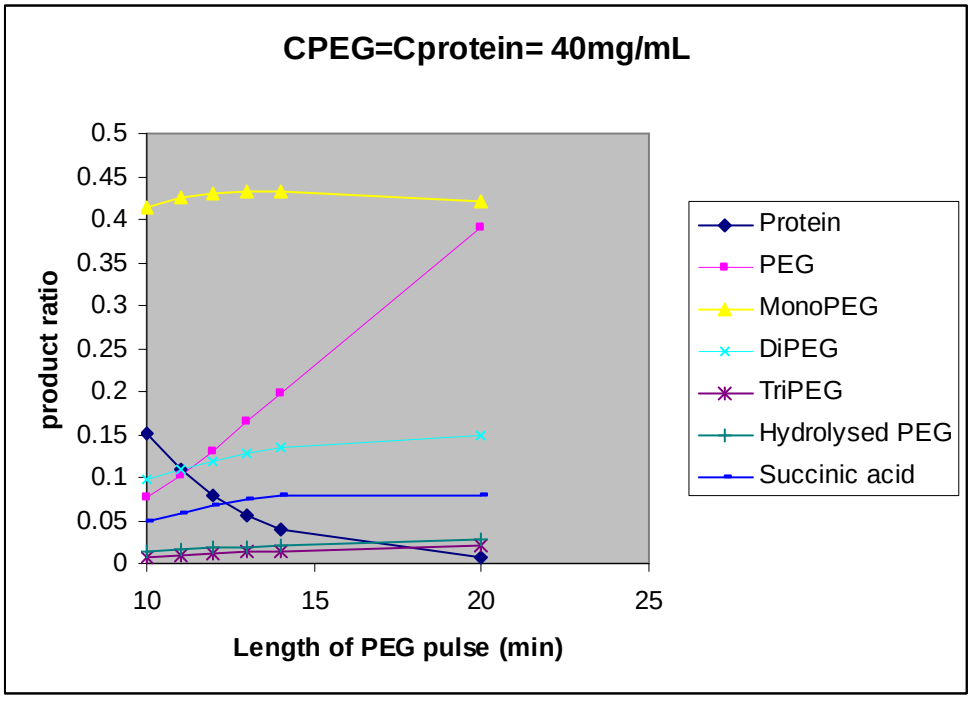


Figure 5-15: product ratio vs pulse length (C_{PEG}= 40mg/mL)

From the Figure 5-15, there is a good ratio of monoPEG, diPEG and also good conversion of protein. Hence small doses of highly concentrated PEG is more effective than longer PEG pulse with low concentrations. In this case equal concentrations of PEG and protein is used (C_{PEG}= $C_{Protein}$= 40mg/mL).

Rate of hydrolysis of PEG

The simulations of axial flow size exclusion column where carried out at different $k_{hydrolysis}$ to check the affect of rate of hydrolysis of PEG on the PEGylation reactions. The results from the simulations are shown in Figure 5-16 and Figure 5-17.

Constant variables:

$C_{protein}$ = C_{PEG} = 40mg/ml

Mass of protein and PEG= 47.124 mg

Superficial velocity U=0.15cm/min

Time t=300min

Length of column= 60 cm

Reaction rate of protein k= 0.003 ml/mg.s

Protein start and finish = 5 to 15 minutes

PEG start and finish = 16 to 26 minutes

From the Figure 5-16 and 5-17, the rate of hydrolysis of PEG does not have a major affect on the overall PEGylation reaction. The increase in rate of hydrolysis of PEG leads to the formation of hydrolysed PEG with not significant change in the mass of other products of PEGylation reaction. In the case of higher hydrolysis rates of PEG,

the feed of PEG needs to be increased to see that there is substantial amount of PEG for PEGylation to take place.

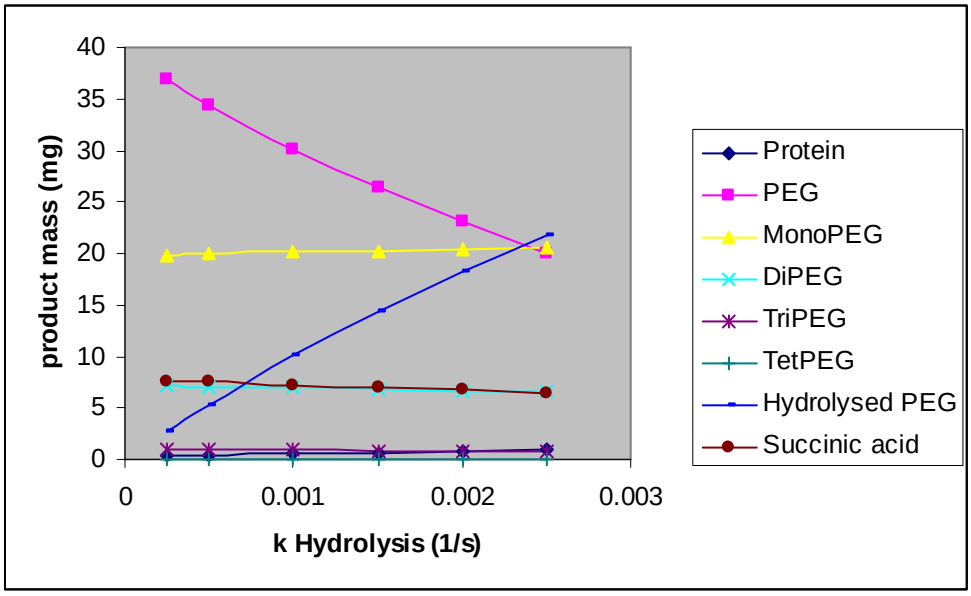


Figure 5-16: product mass vs k hydrolysis

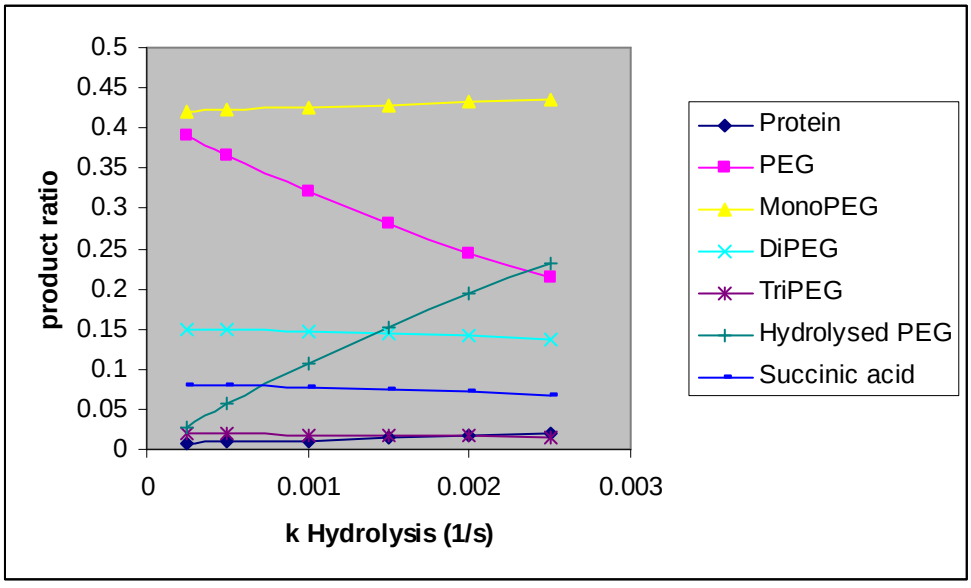


Figure 5-17: product ratio vs k hydrolysis

Product mass and ratio

The product mass of the components is calculated from the peak areas under the chromatogram obtained for each species from the simulated model of the axial flow size exclusion column. The product ratio for all the simulations were obtained by dividing the mass output of the unreacted protein and the PEGylated proteins by the total mass of protein fed in to the simulated axial flow size exclusion column. The product ratios for unreacted PEG, hydrolyzed PEG and succinic acid are calculated by total mass output of these components divided by the total mass of PEG fed in to the simulation model column.

5.4.3 Sample simulation curves

1. Sample curve

The sample curve is obtained from the simulation of axial flow size exclusion reaction model. The variables used to obtain the sample curve (Figure 5-18) are listed below.

Variables

$C_{protein} = C_{PEG} = 40$mg/ml
Mass of protein and PEG= 47.124 mg
Superficial velocity U=0.15cm/min
Time t=300min
Length of column= 60 cm
Reaction rate of protein k= 0.003 ml/mg.s
Protein start and finish = 5 to 16 minutes
PEG start and finish = 16 to 26 minutes
Number of elements the column is divided in to X=round(L./(16.*Dp))

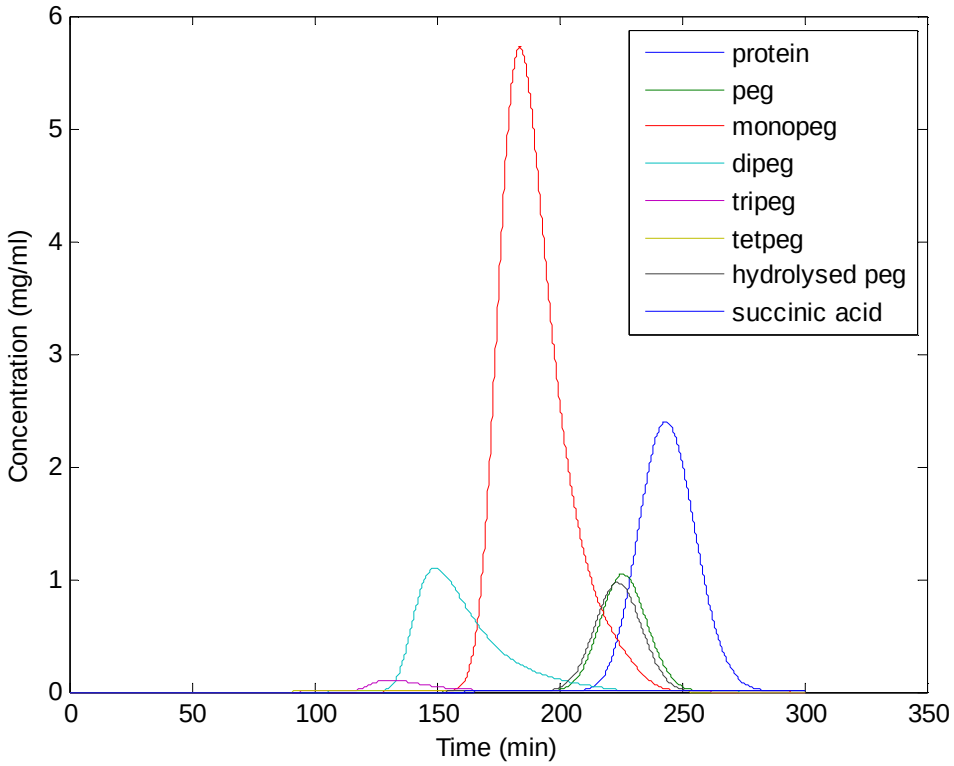


Figure 5-18: sample curve for the simulation of axial flow size exclusion model

2. Variation of PEG pulse

Variables:

$C_{protein} = C_{PEG} = 40$mg/ml

Superficial velocity U=0.15cm/min

Time t=300min

Time divider J= 3

Length of column= 60 cm

Reaction rate of protein k= 0.003 ml/mg.s

Protein start and finish = 5 to 16 minutes

PEG start and finish = 16 to 46 minutes

Number of elements the column is divided in to X=round(L./(16.*Dp))

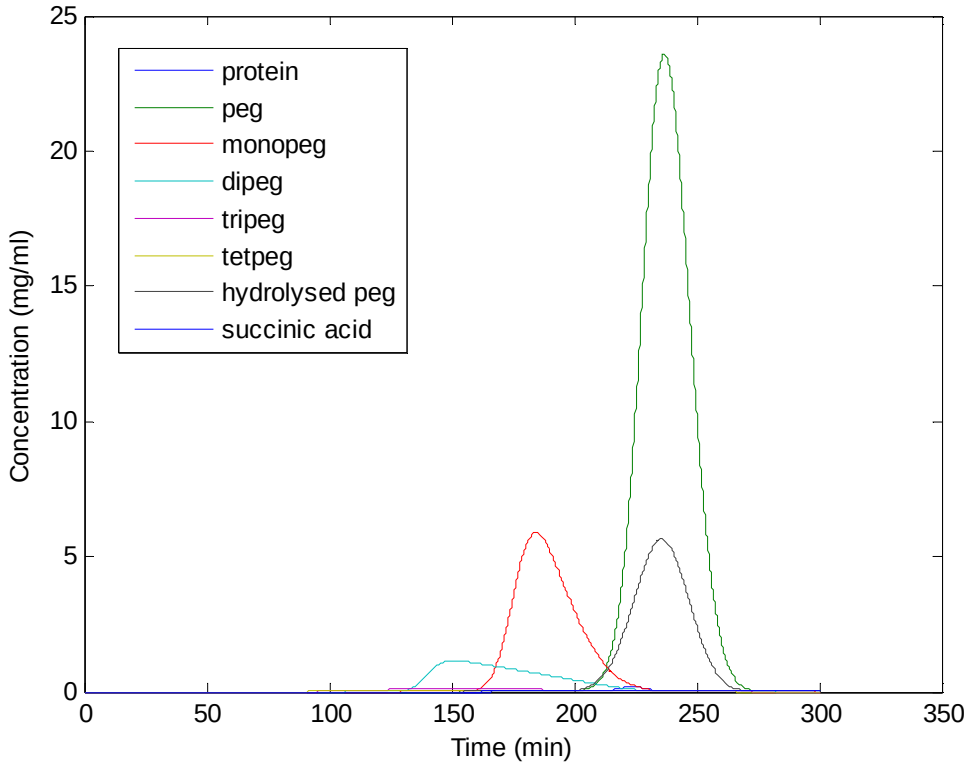


Figure 5-19: variation of PEG pulse length

In the Figure 5-19 the peak height of PEG and hydrolyzed PEG are much higher compared to Figure 5-18 which is a result of increased PEG pulse length.

3. Change in concentration

Variables:

$C_{protein}$ = 40mg/ml

C_{PEG} =.15mg/ml

Superficial velocity U=0.15cm/min

Time t=300min

Length of column= 60 cm

Reaction rate of protein k= 0.003 ml/mg.s

Protein start and finish = 5 to 16 minutes

PEG start and finish = 16 to 46 minutes

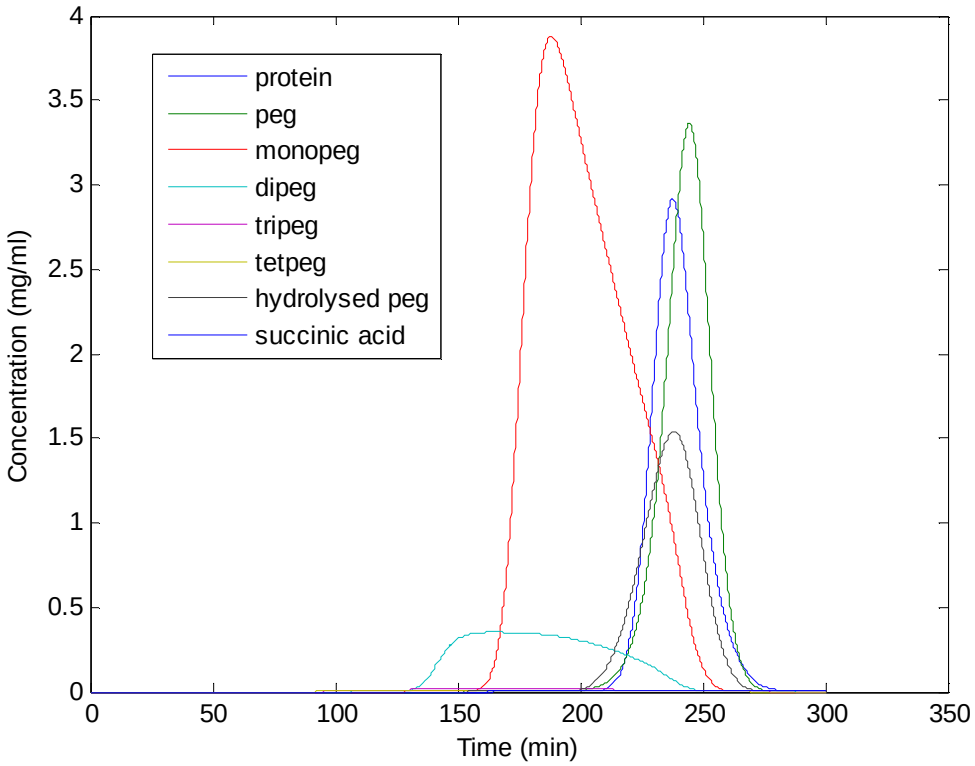


Figure 5-20: change in concentration of PEG

In this case the concentration of the PEG is reduced to 15mg/ml from 40mg/ml hence the amount of hydrolyzed PEG produced is also low with lower conversion of protein.

4. Change in time step

Variables:

$C_{protein}$ = 40mg/ml

C_{PEG} =.15mg/ml

Superficial velocity U=0.15cm/min

Time t=300min

Time divider J= 5

Length of column= 60 cm

Reaction rate of protein k= 0.003 ml/mg.s

Protein start and finish = 5 to 16 minutes

PEG start and finish = 16 to 46 minutes

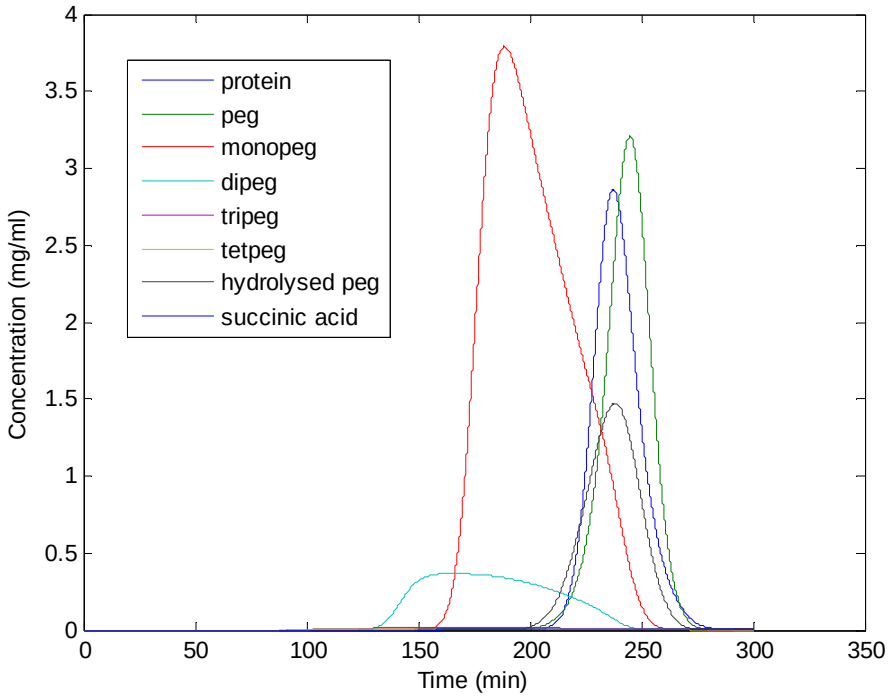


Figure 5-21: Change in time steps at J =5

At J =1

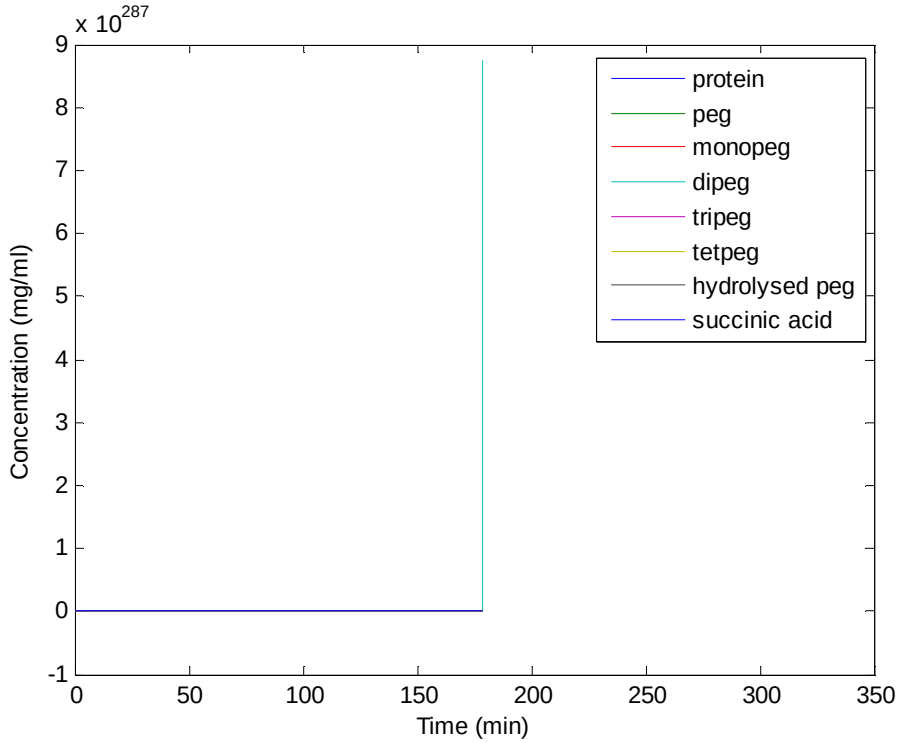


Figure 5-22: Change in time steps at J =1

The time divider J can also increase or decrease the resolution of the curves. In case of Figure 5-21 the resolution is better compared to Figure 5-20. At J=1, is an example of poor resolution.

5. Change in k value

Variables:

$C_{protein}$ = 40mg/ml

C_{PEG} =.15mg/ml

Superficial velocity U=0.15cm/min

Time t=300min

Time divider J= 3

Length of column= 60 cm

Reaction rate of protein k= 0.006 ml/mg.s

Protein start and finish = 5 to 16 minutes

PEG start and finish = 16 to 46 minutes

Number of elements the column is divided in to X=round(L./(16.*Dp))

In the Figure 5-23, more protein is PEGylated compared to Figure 5-20 since the rate of reaction of protein in this case was increased by two times from k = 0.003 ml/mg.s to k = 0.006 ml/mg.s.

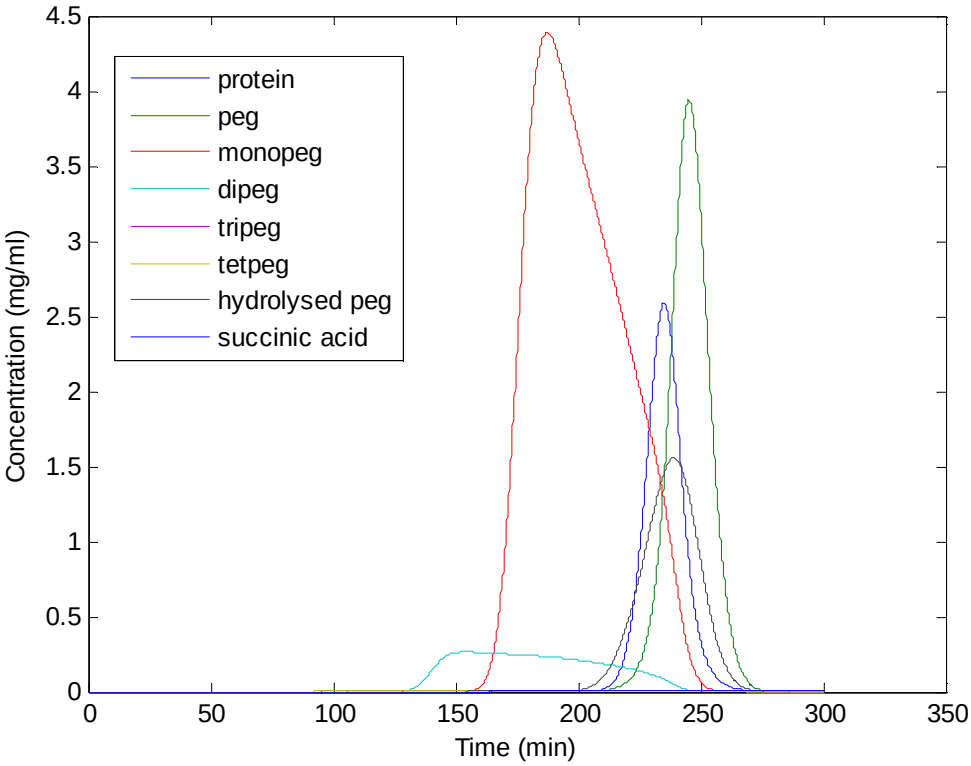


Figure 5-23: change in rate of reaction of protein

6. Change in rate of hydrolysis of PEG

Variables:

$C_{protein}$ = 40mg/ml

C_{PEG} =.15mg/ml

Superficial velocity U=0.15cm/min

Time t=300min

Time divider J= 3

Length of column= 60 cm

Reaction rate of protein k= 0.006 ml/mg.s

Rate of hydrolysis of PEG $k_{hydrolysis}$ = 0.003 1/s

Protein start and finish = 5 to 16 minutes

PEG start and finish = 16 to 46 minutes

Number of elements the column is divided in to X=round(L./(16.*Dp))

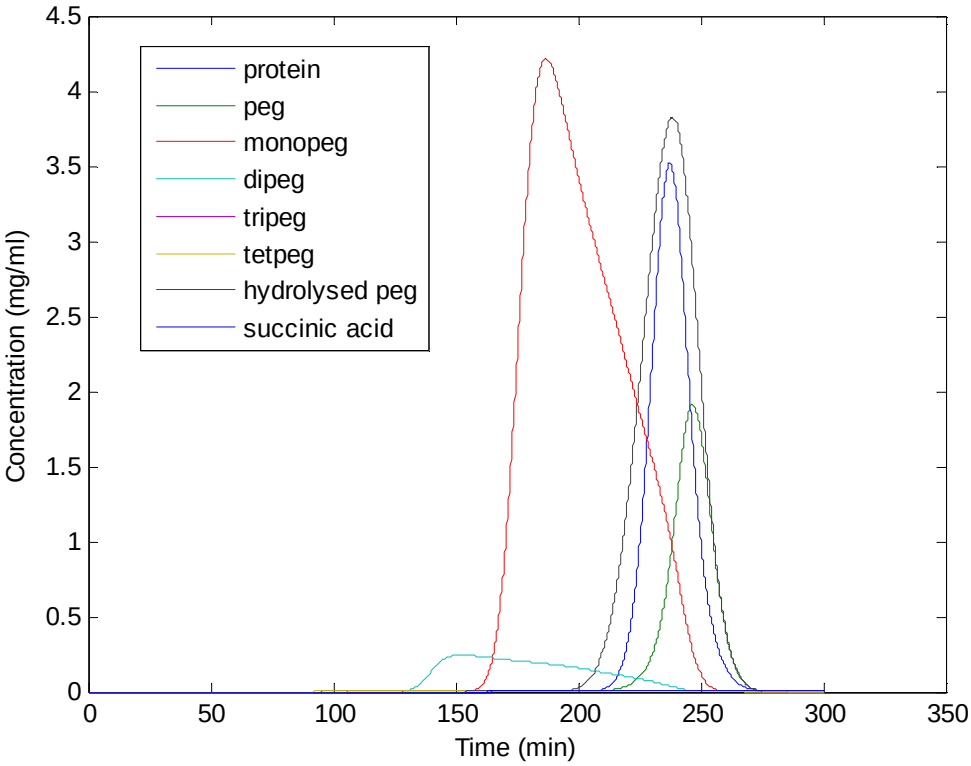


Figure 5-24: change in rate of hydrolysis of PEG

The increase in rate of hydrolysis of PEG rapidly decomposes the active PEG leading to lower conversion of proteins (Figure 5-24). Hence, in the case of higher rate of hydrolysis of PEG it is better to increase to concentration of PEG to make sure that there is adequate amount of active PEG available for PEGylation to take place.

7. Change in resolution

Variables:

$C_{protein}$ = 40mg/ml

C_{PEG} =.15mg/ml

Superficial velocity U=0.15cm/min

Time t=300min

Time divider J= 3

Length of column= 60 cm

Reaction rate of protein k= 0.006 ml/mg.s

Rate of hydrolysis of PEG $k_{hydrolysis}$ = 0.003 1/s

Protein start and finish = 5 to 16 minutes

PEG start and finish = 16 to 46 minutes

Number of elements the column is divided in to X=round(L./(64.*Dp))

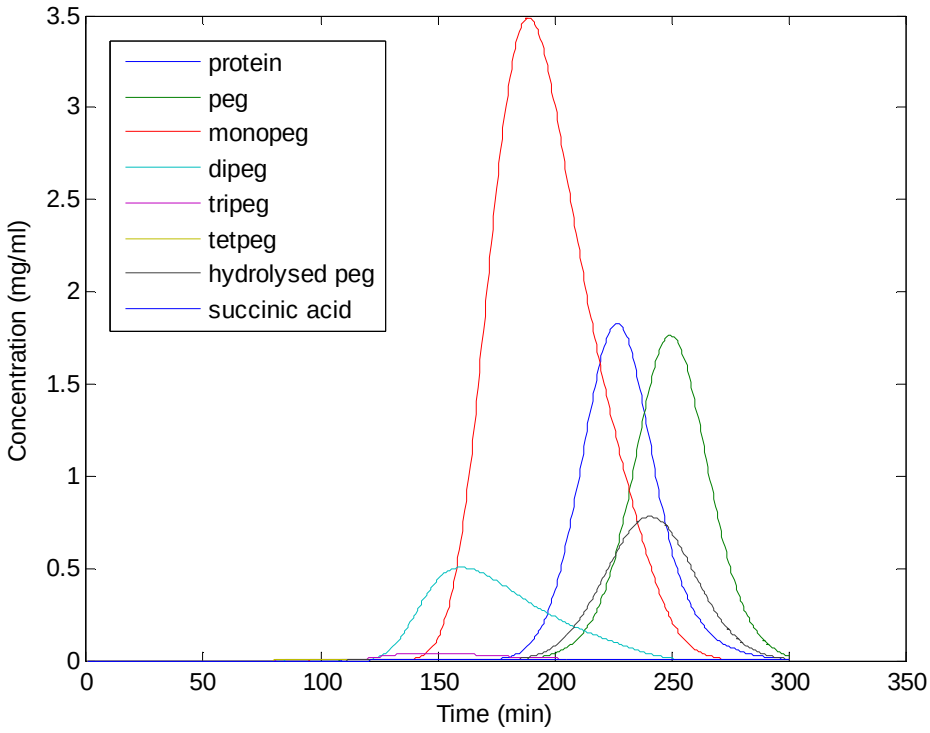


Figure 5-25: change in resolution at X=round(L./(64.*Dp))

At X=round(L./(8.*Dp))

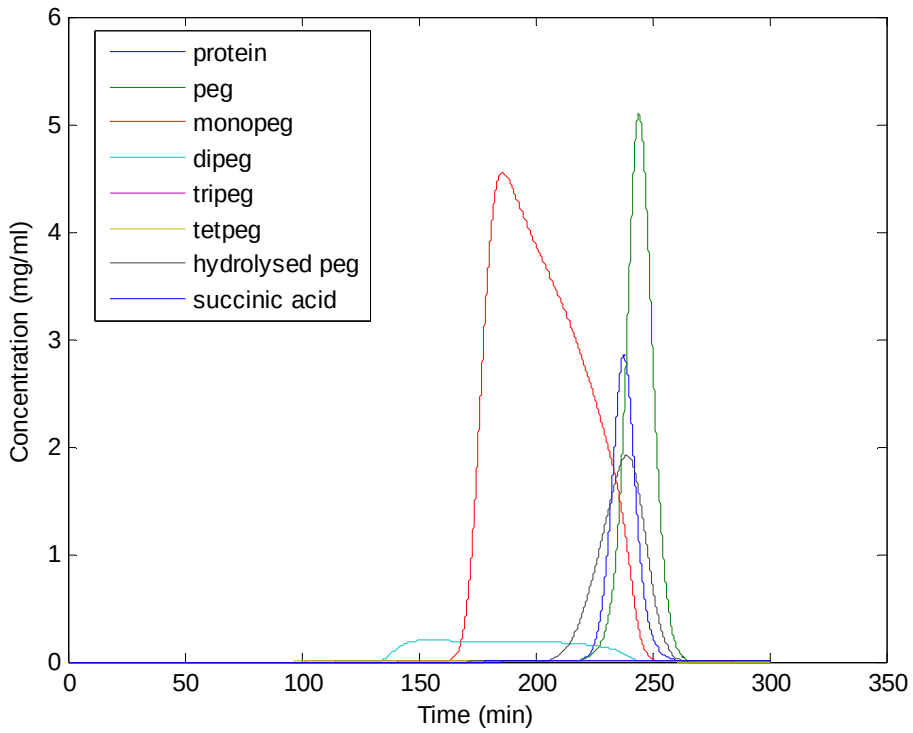


Figure 5-26: change in resolution at X=round(L./(8.*Dp))

The column resolution can be easily varied by varying the facto X (Number of elements the column is divided in to). Figure 5-25 and Figure 5-26 are the examples for the change in resolution.

5.4.4 Discussion from simulation

Controlling product formation is more difficult for batch processes than the axial flow size exclusion reaction process. The separation of products in the latter is much easier. In the axial flow model the PEGylated products move down the column quickly minimizing the possibility of higher degree of PEGylated products whereas it is hard to remove the mono PEGylated products from the batch process.

From the results obtained from the axial flow size exclusion column model, the suggested method for PEGylation is the introduction of highly concentrated PEG with small pulse lengths. This will give a good conversion of protein (Figure 5-27).

Variables

$C_{protein} = C_{PEG}$ = 40mg/ml

Superficial velocity U=0.15cm/min

Time t=300min

Time divider J= 3

Length of column= 60 cm

Reaction rate of protein k= 0.003 ml/mg.s

Protein start and finish = 5 to 16 minutes

PEG start and finish = 16 to 36 minutes

Number of elements the column is divided in to X=round(L./(16.*Dp))

Table 5-12: Mass of reaction components at pulse length of PEG= 20 min

Product	Mass (mg)
Protein in	47.124
PEG in	94.248
Unreacted protein	0.399
Unreacted PEG	36.797
MonoPEG	19.858
Di PEG	7.090
Tri PEG	0.955
Tet PEG	0.085
Hydrolysed PEG	2.708
Succinic acid	7.607

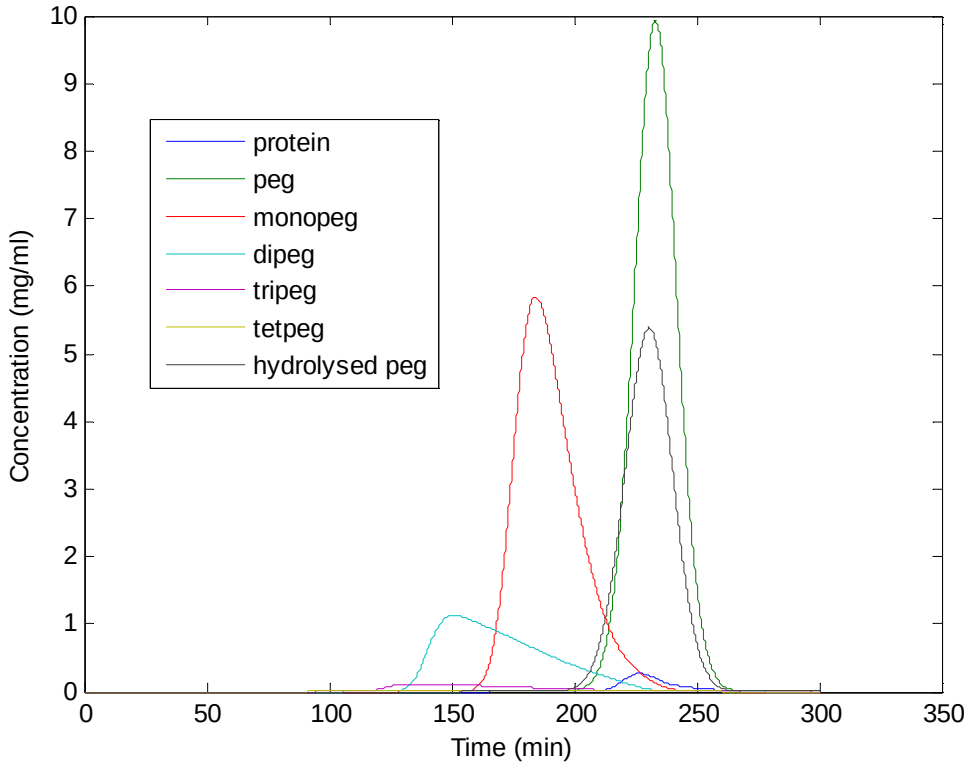


Figure 5-27: Example for the suggested method for PEGylation

Chapter 6

Conclusions and Recommendations

6.1 General findings

Size exclusion PEGylation reaction chromatography was investigated using a model developed by Fee (2005). Column dispersion was neglected and the PEGylation reaction was modelled as second order. The model allowed up to four PEG groups to be attached to a protein and accounted for succinic acid hydrolysis from activated PEG. The model was adapted to simulate α-lactalbumin PEGylation and succinic acid hydrolysis from activated PEG in a batch stirred tank so rate parameters from stirred tank kinetic experiments could be obtained and the model verified. The model was solved using finite differences and simulations run in Matlab. The effect of reaction parameters such as timing, length and concentration of PEG and protein injection, reaction rates, and model resolution on model simulation results was explored.

Succinic acid hydrolysis data showed little difference between using 2kDa and 20kDa PEG in reaction rates indicating that the mechanism for succinic acid hydrolysis from PEG is unaffected by the PEG size or type. A high degree of succinic acid hydrolysis was observed early in both experiments, which suggested either a systematic experimental error or that succinic acid had already hydrolyzed from the PEG prior to the beginning of the reaction. A good model fit was obtained when initial succinic acid hydrolysis was accounted for, showing that the model was suitable for simulating succinic acid hydrolysis from activated PEG.

Analysis of α-lactalbumin PEGylation results showed that PEGylation after one or two PEG groups have been attached is largely sequential. MonoPEG and DiPEG

peaked at 5 minutes followed a second peak for DiPEG at 30 minutes while TriPEG was still forming and had not peaked. Further PEGylation of protein is hindered by the presence of attached PEG groups, either because they form a shield preventing access of the activated PEG to the surface or that residues used for attachment had already been occupied. Reaction rates for native protein and MonoPEG was determined by mathematical analysis using Matlab. Rates for MonoPEG formation were faster than DiPEG formation assuming PEG attachment was sequential. Rates obtained were used in size exclusion PEGylation reaction simulations.

In the simulations it was found that controlling product formation was more difficult for the batch process than the axial flow size exclusion reaction process. In the size exclusion PEGylation simulations it was found that increasing protein concentration increased MonoPEG concentrations and increased the ratio of MonoPEG to starting protein feed concentration. Increasing PEG pulse length and starting PEG concentration initially increased MonoPEG concentration and product ratio until all protein had been PEGylated at which point MonoPEG concentration the product ratio levelled out. Increasing PEG hydrolysis rates did not affect the amount of MonoPEG produced but reduced the activated PEG concentration and increased succinic acid concentration. Optimal conditions for producing MonoPEG were found to be equal concentrations of PEG and protein, with the PEG injection length twice as long as the protein injection, and the PEG injection done immediately after the protein injection.

6.2 Recommendations for future work

The following is recommended for future work:

- Need to identify if succinic acid hydrolysis had occurred prior to batch experiments as reduced activated PEG concentration will impact on PEGylation reaction reducing yields and affecting fitting of models to data.

- Batch PEGylation reaction experiments should be repeated using larger or different types of PEG and different types of protein to investigate if PEGylation reaction results are repeatable, to identify any errors in analysis, and to determine if one model is suitable for a range of PEGylations reactions.

- Perform experiments to verify the optimum size exclusion PEGylation reaction conditions found in the model simulations.

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Appendix A: Matlab models

A.1 Axial flow size exclusion reaction column

```
%Column properties

L=60;                   % length of column (cm)
D=1;                    % diameter of column (cm)
Area=(pi.*D.^2)/4;      % (cm^2)
V=Area.*L;              % volume (cm^3)
Dp=0.01;                % particle diameter (cm)
X=round(L./(16.*Dp));   % number of elements column is divided into
delx=L./X;              % length of an element (cm)
Ve=V./X;                % volume of an element (cm^3)
e=1;                    % void fraction

%Flowrate

U=0.15;                 % superficial velocity (cm/min)
Q=U.*Area;              % flowrate (cm^3/min)

%Time

time=300;               % (min)
res=(Ve.*0.4)./Q;       % residence time (min)
J=3;                    % time divider
delt=res./J;            % change in time for a time step
T=ceil(time./delt);     % total number of time steps

%Component properties

k=0.003;                % reaction rate for protein (ml/mg.s)
k1=0.0025;              % reaction rate for mono PEG (ml/mg.s)
k2=0.002;               % reaction rate for di PEG (ml/mg.s)
k3=0.0016;              % reaction rate for tri PEG (ml/mg.s)
khydr=0.001;            % PEG hydrolysis rate (1/s) [first order]
```

```
%Retarding factors

Kavc=0.6;
KavPEG=0.5;
KavMONO=0.4;
KavDI=0.3;
KavTRI=0.25;
KavQU=0.23;
Kavshydr=0.9;


%Molecular weights

MWpeg=5000;              % (g/mol)
MWs=50;                  % (g/mol)


%Starting concentrations

cfeed=40;                %(mg/ml)
PEGfeed=40;              %(mg/ml)
MONOfeed=0;              %(mg/ml)
DIfeed=0;                %(mg/ml)
TRIfeed=0;               %(mg/ml)
QUfeed=0;                %(mg/ml)


%Boundary conditions


cstart=5;
cfin=15;
PEGstart=16;
PEGfin=36;


%99 percent pure monopeg protein

%cstart=1;
%cfin=9;
%PEGstart=10;
%PEGfin=12;


%Calculating concentrations


for t=1:T+1;
   for x=1:X+1;
      c(t,x)=0;
```

```
      cPEG(t,x)=0;
      cMONO(t,x)=0;
      cDI(t,x)=0;
      cTRI(t,x)=0;
      cQU(t,x)=0;
      cPEGhydr(t,x)=0;
      cshydr(t,x)=0;
   end
end

for t=2:T+1;
   trun(t)=(t-1).*delt;
   for x=1;
      if (trun(t)>cstart & trun(t)<=cfin);
         c(t,x)=cfeed;
      end
      if (trun(t)>PEGstart & trun(t)<=PEGfin);
         cPEG(t,x)=PEGfeed;
      end
   end
end

for t=2:T+1;
   for x=2:X+1;
      c(t,x)=c(t-1,x)+(((c(t-1,x-1)-c(t-1,x)).*Q.*delt)./(Ve.*e.*Kavc))-(k.*c(t-
1,x).*cPEG(t-1,x).*delt);
      cPEG(t,x)=cPEG(t-1,x)+(((cPEG(t-1,x-1)-cPEG(t-
1,x)).*Q.*delt)./(Ve.*e.*KavPEG))-(k.*c(t-1,x).*cPEG(t-1,x).*delt)-(k1.*cPEG(t-
1,x).*cMONO(t-1,x).*delt)-(k2.*cPEG(t-1,x).*cDI(t-1,x).*delt)-(k3.*cPEG(t-
1,x).*cTRI(t-1,x).*delt)-(khydr.*cPEG(t-1,x).*delt);
      cMONO(t,x)=cMONO(t-1,x)+(((cMONO(t-1,x-1)-cMONO(t-
1,x)).*Q.*delt)./(Ve.*e.*KavMONO))+(k.*c(t-1,x).*cPEG(t-1,x).*delt)-(k1.*cPEG(t-
1,x).*cMONO(t-1,x).*delt);
      cDI(t,x)=cDI(t-1,x)+(((cDI(t-1,x-1)-cDI(t-
1,x)).*Q.*delt)./(Ve.*e.*KavDI))+(k1.*cPEG(t-1,x).*cMONO(t-1,x).*delt)-
(k2.*cPEG(t-1,x).*cDI(t-1,x).*delt);
      cTRI(t,x)=cTRI(t-1,x)+(((cTRI(t-1,x-1)-cTRI(t-
1,x)).*Q.*delt)./(Ve.*e.*KavTRI))+(k2.*cPEG(t-1,x).*cDI(t-1,x).*delt)-(k3.*cPEG(t-
1,x).*cTRI(t-1,x).*delt);
      cQU(t,x)=cQU(t-1,x)+(((cQU(t-1,x-1)-cQU(t-
1,x)).*Q.*delt)./(Ve.*e.*KavQU))+(k3.*cPEG(t-1,x).*cTRI(t-1,x).*delt);
      cPEGhydr(t,x)=cPEGhydr(t-1,x)+(((cPEGhydr(t-1,x-1)-cPEGhydr(t-
1,x)).*Q.*delt)./(Ve.*e.*KavPEG))+(khydr.*cPEG(t-1,x).*delt);
      cshydr(t,x)=cshydr(t-1,x)+(((cshydr(t-1,x-1)-cshydr(t-
1,x)).*Q.*delt)./(Ve.*e.*Kavshydr))+(((k1.*cPEG(t-1,x).*cMONO(t-
1,x).*delt)+(k2.*cPEG(t-1,x).*cDI(t-1,x).*delt)+(k3.*cPEG(t-1,x).*cTRI(t-
1,x).*delt)+(khydr.*cPEG(t-1,x).*delt)).*(MWs./(MWs+MWpeg)));
   end
end
```

```
Xgraph=trun(1:T+1);
Yc=c(1:T+1,X+1);
YPEG=cPEG(1:T+1,X+1);
YMONO=cMONO(1:T+1,X+1);
YDI=cDI(1:T+1,X+1);
YTRI=cTRI(1:T+1,X+1);
YQU=cQU(1:T+1,X+1);
YPEGhydr=cPEGhydr(1:T+1,X+1);
Yshydr=cshydr(1:T+1,X+1);


Mc=mean(Yc).*time.*Q;
MPEG=mean(YPEG).*time.*Q;
MMONO=mean(YMONO).*time.*Q;
MDI=mean(YDI).*time.*Q;
MTRI=mean(YTRI).*time.*Q;
MQU=mean(YQU).*time.*Q;
MPEGhydr=mean(YPEGhydr).*time.*Q;
Mshydr=mean(Yshydr).*time.*Q;


Massout=Mc+MPEG+MMONO+MDI+MTRI+MQU+MPEGhydr+Mshydr;
Massin=((cfin-cstart).*Q.*cfeed)+((PEGfin-PEGstart).*Q.*PEGfeed);


%Figure


plot(Xgraph,Yc,Xgraph,YPEG,Xgraph,YMONO,Xgraph,YDI,Xgraph,YTRI,Xgraph,
YQU,Xgraph,YPEGhydr,Xgraph,Yshydr);
ylabel('Concentration (mg/ml)');
xlabel('Time (min)');
legend('c','peg','monopeg','dipeg','tripeg','tetpeg','hydrolysed peg','succinic acid');


%Purity of Monopeg


%Peakstart=input('Peak start? ');
%Peakfinish=input('Peak finish? ');

%PeakVol=(Peakstart-Peakfinish).*Q;

%Ps=round(Peakstart./delt);
%Pf=round(Peakfinish./delt);

%cmass=mean(c(Ps:Pf, X+1)).*PeakVol;
```

```
%PEGmass=mean(cPEG(Ps:Pf, X+1)).*PeakVol;
%MONOmass=mean(cMONO(Ps:Pf, X+1)).*PeakVol;
%DImass=mean(cDI(Ps:Pf, X+1)).*PeakVol;
%TRImass=mean(cTRI(Ps:Pf, X+1)).*PeakVol;
%QUmass=mean(cQU(Ps:Pf, X+1)).*PeakVol;
%PEGhydrmass=mean(cPEGhydr(Ps:Pf, X+1)).*PeakVol;
%shydrmass=mean(cshydr(Ps:Pf, X+1)).*PeakVol;


%Masspeak=cmass+PEGmass+MONOmass+DImass+TRImass+QUmass+PEGhydr
mass+shydrmass;

%MONOpurity=MONOmass./Masspeak



%Save results to a datafile that can be imported into Microsoft Excel

%Ex(1:T+1,1)=Xgraph;
%Ex(1:T+1,2)=Yc;
%Ex(1:T+1,3)=YPEG;
%Ex(1:T+1,4)=YMONO;
%Ex(1:T+1,5)=YDI;
%Ex(1:T+1,6)=YTRI;
%Ex(1:T+1,7)=YQU;
%Ex(1:T+1,8)=YPEGhydr;

%csvwrite('peg.dat',Ex);

%Ex(1,1)=((cfin-cstart).*Q.*cfeed);                % total mass of feed protein (mg)
%Ex(2,1)=((PEGfin-PEGstart).*Q.*PEGfeed);      % total mass of feed PEG (mg)
%Ex(3,1)=Massin;                  % total mass fed in to the column (mg)
%Ex(4,1)=Massout;                 % total mass of the products (mg)
%Ex(5,1)=cfeed;                   % feed concentration of protein (mg/ml)
%Ex(6,1)=cfin-cstart;             % pulse length of protein
%Ex(7,1)=PEGfeed;                 % feed concentration of PEG (mg/ml)
%Ex(8,1)=PEGfin-PEGstart;         % pulse length of PEG


%Ex(1,2)=Mc;                      % mass of unreacted protein (mg)
%Ex(2,2)=MPEG;                    % mass of unreacted PEG (mg)
%Ex(3,2)=MMONO;                   %  mass of mono PEG (mg)
%Ex(4,2)=MDI;                     % mass of di PEG (mg)
%Ex(5,2)=MTRI;                    % mass of tri PEG (mg)
%Ex(6,2)=MQU;                     % mass of tetra PEG (mg)
%Ex(7,2)=MPEGhydr;                % mass of hydrolyzed PEG (mg)
%Ex(8,2)=Mshydr;                  % mass of succinic acid (mg)
```

```
% Product ratio of components


%Ex(1,3)=Mc./((cfin-cstart).*Q.*cfeed);
%Ex(2,3)=MPEG./((PEGfin-PEGstart).*Q.*PEGfeed);
%Ex(3,3)=MMONO./((cfin-cstart).*Q.*cfeed);
%Ex(4,3)=MDI./((cfin-cstart).*Q.*cfeed);
%Ex(5,3)=MTRI./((cfin-cstart).*Q.*cfeed);
%Ex(6,3)=MQU./((cfin-cstart).*Q.*cfeed);
%Ex(7,3)=MPEGhydr./((PEGfin-PEGstart).*Q.*PEGfeed);
%Ex(8,3)=Mshydr./((PEGfin-PEGstart).*Q.*PEGfeed);

%csvwrite('pegmass.dat',Ex);


%Doing animation


figure;


Step=100;                          % number of steps for graphing

S=(T+1)./Step;

for x=1:X+1;
   dist(x)=(x-1).*delx;
end

for s=1:Step;
   ts=round(s.*S);
   AXgraph=dist(1:X+1);
   AYc=c(ts,1:X+1);
   AYPEG=cPEG(ts,1:X+1);
   AYMONO=cMONO(ts,1:X+1);
   AYDI=cDI(ts,1:X+1);
   AYTRI=cTRI(ts,1:X+1);
   AYQU=cQU(ts,1:X+1);
   AYPEGhydr=cPEGhydr(ts,1:X+1);
   AYshydr=cshydr(ts,1:X+1);

plot(AXgraph,AYc,AXgraph,AYPEG,AXgraph,AYMONO,AXgraph,AYDI,AXgrap
h,AYTRI,AXgraph,AYQU,AXgraph,AYPEGhydr);
   axis([0 60 0 50]);
   ylabel('Concentration (mg/ml)');
   xlabel('Distance (cm)');
   legend('c','peg','monopeg','dipeg','tripeg','tetpeg','hydrolysed peg');
   pause(0.1);
end
```

A.2 Continuous stirred batch model

```
%Batch simulation model

%Volume of beaker

V=25;                       % (ml)

%Time

time=180;                   % (min)
delt=0.01;                  % change in time for a time step
T=ceil(time./delt);         % total number of time steps

%Component properties

k=0.003;                    % reaction rate for protein (ml/mg.s)
k1=0.0025;                  % reaction rate for mono PEG (ml/mg.s)
k2=0.002;                   % reaction rate for di PEG (ml/mg.s)
k3=0.0016;                  % reaction rate for tri PEG (ml/mg.s)
khydr=0.001;                % PEG hydrolysis rate (1/s) [first order]
%Molecular weights

MWpeg=5000;                 % (g/mol)
MWs=50;                     % (g/mol)


%Boundry conditions


for t=1:T+1;

      c(t)=0;
      cPEG(t)=0;
      cMONO(t)=0;
      cDI(t)=0;
      cTRI(t)=0;
      cTET(t)=0;
      cPEGhydr(t)=0;
      cshydr(t)=0;

end


%Starting concentrations

for t=1;
   c(t)=20;                 % (mg/ml)
   cPEG(t)=40;              % (mg/ml)
```

```
   cMONO(t)=0;              % (mg/ml)
   cDI(t)=0;                % (mg/ml)
   cTRI(t)=0;               % (mg/ml)
   cTET(t)=0;               % (mg/ml)
   cPEGhydr(t)=0;           % (mg/ml)
   cshydr(t)=0;             % (mg/ml)
end



%Calculating concentrations


for t=2:T+1;
      trun(t)=(t-1).*delt;
      c(t)=c(t-1)-(k.*c(t-1).*cPEG(t-1).*delt);
      cPEG(t)=cPEG(t-1)-(k.*c(t-1).*cPEG(t-1).*delt)-(k1.*cPEG(t-1).*cMONO(t-
1).*delt)-(k2.*cPEG(t-1).*cDI(t-1).*delt)-(k3.*cPEG(t-1).*cTRI(t-1).*delt)-
(khydr.*cPEG(t-1).*delt);
      cMONO(t)=cMONO(t-1)+(k.*c(t-1).*cPEG(t-1).*delt)-(k1.*cPEG(t-
1).*cMONO(t-1).*delt);
      cDI(t)=cDI(t-1)+(k1.*cPEG(t-1).*cMONO(t-1).*delt)-(k2.*cPEG(t-1).*cDI(t-
1).*delt);
      cTRI(t)=cTRI(t-1)+(k2.*cPEG(t-1).*cDI(t-1).*delt)-(k3.*cPEG(t-1).*cTRI(t-
1).*delt);
      cTET(t)=cTET(t-1)+(k3.*cPEG(t-1).*cTRI(t-1).*delt);
      cPEGhydr(t)=cPEGhydr(t-1)+(khydr.*cPEG(t-1).*delt);
      cshydr(t)=cshydr(t-1)+(((k1.*cPEG(t-1).*cMONO(t-1).*delt)+(k2.*cPEG(t-
1).*cDI(t-1).*delt)+(k3.*cPEG(t-1).*cTRI(t-1).*delt)+(khydr.*cPEG(t-
1).*delt.*(MWs./(MWs+MWpeg)))));

end

Xgraph=trun(1:T+1);
Yc=c(1:T+1);
YPEG=cPEG(1:T+1);
YMONO=cMONO(1:T+1);
YDI=cDI(1:T+1);
YTRI=cTRI(1:T+1);
YTET=cTET(1:T+1);
YPEGhydr=cPEGhydr(1:T+1);
Yshydr=cshydr(1:T+1);


figure;
plot(Xgraph,Yc,Xgraph,YPEG,Xgraph,YMONO,Xgraph,YDI,Xgraph,YTRI,Xgraph,
YTET,Xgraph,YPEGhydr,Xgraph,Yshydr);
ylabel('Concentration (mg/ml)');
xlabel('Time (min)');
legend('c','peg','monopeg','dipeg','tripeg','tetpeg','hydrolysed peg','succinic acid');
```

```
% Mass of products

Mc=c(1+T);
MPEG=cPEG(1+T);
MMONO=cMONO(1+T);
MDI=cDI(1+T);
MTRI=cTRI(1+T);
MTET=cTET(1+T);
MPEGhydr=cPEGhydr(1+T);
Mshydr=cshydr(1+T);


%Save results to a datafile that can be imported into Microsoft Excel

Ex(1:T+1,1)=Xgraph;
Ex(1:T+1,2)=Yc;
Ex(1:T+1,3)=YPEG;
Ex(1:T+1,4)=YMONO;
Ex(1:T+1,5)=YDI;
Ex(1:T+1,6)=YTRI;
Ex(1:T+1,7)=YTET;
Ex(1:T+1,8)=YPEGhydr;
Ex(1:T+1,9)=Yshydr;

csvwrite('peg.dat',Ex);


%Ratio MONOPEG to DIPEG

%R=MMONO./MDI;
%Conv=(c(1)-c(T+1))./c(1);
%P=Conv.*100;

%Ex(1,1)=cPEG(1);
%Ex(2,1)=MPEG;
%Ex(3,1)=MMONO;
%Ex(4,1)=MDI;
%Ex(5,1)=MTRI;
%Ex(6,1)=MTET;
%Ex(7,1)=MPEGhydr;
%Ex(8,1)=Mshydr;
%Ex(9,1)=Mc;
%Ex(10,1)=P;


%csvwrite('Batch.dat',Ex);
```

Appendix B

Varied protein concentration

Table B-1: results of simulation by varying the concentration of proteins

Concentration of protein (mg/mL)	5	10	15	20	25	30	35	40
Protein	0.031	0.102	0.262	0.604	1.280	2.490	4.425	7.185
PEG	36.828	29.723	23.289	17.585	12.71	8.754	5.747	3.616
MonoPEG	1.783	3.891	6.303	8.975	11.814	14.662	17.313	19.592
DiPEG	1.259	2.368	3.298	4.016	4.495	4.73	4.758	4.650
TriPEG	0.236	0.393	0.483	0.516	0.508	0.475	0.433	0.394
TetPEG	0.025	0.037	0.040	0.038	0.033	0.029	0.025	0.022
Hydrolysed PEG	1.979	1.706	1.465	1.255	1.076	0.927	0.807	0.711
Succinic acid	0.966	1.910	2.738	3.312	3.519	3.340	2.871	2.273

Table B-2: product ratio of components at varied concentration of proteins

Mass	5	10	15	20	25	30	35	40
Protein	0.005	0.008	0.014	0.025	0.043	0.070	0.107	0.152
PEG	0.781	0.630	0.494	0.373	0.269	0.185	0.121	0.076
MonoPEG	0.302	0.330	0.356	0.380	0.401	0.415	0.420	0.416
DiPEG	0.214	0.201	0.186	0.170	0.152	0.133	0.115	0.098
TriPEG	0.040	0.033	0.027	0.022	0.017	0.013	0.010	0.008
TetPEG $x10^{-3}$	4.4	3.2	2.3	1.6	1.139	0.818	0.61	0.469
Hydrolysed PEG $x10^{-3}$	42.009	36.212	31.1	26.641	22.843	19.683	17.12	15.093
Succinic acid $x10^{-3}$	20.498	40.542	58.11	70.285	74.689	70.884	60.92	48.251

Increasing PEG pulse

Table B-3: results of simulations at different PEG pulse length (C_{PEG}= 10mg/mL)

Pulse length (min)	10	20	30	40	50	60	70
Protein	34.067	22.553	14.816	11.822	11.158	11.071	11.063
PEG	0.131	0.770	4.153	11.956	22.451	33.472	44.368
MonoPEG	7.920	14.544	18.613	19.966	20.223	20.254	20.257
DiPEG	0.452	1.207	1.970	2.406	2.526	2.543	2.544
TriPEG	0.010	0.038	0.081	0.119	0.134	0.136	0.137
TetPEG $x10^{-3}$	0.151	0.785	2.072	3.724	4.656	4.862	4.884
Hydrolysed PEG	0.115	0.393	0.830	1.365	1.938	2.508	3.065
Succinic acid	0.075	0.624	2.112	3.406	3.842	3.911	3.918

Table B-4: results of simulations at different PEG pulse length (C_{PEG}= 20mg/mL)

Pulse length (min)	10	30	50	70	90
Protein	23.044	3.0916	2.5256	2.5226	2.5226
PEG	0.446	22.13	65.982	110.06	139.45
MonoPEG	13.712	21.953	21.969	21.969	21.969
DiPEG	1.547	4.886	5.080	5.080	5.080
TriPEG	0.068	0.426	0.482	0.483	0.483

Table B-5: product ratios at different PEG pulse length (C_{PEG}= 10mg/mL)

Pulse length (min)	10	20	30	40	50	60	70
Protein	0.722	0.478	0.314	0.250	0.236	0.235	0.234
PEG	0.011	0.032	0.117	0.253	0.381	0.473	0.538
MonoPEG	0.168	0.308	0.395	0.423	0.429	0.429	0.429
DiPEG	0.009	0.025	0.041	0.051	0.053	0.054	0.054
TriPEGx 10^{-3}	0.219	0.823	1.728	2.53	2.846	2.9	2.9

Table B-6: product ratios at different PEG pulse length (C_{PEG}= 20mg/mL)

Pulse length (min)	10	30	50	70	90
Protein	0.489	0.065	0.053	0.053	0.053
PEG	0.018	0.313	0.560	0.667	0.657
MonoPEG	0.291	0.465	0.466	0.466	0.466
DiPEG	0.033	0.103	0.107	0.107	0.107
TriPEG	0.001	0.009	0.010	0.010	0.010

Table B-7: results of simulations at different PEG pulse length (C_{PEG}= $C_{Protein}$= 40mg/mL)

Mass	10	11	12	13	14	20
Protein	7.185	5.202	3.698	2.607	1.843	0.398
PEG	3.616	5.298	7.4503	10.056	13.068	36.797
MonoPEG	19.592	20.087	20.338	20.413	20.381	19.858
DiPEG	4.650	5.176	5.641	6.032	6.344	7.089
TriPEG	0.394	0.467	0.542	0.617	0.688	0.954
TetPEG	0.022	0.027	0.033	0.039	0.046	0.084
Hydrolysed PEG	0.711	0.862	1.029	1.208	1.399	2.707
Succinic acid	2.273	3.041	3.843	4.619	5.321	7.607

Table B-8: product ratios at different PEG pulse length (C_{PEG}= 40mg/mL)

Pulse length (min)	10	11	12	13	14	20
Protein	0.152	0.110	0.078	0.055	0.039	0.008
PEG	0.076	0.102	0.131	0.164	0.198	0.390
MonoPEG	0.415	0.426	0.431	0.433	0.432	0.421
DiPEG	0.098	0.109	0.119	0.128	0.134	0.150
TriPEG	0.008	0.009	0.011	0.013	0.014	0.020
TetPEG $x10^{-3}$	0.469	0.581	0.707	0.846	0.993	1.802
Hydrolysed PEG	0.015	0.016	0.018	0.019	0.021	0.028
Succinic acid	0.048	0.058	0.068	0.075	0.080	0.080

Rate of hydrolysis of PEG

Table B-9: results of simulations at different k hydrolysis

K hydrolysis (1/s)	0.00025	0.0005	0.001	0.0015	0.002	0.0025
Protein	0.398	0.443	0.549	0.677	0.830	1.010
PEG	36.797	34.458	30.18	26.392	23.043	20.089
MonoPEG	19.858	19.939	20.098	20.253	20.401	20.539
DiPEG	7.089	7.036	6.923	6.801	6.670	6.532
TriPEG	0.954	0.933	0.892	0.850	0.808	0.767
TetPEG	0.085	0.081	0.075	0.068	0.063	0.058
Hydrolysed PEG	2.707	5.279	10.044	14.349	18.246	21.778
Succinic acid	7.606	7.498	7.260	6.995	6.706	6.398

Table B-10: product ratios at different k hydrolysis of PEG

K hydrolysis (1/s)	0.00025	0.0005	0.001	0.0015	0.002	0.0025
Protein	0.008	0.009	0.011	0.014	0.017	0.021
PEG	0.390	0.365	0.320	0.280	0.244	0.213
MonoPEG	0.421	0.423	0.426	0.429	0.432	0.435
DiPEG	0.150	0.149	0.146	0.144	0.141	0.138
TriPEG	0.020	0.019	0.018	0.018	0.017	0.016
TetPEG $x10^{-3}$	1.802	1.729	1.59	1.46	1.34	1.23
Hydrolysed PEG	0.028	0.056	0.106	0.152	0.193	0.231
Succinic acid	0.080	0.079	0.077	0.074	0.071	0.067