STUDIES ON SITE-SPECIFIC PEGYLATION OF URICASE FROM *BACILLUS FASTIDIOUS* USING MPEG-DERIVATIVES

Thesis
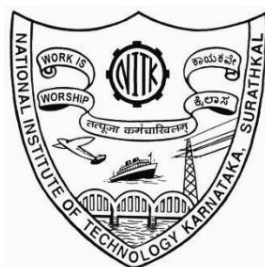
Submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

by

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D E C L A R A T I O N

I hereby *declare* that the Thesis entitled **"Studies on site-specific PEGylation of uricase from *Bacillus fastidious* using mPEG-derivatives"** which is being submitted to the **National Institute of Technology Karnataka, Surathkal** in partial fulfillment of the requirements for the award of the Degree of **Doctor of Philosophy** in the Department of Chemical Engineering, is a *bonafide report of the research work carried out by me*. The material contained in this Thesis has not been submitted to any University or Institution for the award of any degree.

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CERTIFICATE

This is to certify that the Thesis entitled **"Studies on site-specific PEGylation of uricase from *Bacillus fastidious* using mPEG-derivatives"** submitted by **Ms. Pooja Nanda (Register Number: 121161CH12F02)** as the record of the research work carried out by her, is *accepted as the Thesis submission* in partial fulfillment of the requirements for the award of degree of **Doctor of Philosophy**.

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DEDICATED TO MY MOTHER

ABSTRACT

Uricase (Urate oxidase) is a therapeutic enzyme which is administered for the treatment of hyperuricemic and gout patients. Uricases are considered supreme and most effective in the treatment of refractory gout (Yang et al. 2012). It is presently administered in its randomly conjugated/PEGylated form as Uricase-Polyethylene glycol (PEG) conjugate, however, it suffers from significant efficacy-related shortcomings. The present randomly PEGylated uricase formulations available in the market are Rasburicase and Pegloticase. Rasburicase ((Fasturtec[®]/ Elitek[®]) (a recombinant uricase from *Aspergillus flavus*) has a monthly dose of 10 mg/kg body weight and elicited an immune response (Vogt, 2005). Pegloticase (Krystexxa[®]) (recombinant mammalian uricase modified with methoxy-PEG) has a biweekly dosage of 0.14 mg/kg body weight (Sclesinger, 2011) and elicited an immune response against mPEG (Yue et al. 2008; Ganson et al. 2006).

Considering a very interesting case of site-specific PEGylated Interferon α-a, only 4 µg of weekly dosage was sufficient for the management of chronic Hepatitis C with reduced immunogenicity (Foster, 2010; Rodriguez-Torres et al. 2009). Hence, site-specific PEGylated uricase can prove to be an efficient alternate PEG therapeutic to overcome the demerits of the existing uricase therapeutics.

The present work encompassed the development of site-specific PEGylated uricase conjugates following the implementation of two different second generation PEGylation strategies namely thiol and N-terminal PEGylation. mPEG-maleimide and mPEG-propionaldehyde were used as PEGylating reagents for thiol and N-terminal PEGylation respectively. The PEGylation reaction conditions which influenced the yield of the site-specific uricase conjugates were optimized to achieve a higher conjugate yield and productivity. The uricase conjugates obtained were purified using ultrafiltration, gel filtration chromatography and size exclusion fast protein liquid chromatography (SE-FPLC). The purified uricase conjugates were characterized by their residual activity, the degree of modification, molecular weight, size, conformational changes, storage stability, kinetic and immunological properties.

This is the first report on synthesis of site-specific uricase conjugates and optimization of PEGylation reaction conditions for their production. The PEGylated uricase conjugates obtained by both thiol and N-terminal PEGylation strategies possessed better storage stability and residual activities in comparison to the residual activities possessed by PEGylated uricase conjugates reported till date. The site-specific uricase conjugates also displayed a 60-70 % reduction in immunogenicity compared to native uricase. The conjugates synthesized in the present study appeared to have beneficial and long-acting uricolytic effects for curing hyperuricemia and gout in comparison to the random PEGylated uricase. This site-specific uricase can be a potential conjugate for further studies related to characterization and immunological studies.

Keywords: Uricase, site-specific PEGylation, stability, residual activity, immunogenicity

LIST OF ABBREVATIONS

^{1}H NMR	Proton Nuclear Magnetic Resonance
ACN	Acetonitrile
ALD	Aldehyde
ANOVA	Analysis of variance
CD	Circular dichroism
CM	Carboxymethyl
CPCSEA	Committee for the Purpose of Control and Supervision of Experiments on Animals
DEAE	Dimethylamino ethyl
DEPE	Diethyl aminopropyl
DTNB	5,5-dithio-bis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
EC	Enzyme commission
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immune sorbate assay
EMEA	European Medicines Agency
EPR	Enhanced permeation and retention
FDA	Food and Drug Association
GCSF	Granulocyte-colony stimulating factor
GRAS	Generally Recognized as Safe
HCl	Hydrochloric acid
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HRP	Horse radish peroxidase
IEC	Ion-exchange chromatography
IFN	Interferon α-2a
Ig	Immunoglobulin
KMC	Kasturba Medical College

mPEG	Methoxy polyethylene glycol
mPEG-mal	Methoxy polyethylene glycol-maleimide
mPEG-np	Methoxy(polyethylene glycol)-*p*-nitrophenyl carbonate
mPEG-prop	Methoxy polyethylene glycol-propionaldehyde
MWCO	Molecular weight cutoff
NHS	N-hydroxy succinimidyl
NPC	Nitrophenyl carbonate group
-Osu	Succinimidyl esters
OVAT	One variable at a time
PBS	Phosphate buffered saline
PDB	Protein Data Bank
PDI	Polydispersity index
PEG	Polyethylene glycol
PEG-IA	PEG-iodoacetamide
PEG-OPDS	PEG-ortho pyridyl disulfide
PEG-VS	PEG-vinyl sulfone
PSA	Polysialic acid
RP-HPLC	Reverse Phase-High Performance Liquid Chromatography
RPM	Rotations per minute
RT	Room Temperature
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide electrophoresis
SE-FPLC	Size-Exclusion-Fast Protein Liquid Chromatography
SE-HPLC	Size-Exclusion-High Performance Liquid Chromatography
SEM	Standard error of mean
SPB	Sodium phosphate buffer

SSP	Site-specific PEGylation
TCEP	Tris(2-carboxyethyl)phosphine
TFA	Trifluro acetic acid
TLS	Tumor lysis syndrome
TNBS	Tri-nitro benzene sulfonic acid
Uc	Uricase from *Bacillus fastidious*
UV	Ultra-violet
WFI	Water for Injection

NOMENCLATURE

Symbol	Description
°C	Celsius
μg	Microgram
Å	Angstrom
Đ	Heterogeneity index
hr	Hour
kDa	Kilo Dalton
kg	Kilogram
mg	Milligram
min	Minute
mL	Milli liter
Mol	Molar
N	Normal
ng	Nanogram
-NH$_2$	Amine group
Nm	Nanometer
R^2	Correlation coefficient
U/mg	Units per milligram
U/mL	Units per milli liter
V	Volt
v	Volume

1 Introduction

1.1 Background of the research

Human being is always at a risk of acquiring or developing diseases and health disorders in the form of metabolic, cancerous, neurodegenerative, autoimmune disorders. It is become indispensable to treat these disorders with the aid of therapeutic drugs and proteins (particularly enzymes, antibodies and peptides). Many proteins are recognized to be directly or indirectly involved in the functioning of various metabolic pathways, hence they can be considered as potential sources in addressing disease development. When a protein interacts with a cellular target, it alters dysfunctional cellular processes and thus can prove to be potentially beneficial as a therapeutic agent. However, clinical applications of several of such proteins are inadequate because of their diminutive plasma half-life, immunogenicity, *invivo* proteolytic decay, reduced solubility (Roberts et al. 2002) and high price (Yoshioka et al. 2011).

A number of drug delivery systems have been established in the recent ages to overcome the inherent demerits of the therapeutic agents and to enhance the pharmaceutical properties of therapeutic agents. Drug delivery systems offer many advantages like the ability to transport a drug molecule more selectively to a specific site, decreased variability in systemic drug concentrations, less frequent drug dosing, consistent rate of absorption and reductions in the generation of toxic metabolites (Robinson and Mauger, 1991). Drug delivery systems are developed based on different strategies like manipulation of amino acid chains in a therapeutic protein molecule, a fusion of the therapeutic agent with serum proteins such as albumin and immunoglobulin and preparation of complexes by conjugating the therapeutic proteins with natural or synthetic polymers. Usage of these drug delivery strategies can help in achieving desired encapsulation and slow drug release (Filpula and Zhao, 2008).

Out of many types of drug delivery systems, delivery of therapeutics using various polymers is of emerging interest in pharmaceutical chemistry. This is predominantly for the delivery of synthetic and natural drugs like peptides, enzymes and oligonucleotides (Duncan et al. 2003). Various polymers like biodegradable/non-

degradable, synthetic/natural polymers are being used for the development of micelles and polymersomes (polymeric vesicles) as drug delivery vehicles. The potential advantages of developing polymer therapeutics are: protection of labile drugs from proteolytic or chemical degradation, reduction in drug induced-immunogenicity, decreased antibody recognition, increased plasma half-life and new prospects of drug targeting (Veronese and Morpuro, 1999). In order to develop new polymeric carriers for the purpose of drug delivery, the suitability of many polymers have been tested with respect to their biocompatibility and efficacy. For example polyethylene glycol (PEG) (Abuchowski et al. 1977), polysialic acid (Fernandes and Gregoriadis, 2001), poly-acryloyl-morphine (Schiavon, 2000), hydroxyethyl starch (Treetharnmathurot et al. 2009), poly-2-methacryloyloxyethyl phosphoryl-choline (Lewis et al. 2008), hyaluronic acid (Oh et al. 2010), poly-vinylpyrrolidone (Kaneda et al. 2004), chitosan (Qian et al. 2006), silk fibroin (Zhang, 2005), poly(*N*-(2-hydroxypropyl) meth acrylamide (Tao et al. 2009) etc have been tested. Potential polymers for drug delivery systems have been verified for the synthesis of polymer-protein conjugates as reported in literature.

Along with the variety of polymer used, many conjugation chemistries have been explored with regard to the synthesis of polymer-protein conjugates. Broadly, the conjugation interactions can be classified as the first generation (random) and second generation (site-specific) conjugation. The first generation conjugates (random) can be prepared by attachment of polymers arbitrarily to surface accessible amino acids of a protein (for example, attachment to the lysine residues). However, this conjugation approach has less selectivity, limited applicability and is exclusive to polymers with lesser molar mass (Roberts et al. 2002). Random conjugation also results in loss of biological activity and unpredictable *invivo* behavior (Pasut and Veronese, 2007). Therefore strategies for second generation conjugation, (the site-specific approach of protein modification) which depend on the terminal functional group of polymer and target specific sites on the protein surface have gained enough importance in the recent years (Zalipsky, 1995). Site-specific conjugation has added advantages like enhanced control over the ligation process, minimal loss in biological activity, robustness, applicability to a wide range of labels and modifications, reduced immunogenicity, homogeneity of conjugates and reduced dosage of drugs.

Among all the polymers mentioned earlier, PEG is a highly investigated polymer (by the process named PEGylation) for the covalent modification of biological molecules (Roberts et al. 2002). However, randomly conjugated PEGylated therapeutics have been reported to be associated with many shortcomings like reduced bioactivity, adverse immunological responses, accumulation of PEG in several internal organs leading to vacuoles formation. In order to beat these shortcomings, site-specific PEGylation strategies are being employed as promising alternatives in which specific amino acids are coupled with terminally functionalized PEG polymers. Site-specific PEGylated conjugates are established to retain their original biological activity, display reduced immunogenicity, augmented plasma half-life and exhibit negligible organ accumulation in comparison to randomly PEGylated proteins (Foster et al. 2010). Also, it reduces the PEG-based immunogenicity as the number of PEG strands are attached to the protein molecule is very less (Barz et al. 2011).

Given that the immunogenicity of PEG has been proven, in view of the increasing regulatory requirements in the existing pharmaceutical industry, the reproducibility of site-specific PEGylated protein in terms of its yield is very important. The process development and the scale-up technology for the production of site-specific PEGylated protein therapeutics are of foremost prominence. The present study deals with the development of site-specific PEGylation technology for Uricase enzyme (EC 1.7.3.3; Class: oxidoreductases).

Uricase catalyzes the oxidation of uric acid to allantoin and thus supports the purine degradation pathway. Uricase is administered as a therapeutic protein for the treatment of hyperuricemia and gout. Uricase from microbial and animal origin is highly antigenic upon administered in patients and its prolonged usage consequently increases the risk of allergic responses and anaphylactic shock (Bomalaski et al. 2002). Native/unmodified uricase from *Aspergillus flavus* commercially exists for clinical use particularly in France and Italy under the brand name of Uricozyme® (Bomalaski et al. 2002). A recombinant variety of uricase named as Rasburicase® is available and is effective in the treatment of acute tumor lysis syndrome (Cannella and Mikuls, 2005). Pegloticase (Krystexxa®, formerly Puricase) is the randomly PEGylated uricase prescribed for the treatment of severe, refractory chronic gout which was previously developed by Savient Pharmaceuticals and currently manufactured by Crealta

Pharmaceuticals (USA). Pegsiticase (Uricase-PEG 20) is a modified pegylated recombinant uricase derived from *Candida utilis* is under development.

Though PEGylated uricase is a highly successful therapeutic biologic, there are several limitations associated with it. For example, the commercial available PEGylated uricase formulations like Rasburicase and Pegloticase have high dosage requirements. Rasburicase has a dose of 0.15-0.2 mg/kg once a day for 5 days (McDonnell, 2006) and rapidly elicited an immune response (Vogt, 2005). Pegloticase has a biweekly dose of 0.14 mg/kg body weight (Schlesinger, 2011), which is approximately 1.12 mg/kg body weight per month and elicited a quick immune response against methoxy-PEG (mPEG) (Yue et al. 2008). Considering a very interesting case of site-specific PEGylated Interferon α-a, only 4 μg of weekly dosage is sufficient for the treatment of chronic Hepatitis C with reduced immunogenicity (Foster, 2010; Rodriguez-Torres et al. 2009). Hence, development of site-specific PEGylated uricase can result in an improved form of uricase formulation for the treatment of gout.

The development of site-specific PEGylated uricase requires elaborate and in-depth knowledge about the existing randomly PEGylated uricase conjugates, primarily with respect to immunogenicity. The antigenicity of randomly PEGylated uricase was reported by Ganson et al. in 2006. Interestingly in their study, antibodies were produced against PEG itself rather than uricase, which indicates that PEG immunogenicity earns further exploration. It also has prospective implications for other PEGylated therapeutic representatives in clinical use. It can be concluded that reduction in the number of PEG strands attached can significantly reduce the PEG-induced immunogenicity, which can be accomplished through site-specific PEGylation. Yang et al. (2013) have reported that Pegloticase (recombinant mammalian uricase modified with mPEG), after sustained treatment for three months at a biweekly therapeutic dose of 0.14 mg/kg body weight had prompted an immune response against mPEG in nearly 20% of patients. Hence, site-specific PEGylated uricase can prove to be an efficient therapeutic conjugate capable of overcoming the demerits of the existing randomly PEGylated uricase therapeutics.

Based on the literature survey, it was observed that there is no data or reports based on the site-specific PEGylation of uricase using PEGylation reagents used in

the current study. In the present work PEGylating reagents namely mPEG-maleimide and mPEG-propionaldehyde were employed to PEGylate uricase obtained from *Bacillus fastidious* in a site-specific manner. PEGylation reaction conditions influencing the yield of PEGylated uricase conjugates were optimized to achieve a higher conjugate yield and productivity. The uricase conjugates thus obtained were purified using ultrafiltration, size exclusion-high performance chromatography and size exclusion-fast protein liquid chromatography and analyzed by SDS-PAGE. The purified conjugates obtained were further characterized for their residual activity, degree of modification, molecular weight, size, structural and conformational changes, storage stability, kinetic and immunological properties.

1.2 Hypothesis

Site-specific modification of uricase using polymers (like Polyethylene glycol) with required standards for pharmaceutical applications can prove to be a potent solution to overcome the demerits of randomly conjugated uricase therapeutics. In the present study, it is indicated that the problems encountered by the commercially available uricase therapies like low storage stability, high dosage levels, post-PEGylation immunogenicity and post-PEGylation loss of uricolytic activity can be overcome by PEGylating uricase in a site-specific manner.

The present thesis has been categorized into five chapters namely:

Chapter 1 presents the **Introduction** which discusses the background of research, need for the study and problem statement.

Chapter 2 presents detailed **Literature Review** which summarizes the relevant literature study carried out during the current research highlighting the main research gaps. This chapter is followed by the description of scope and objectives of the study, which were framed based on the key research questions.

Chapter 3 presents the **Materials and Methods** which describes and lists the chemicals, equipment, experimental methodologies and analytical procedures adopted to accomplish the set objectives.

Chapter 4 on **Results and Discussion** presents detailed discussion dealing with the obtained results and justification reinforced by the findings of this study with relevant literature support.

Chapter 5 deals with **Summary and Conclusions** of the present work along with the **future scope** for research.

2.1 Therapeutic enzymes and their limitations*:*

Therapeutic enzymes are used as medicine in their isolated or adjunct forms to treat many diseases effectively without several side-effects (McGrath and Walsh 2005). Enzymes as drugs have two significant features that differentiate them from all other types of drugs, primarily, they bind to their targets in a specific manner with great affinity and secondly they act as catalysts to convert the target molecules to explicit products. These two features qualify the therapeutic enzymes as potent drugs capable of altering the biochemistry of various pathways in the human body (Vellard, 2003). Additionally, an enzyme drug far exceeds synthetic drug in many contexts like reaction specificity, catalytic efficiency, and capability to operate under mild conditions of temperature and pH. These characteristics have resulted in the inquisitive development of many new enzyme drugs for treating a wide variety of diseases, where they are required in a relatively smaller quantities but with a higher degrees of purity and specificity.

A few examples of commercial therapeutic enzymes used for treating diseases are as follows: asparaginase (catalyzes the conversion of asparagine to aspartate and is used in the treatment of leukemia); collagenase (catalyzes collagen hydrolysis and used in the treatment of skin ulcers); hyaluronidase (catalyzes hyaluronate hydrolysis and used in the treatment of heart related aliments); lysosome (catalyzes the bacterial cell lysis and hence applied as an antibiotic); uricase (catalyzes conversion of uric acid to allantoin and hence used to treat hyperuricemia); β-lactamase (catalyzes the conversion of penicillin to penicilloate and thus diminishes penicillin allergy); streptokinase (catalyze the conversion of plasminogen to plasmin and used for dissolving blood clots) etc.

However, clinical applications of many of these proteins are limited owing to properties like short plasma half-life, immunogenicity, proteolytic decay, low solubility (Roberts et al. 2002) and high expense (Yoshioka et al. 2011). Additionally, therapeutic enzymes of animal and microbial origin are recognized as foreign antigenic bodies by the human immune system; they can elicit an immune response and consequently cause severe allergic reactions upon persistent dosage.

2.2 Progress in the development of various drug delivery systems for protein delivery:

Drug discovery and progress is a time-consuming, arduous and expensive process. The average budget for the developmental of a new drug (a new chemical entity) from a research laboratory to the patients is a staggering number of around $400 to $650 million (Bangham, 1964) and the entire process takes up to 14 years to complete (Nishikawa, 2002). It becomes imperative to scrutinize the entire drug development process, ascertain steps where changes can be incorporated in order to ameliorate and preserve the drug efficacy. A plethora of drug delivery systems has been established in the recent years to surpass the demerits of conventional drugs and enhance their pharmacological properties.

Delivery of a therapeutic protein to the systemic circulation and eventually to the site of action to produce a desired pharmacological effect is the ultimate goal of protein drug delivery. Implementation of these drug delivery strategies ensures desired encapsulation and slow drug release (Kodera et al. 1998; Filpula and Zhao, 2008). These protein drug delivery systems have been developed by strategies such as fusion with serum proteins like albumin/immunoglobulin, preparation of complexes by conjugation of proteins with natural or synthetic polymers, manipulation of amino acid chains in protein molecules etc.

Since structure dictates function, a simple alteration in the structure of a protein can directly trigger its functional changes such as signaling, targeting, catalysis, catabolism, modification of circulation time in the body and immunogenicity. There are diverse chemical reactions that modify the primary sequence of protein, namely phosphorylation (Hunter, 1995), acylation, methylation (Wood and Shilatifard, 2004), glycosylation (Varki, 1999) and sulfonylation/sulfation (Hemmerich, 2004). Many proteins in the body are present in its conjugated forms and investigators are now trying to replicate the same phenomenon for the modification of proteins from other sources using conjugation techniques with conjugating agents like Polyethylene glycol and other biocompatible polymers.

2.3 PEGylation:

PEGylation is a process of attaching PEG molecule [HO-$(CH_2CH_2O)_n$] to proteins, peptides and small molecules, is a highly successful strategy for improving their pharmaceutical properties. PEGylation was first validated in the year 1970 by Davies and Abuchowsky, who had modified albumin and catalase enzyme with PEG (Veronese and Pasut, 2005). Knop et al (2010) have listed the advantages and disadvantages of employing PEG as a conjugating agent for proteins and small molecule delivery, which are as follows:

Advantages

a) Molar mass: The molar mass of the PEGylating reagent is established to be a significant contributing factor towards the biocompatibility, stealth behavior, uniform *invivo* effects and kidney ultrafiltration rate. PEGylation reagents with a molar mass in the range of 20-50 kDa are frequently employed for the conjugation of low-molar-mass drugs such as small molecules, oligonucleotides, and siRNA. PEG reagents with lower molar masses (in the range of 1-5 kDa) are used for the conjugation of larger drug molecules such as antibodies/nanoparticulate systems, resulting in rapid renal clearance being circumvented size augmentation of the conjugates above the renal clearance threshold. In this way, opsonization, subsequent elimination by the RES (reticuloendothelial system), enzymatic degradation and cationic charges can be evaded. PEG (molecular weight 4 kDa) can be safely administrated intravenously at a dose level of 16 g/kg body weight in rats, guinea pigs, rabbits and monkeys (Carpenter et al., 1971).

b) Polydispersity Index: The polydispersity index (PDI) is the measure of the distribution of molecular mass in a given polymer sample and is calculated as the ratio of weight average molecular weight to the number average molecular weight. It is important to use reagents of narrow PDI range as highly polydisperse PEGylation reagents are associated with problems like heterogeneity, probability of cross-linking, occurrence of structurally determined fluctuations in quality, challenging analytics,

and product heterogeneity. The pharmacokinetic and biodistribution of such polymers cannot be expected to be uniform. Higher PDI of PEG reagents leads to a higher polydispersity of the PEGylated conjugates. PDI value of below 1.1 for the PEGylating agent results in an homogeneous PEGylated conjugate and homogeneity results in high reproducibility in terms of plasma half-life and lower immunogenicity of the carrier (Pasut and Veronese, 2007).

c) Solubility: PEGylating agents exhibit a very high solubility in organic solvents which facilitates end-group modifications of the proteins in a relatively easier manner. PEG is soluble in water and has a low intrinsic toxicity, which makes it completely suited for biological applications. Many therapeutic proteins possess low solubility and limited solubility at physiological pH, which are major drawbacks in terms of its applicability (Yang, 2013). PEGylated enzymes also allow bioconversion of substrates with low water solubility.

d) Reduced Immunogenicity: Masking the protein surface by PEG strands reduces the interaction of PEGylated drug within the circulatory system and results in reduced immunogenicity and antigenicity. Also, it results in masking of active sites of the enzyme. Through PEGylation, charges in the carrier systems are shielded resulting in decreased zeta-potential and lesser charge-induced interactions within the body. Consequently, conjugate recognition by the immune system induced opsonization is suppressed. Additionally, PEG has FDA (Food and Drug Association) approval for its usage in pharmaceutical products (Pasut and Veronese 2007) and has designated PEG as "Generally Recognized as Safe" (GRAS) compound.

e) Stability: PEGylation provides greater physical as well as thermal stability and additionally prevents drug aggregation at physiological conditions and during storage. PEGylation results in the formation of a "conformational cloud" around the protein molecule as a result of the steric hindrance/masking of surface charges caused by hydrophilic PEG strands. Enhanced thermostability and pH stability upon PEGylation have been reported by several researchers.

Disadvantages:

a) There are a few reports concerning PEG induced immunogenicity in the body. (Ganson et al. 2006)

b) An antagonism arises due to the non-biodegradability of PEG (Ulbricht et al. 2014)

c) Although PEG is the gold standard of polymers for drug delivery, it displays well-known sensitivity against oxidative degradation (Ulbricht et al. 2014).

d) Usage of PEG protein increases vacuole formation in animal tissues (liver and liver) (Bendele et al. 1998).

2.4 PEGylated proteins:

The most relevant feature changes in a protein molecule after PEGylation are size enlargement, surface charge alteration, protein surface cum glycosylation function masking and epitope shielding. Size enlargement delays kidney ultra-filtration and promotes drug accumulation into permeable tissues by the passive enhanced permeation and retention mechanism (EPR) (Caliceti and Veronese. 2003). Epitope shielding also enhances thermal stability, solubility and at the same time diminishes immunogenicity of the protein and consequently decreases the probability of inducing allergic reactions.

PEG is the most comprehensively investigated polymer for the covalent binding of biological molecules for various biotechnological applications (Roberts et al. 2002). However, PEGylated therapeutics have been reported to be associated with many shortcomings like adverse immunological responses, accumulation of PEG in some internal organs (Yamaoka et al. 1994) and formation of vacuoles (Viegas et al. 2011). Additionally, advancement in PEG-protein binding techniques like site-specific PEGylation is under investigation. Due to these shortcomings, various other polymers are now being investigated as alternative stealth polymers (Barz et al. 2011). **Table 2.1** gives a review of various commercially available PEGylated therapeutic proteins

Table 2.1: A review of various commercial PEGylated therapeutic proteins

Sl no	Protein	PEGylated conjugate (Brand Name)	Indication	Strategy of PEGylation	Year of approval	Reference
1	Adenosine deaminase	Pegademase®	Severe combined immunodeficiency	First generation	1990	Levy et al. 1988
2	Asparaginase	Oncaspar®	Leukemia	First generation	1990	Graham, 2003
3	Interferon alfa-2a	Pegasys®	Hepatitis C	First generation	2002	Wang et al., 2002
4	Interferon alfa-2b	Pegintron®	Hepatitis C	First generation	2000	Monkarsh et al., 2000
5	Granulocyte-CSF	Neulasta®	Neutropenia	Second generation	2002	Hak et al., 2004
6	Human growth hormone antagonist	Somavert®	Acromegaly		2002	Roelfsema et al. 2006
7	anti-vascular endothelial growth factor aptamer	Macugen®	Neovascular age-related macular degeneration		2004	Pasut and Veronese, 2007

Sl no	Protein	PEGylated conjugate (Brand Name)	Indication	Strategy of PEGylation	Year of approval	Reference
8	Epoetin beta	Mircera[®]	Anemia with chronic kidney disease		2007	Macdougall et al., 2005
9	Anti-TNFα-Fab-C terminal	Cimzia[®]	Rheumatoid arthritis	Second generation	2008	Blick and Curran. 2007
10	Uricase	Krystexxa[®]	Gout	First generation	2010	Sherman et al., 2008
11	Analog of erythropoietin	Omontys[®]	Anemia with chronic kidney disease	Second generation	2012	Fishbane et al., 2013

2.5 Development and modes of PEGylation process:

The production of PEGylated proteins involves covalent attachment of one or more PEG molecules to a native/unmodified protein and purification of the reaction mixture to recover the final PEGylated product (Veronese 2001). There are different strategies for PEGylation, which were evolved based on the type of group on protein surface.

2.5.1 First generation of PEGylation:

The potential of many PEGylating reagents have been recognized for efficient synthesis of PEG-protein conjugates through covalent bonding by random attachment of PEG strands to abundant and surface accessible amino acids of a protein molecule

by an approach known as ′First generation PEGylation′. In the first generation PEGylation, amino groups (mainly the α-amino/ε-amino groups) of the lysine residues are usual sites of PEG linkage (Veronese, 2001). Lysine residues on the protein surfaces are relatively abundant and are easily accessible for PEGylation.

Commonly used PEGylating agents are broadly categorized into two classes namely: Alkylating and acylating agents. Alkylating reagents do not modify the charge of the amino residues on the protein molecule, since the positive charge of amino group is not disturbed the biological activity of protein can be retained (Veronese, 2001). Few examples of alkylating PEGylating reagents are a) PEG aldehyde (for α and ε amino acids), b) tersylated PEG and c) PEG-epoxide. PEG-aldehyde gives a permanent linkage after Schiff base formation followed by a cyanoborohydride reduction which is a convenient way of PEGylation. PEG-tresyl chloride activation is an alternate method to maintain positive charge (Delgado et al. 1999).

Acylating PEGs are hydroxy succinimidyl esters (-Osu) of carboxylated PEGs in which the terminal PEG hydroxyl group is activated by chloroformates/carbonyl imidazole. A few examples of acylating agents are a) PEG-oxycarbonylimidazol, b) PEG-benzotriazole carbonate, c) PEG-p-nitrophenyl carbonate, d) PEG-triphenyl carbonate, e) PEG-oxy carbonyl imidazole, f) PEG-benzotriazole carbonate etc. These reagents exhibit lower reaction rates than the –Osu activated PEGs and these properties allow exploitation of different reactivity among the amino groups of the macromolecules by stopping the reactions at desired intervals of modifications. Reactive PEGylating agents like PEG-carboxylates and PEG-carbonates can also target another type of nucleophilic groups found in proteins such as the side chains of serine, threonine, tyrosine and histidine (Sivakolundu and Mabrouk, 2003;Orsatti and Veronese, 1999).

However, first generation PEGylation exhibits numerous shortcomings which are as follows: a) lack of selectivity, b) applicability only to polymers of low molar mass (Roberts et al. 2002; Kozlowski and Harris. 2001), c) side reactions and generation of byproducts, d) risk of diol contamination in the PEGylating reagents (when PEG is used in excess), e) loss of biological activity and unpredictable *invivo* behavior exhibited by the conjugates (Pasut and Veronese, 2007), f)

heterogeneity/non-uniformity of the PEGylation site results in the production of multi-PEGylated conjugates. The heterogeneous conjugates display varying specific activities and results in non-uniform immunological implications *invivo*. First generation requires difficult purification process and consequent impeded reproducibility of uniform conjugates at the synthesis level itself (Wang et al. 2002). Lack of selectivity is a major shortcoming as the reactive groups may be buried within the core tertiary structure of the protein or may be sterically hindered resulting in the synthesis of heterogeneous PEGylated proteins of varying molecular weights (Bentley et al. 1999).

It is therefore, essential to choose a modification chemistry, which reduces the complexity of PEG-protein conjugates, in order to make the purification process simpler and improve reproducibility of uniform conjugates starting from the initial synthesis levels (Wang et al. 2002). Few examples of PEGylated products developed through first generation of PEGylation are: Adagen® (PEGylated Adenosine deaminase-Sigma-Tau Pharmaceuticals, Inc. Italy), Oncaspar® (PEGylated L-Asparaginase-Sigma-Tau Pharmaceuticals, Inc. Italy), Krystexxa® (PEGylated Uricase-Crealta Pharmaceuticals, LLC.USA), PEGasys® (PEGylated Interferon alpha 2a-Genentech Inc.-USA), Somavert® (PEGylated hGH antagonist- Pfizer-USA and Pharmacia & Upjohn-USA) etc.

2.5.2 Second generation of PEGylation:

In order to overcome the disadvantages of first generation/random conjugation, innovative strategies that rely on the exploitation of less abundant and chemo-selective anchors have been explored (Kochendoerfer, 2005). This is known as 'second generation PEGylation', which involves site-specific conjugation of PEGylation reagent to one specific type of amino acid. This can be accomplished by terminal functionalization of PEG with suitable reactive groups and then binding it to specific sites on the protein surface. The advantages of this approach are as follows: a) enhanced control over the ligation process, b) no or negligible loss of biological activity, c) applicability to a wide range of labels and modifications, d) conjugate homogeneity, e) requirement of very minute quantity of polymer which will lower the

immunogenicity etc. This can be achieved by employing PEG derivatives with terminal functional groups which can specifically target a single type of amino acid residue. Few examples of PEGylated products developed by the second generation of PEGylation are PEGIntron® (PEGylated Interferon alpha 2b-Histidine-Schering-Plough Corporation-USA), Cimzia® (Anti-TNFα-Fab-C terminal, UCB group of Companies, Belgium), Neulasta® (PEGylated Granulocyte-CSF- Amgen- USA), Mircera® (PEGylated erythropoietin receptor activator-Roche-Switzerland).

Various approaches of site-specific conjugation manner are as follows: (a) N-terminal modification, (b) C-terminal modification, (c) thiol group modification, (d) arginine modification, (e) modification at glycosylation sites, (f) transglutaminase-mediated enzymatic modification, (g) modification at the histidine tags etc. **Figure 1.1** represents various sites on a protein molecule which can be used for PEGylation. **Table 2.2** represents various strategies and respective PEG derivatives employed for site-specific PEGylation of proteins. **Table 2.3** indicates reaction conditions used for the synthesis of a few site-specific PEGylated therapeutic proteins via Thiol PEGylation

Figure 1.1: Various sites on a protein molecule which can prove to be potential sites for PEGylation

Table 2.2: Various strategies and respective PEG derivatives employed for random and site-specific PEGylation of proteins

Sl no	Site-Specific PEGylation Strategy	Target Functional group	PEG Derivative used
1	Amino group PEGylation	NH_2-R (amino group)	1) **Alkylating PEGs:** a)PEG-Aldehyde b) Tersylated PEG c) PEG-Epoxide 2) **Acylating PEGs:** a) PEG-oxycarbonylimidazol b)PEG-benzotriazole carbonate c)PEG-p-nitrophenyl carbonate d)PEG-triphenyl carbonate e)PEG-oxy carbonyl imidazole f)PEG-benzotriazole carbonate
2	N-Terminal PEGylation	α-NH_2 of N-Terminus	1) mPEG-Aldehyde 2) mPEG-Propionaldehyde 3)mPEG-Hydrazine
3	C-terminal PEGylation	a)Addition of cysteine residue to the C-terminal end b)C-Terminal hydrazide/ aminooxy groups of the protein	1) mPEG –maleimide 2)Benzaldehyde PEG Derivatives 3) maleimide PEG Polymers
4	Thiol-reactive PEGylation	HS-R/ Cysteine residues	1) m PEG-ortho pyridyl disulfide 2) m PEG-Maleimide 3) m PEG-vinyl sulfone 4) m PEG-iodoacetamide
5	Arginine PEGylation	NH_2-NH-C-NH-R	m PEG-phenylglioxale

6	Transglutaminase-mediated PEGylation	These catalyze acyl transfer between a γ-carboxamide group of glutaminyl and a primary amine (Є amino group)	Microbial Glutaminase enzyme

i) N-terminal and C-terminal modification

The N-terminal amino group and the C-terminal carboxylic acid can be used as potential sites for conjugation with aldehyde functionalized PEG reagents (Fee and Damodaran, 2012). N-terminal PEGylation guarantees a higher degree of homogeneity of the final PEGylated product (where it is possible to direct the coupling reaction towards the N-terminus of the protein). This selectivity is probable by taking advantage of the different pKa values between the ε-amino group of lysine and the α-amino group of the N-terminus. By lowering the pH of the reaction mixture to 5-6, all the ε-amino groups in the proteins will tend to be protonated, whereas the α-amino group still partially remains as a free base for coupling with activated PEG reagents. In these reactions, an unstable Schiff's base is formed (as an intermediate) which in turn is reduced to a stable secondary amine. This method works efficiently when less reactive PEG aldehydes like PEG-acetaldehyde, PEG-benzaldehyde and PEG-propionaldehyde are employed for PEGylation. This method has been successfully applied to manufacture several PEGylated conjugates including Neulasta®, (N-terminal monoPEGylated granulocyte colony stimulating factor (GCSF). **Table 2.3** represents a few reports on the N-terminal PEGylation of protein molecules.

C-terminal PEGylation is performed by direct coupling of PEG-NH$_2$ to activated carboxylic groups of proteins. There are reports on incorporation of cysteine residues on the C-terminal with cysteine residues using site-specific mutagenesis followed by PEGylation using the thiol strategy (Gao et al. 2010).

Table 2.3: A few reports on N-terminal PEGylation of protein molecules

Sl no	Protein	PEGylating reagent	Reducing agent used	Reaction conditions	Reaction duration	References
1	Salmon Calcitonin	mPEG-succinimidyl carbonate		0.1 M SP buffer, pH 8.0, 4°C	1 hr	Lee et al. 1999
2	human interferon (IFN)-b-1a	20-kDa PEG aldehyde	5 mM sodium cyanoborohydride	5 mM sodium phosphate, pH 6.0	20 hrs	Pepinsky et al., 2001
3	Type I soluble tumor necrosis factor receptor	PEG-aldehyde (2, k and 20 kDa)				Kerwin et al., 2002
4	Rh granulocyte colony stimulating factor	PEG-aldehyde (6 kDa)	20 mM sodium cyanoborohydride	100 mM sodium acetate, pH 5	1 hr	Kinstler et al., 2002
5	Epidermal Growth Factor	PEG-propionaldehyde (2 and 5 kDa)	2.5 mM sodium borohydride	50 mM sodium acetate buffer, pH 5.5, RT	24 hrs	Lee et al., 2002
6	Lysine-deficient mutant tumor necrosis factor-a	Linear PEG-aldehyde (5 and 20 kDa) and branched PEG (10 and 40 kDa)		PBS, 37°C	30 min	Yoshioka et al., 2003
7	Octreotide	Succinimidyl	20 mM	0.1 M	Overnight	Na et al.,

Sl no	Protein	PEGylating reagent	Reducing agent used	Reaction conditions	Reaction duration	References
		propionate-mPEG (2 kDa) and butyraldehyde-mPEG (2 and 5 kDa)	sodium cyanoborohydride	acetate buffer, pH 5, 4°C		2005
8	Rh granulocyte colony stimulating factor	PEG-aldehyde (5 and 20 kDa)		100 mM sodium phosphate, pH 6.9, 37°C	48 hrs	Rajan et al., 2006
9	Recombinant Interferon α-2a	mPEG aldehyde (5, 10, and 20 kDa)	sodium cyanoborohydride	100 mM sodium phosphate, pH 5, 4°C	24 hrs	Lee et al., 2007
10	Rh keratinocyte growth factor 2	PEG-phenyl-isothiocyanate (20 kDa)		sodium acetate solution, pH 6.2, 4°C	4 hrs	Huang et al., 2009
11	Rh granulocyte colony stimulating factor	mPEG-ALD (10 kDa, 20 kDa, and 30 kDa)	sodium cyanoborohydride	20mM sodium acetate buffer, pH 4.5, 4 ∘C,	20 hrs	Zhai et al., 2009
12	Rh interleukin-1 receptor antagonist	mPEG-propionaldehyde (5 kDa)	sodium cyanoborohydride	0.04 M sodium acetate, pH 5,	Overnight	Yu et al., 2009

Sl no	Protein	PEGylating reagent	Reducing agent used	Reaction conditions	Reaction duration	References
				4°C		
13	Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand	mPEG-propionaldehyde (5 kDa)	sodium cyanoborohydride	50 mmol/L acetate buffer , pH 5.0	12 hrs	Chae et al, 2010
14	Human growth hormone	mPEG-propionaldehyde (20.7 kDa)	sodium cyanoborohydride	100 mM sodium phosphate buffer, pH 5.1,	12 hrs	Pai et al., 2011
15	Rh granulocyte colony stimulating factor	mPEG-propionaldehyde (20 kDa)	sodium cyanoborohydride	100 mM sodium Phosphate buffer, 20-25°C	1-5 hr	Tiwari et al., 2011
16	Staphylokinase	mPEG-propionaldehyde (20 kDa)	sodium cyanoborohydride	50 mMsodium acetate buffer, pH 5.0 at 4°C	1 hr	Wang et al., 2011
17	Porcine prothrombin	PEG-phenyl-isothiocyanate (40 kDa)		sodium acetate solution, pH 6.2 at 25°C	2 h	Zhou and Chen, 2011
18	Keratinocyte growth factor	mPEG-butyraldehyde	sodium cyanoborohydride	20 mM sodium		Huang et al., 2012

Sl no	Protein	PEGylating reagent	Reducing agent used	Reaction conditions	Reaction duration	References
		(20 kDa)		phosphate buffer, pH 6.0		
19	Rh methionyl-granulocyte colony stimulating factor	mPEG-propionaldehyde (20 kDa)	sodium cyanoborohydride	100 mM phosphate buffer, pH 5	Overnight	Natalello et al., 2012
20	Lysozyme	mPEG-aldehyde (5 and 10 kDa)	sodium cyanoborohydride	25 mM sodium phosphate buffer pH 7.2, RT	3.5 hrs	Maiser et al., 2012
21	Transferrin, tumor targeting factor	Di-aldehyde PEG (3.4, 5, and 10 kDa)	Sodium cyanoborohydride	50 mM acetate buffer, pH 5.0	3 hrs	Kim et al., 2012
22	Staphylokinase dimers	mPEG-propionaldehyde (20 kDa)	Sodium cyanoborohydride	20 mM sodium acetate buffer, pH 5.0, 4 ℃	overnight	Liu et al., 2012
23	Fibroblast growth factor-21	mPEG-propionaldehyde (20 kDa)	Sodium cyanoborohydride	50 mM sodium phosphate buffer pH 6.0, RT, 4 ℃	8 hrs	Ye et al., 2015

Sl no	Protein	PEGylating reagent	Reducing agent used	Reaction conditions	Reaction duration	References
24	Interferon (IFN) beta-1a	mPEG-butyraldehyde (20 kDa)	sodium cyanoborohydride	10 mM sodium acetate, pH 5.0, 22°C	22 hrs	Korzhavin et al, 2015
25	Recombinant lidamycin apoprotein of lidamycin	mPEG-propionaldehyde derivative (Mw 20 kDa)	sodium borohydride	50 mM sodium acetate buffer, pH 5.5, RT	24 hrs	Li et al., 2015

ii) Thiol group modification:

Cysteine residues are apposite targets for achieving site-specific modification of proteins. They are present in free form at a relatively low natural abundance in comparison to the oxidized cysteine species and also other amino acids. This advantage offers a good scope for obtaining mono-PEGylated conjugates. Cysteine residues can be modified selectively, rapidly, quantitatively either in a reversible/irreversible fashion (Colonna et al. 2008). There are several examples of the insertion of cysteines at desired positions in a protein sequence by genetic engineering for site-specific conjugation (Xian et al. 1999).

PEG-Maleimide (PEG-Mal), PEG-vinyl sulfone (PEG-VS), PEG-iodoacetamide (PEG-IA) and PEG-ortho pyridyl disulfide (PEG-OPDS) derivatives can be used for site-specific conjugation at the cysteine residue.

Reducing agents like dithiothreitol (DTT) and tris(2-carboxyethyl)phosphine (TCEP) can be used to reduce the uricase homotertramer into single subunits for exposure of hidden thiol (-SH) groups from the protein cleft. The free thiol groups now become accessible to mPEG-mal chains for PEGylation reaction. DTT has a low redox potential (-0.33 volts at pH 7) and is capable of maintaining monothiol groups

completely in the reduced state and reduces disulfides quantitatively (Cleland et al. 1963) and hence is preferred for reduction process in comparison to TCEP.

iii) Arginine Modification:

Only a few examples of polypeptide PEGylation at the level of arginine were reported based on the use of PEG-dioxo compound (Mero et al. 2011). In these reactions, PEG-dioxo compound are preferred which require a longer reaction time which adversely affects the protein stability.

iv) Glutamine Enzymatic PEGylation:

Several proteins have been significantly modified using TGase, for example, human growth hormone, recombinant human interluekin-2 and granulocyte-colony stimulating factor. Transglutaminase enzyme (TGase) catalyzes an acyl transfer between the γ-carboxamide group of glutaminyl residue (acyl donor) and primary amine (acyl acceptor) (Fontana et al. 2008).

v) Histidine Tag PEGylation:

Cong et al. (2012) have pronounced covalent conjugation of PEG in a site-specific manner to a polyhistidine tag (His-tag) on an antibody molecule. In their report, PEGylation was attained with a domain antibody (dAb), which had a 6-histidine His-tag on the C-terminus (dAb-His(6)) and interferon α-2a with an 8-histidine His-tag on the N-terminus (His(8)-IFN). The site of PEGylation at the His-tag for both dAb-His(6)-PEG and PEG-His(8)-IFN was confirmed by digestion, chromatographic and mass-spectral studies. Kim et al (2013) have developed a conjugation strategy through complementary interactions between a His-tag and a Ni^{2+} complex of nitrilotriacetic acid to improve the half-life of therapeutic proteins in the blood following systemic administration *invivo*.

vi) Disulfide bridging modification:

Balan et al. (2007) developed a strategy of cysteine PEGylation which exploits the latent conjugation selectivity of the two sulfur atoms that are derived from the ubiquitous disulfide bonds of proteins. This strategy involves PEGylation using a mono and bis-sulfone terminated PEG reagent that can insert a three carbon bridge that connects to the two sulfur atoms that form the disulfide bond. Their studies suggested that peptides, proteins, enzymes and antibody fragments can be PEGylated in a site-specific manner across a native disulfide bond using a three-carbon bridge without destroying their tertiary structure or abolishing their biological activity.

vii) Site-specific PEGylation of proteins containing unnatural amino acids:

Deiters et al (2004) have reported the site-specific incorporation of *p*-azidophenylalanine into proteins in yeast, wherein the azido group was used in a mild cycloaddition reaction with an alkyne-derivatized PEG reagent to afford selectively PEGylated protein.

2.6 Site-specific PEGylation of few therapeutic proteins using Thiol PEGylation:

Cysteine amino acid is typically involved in the formation of disulfide bridges with a second cysteine residue for the formation of cystine entity. Cysteine is rarely present in proteins in its reduced form. Even when naturally present, its reactivity in the native protein can be significantly lesser owing to its positioning in a less accessible hydrophobic cleft (Arakawa et al. 1993).

Thiol PEGylation is not restricted to protein or peptide containing free cysteine but it can also be applied to proteins/peptides devoid of a free cysteine molecule. This can be achieved by employing genetic engineering technologies like site directed mutagenesis, substitution of cysteine with other moieties and usage of linkers like 2-iminothiolane (Pasut and Veronese. 2012). **Table 2.4** represents a review of various therapeutic proteins and peptides modified by thiol PEGylation. The ratio of protein

and polymer reacted, reaction buffers used, reaction conditions, EDTA and reducing agent concentrations have also been indicated in the **Table 2.4.**

Table 2.4: Reaction conditions used for the synthesis of a few site-specific PEGylated therapeutic proteins via Thiol PEGylation

Sl no	Protein	Polymer and Mol Wt	Protein: PEG Ratio	pH, time and Temperature	EDTA Conc	Reductant	Reference
1	Papain	mPEG-glutaryl-S-SPy		5.0		DTT	Azarkan et al. 1995
2	Staphylokinase variant, with Ser in position 3 mutated into Cys	PEG-maleimide (5, 10 and 20 kDa)	1:3	pH 7.9, at RT, 1 hr		Dithiothreitol and desalting on Sephadex G25	Collen et al. 2000
3	Anti-Tac(Fv)-PE38 (LMB-2), a recombinant immunotoxin	mPEG-mal 5 and 20 kDa		25°C, 12 hrs	1 mM		Tsutsumi et al. 2000
4	Subunit of a voltage-gated potassium channel	mPEG-mal 40kDa	20 mM of PEG in the buffer	pH 7.2, 15 min, 3,6 and 16-19hrs	2 mM		Lu et al, 2001

Sl no	Protein	Polymer and Mol Wt	Protein: PEG Ratio	pH, time and Temperature	EDTA Conc	Reductant	Reference
5	Interferon α2a	mPEG-mal (5,10,20 and 40)	1:15-20	8.5, RT, 1 hr		10-15 molar excess TCEP	Rosendahl et al, 2005
6	Granulocyte-Macrophage Colony-Stimulating Factor	mPEG-mal (5 kDa)	1:20	pH 8.0 at RT, 1.5 Hrs		15 fold TCEP	Doherty et al, 2005
8	Wild type Epo (BV Epo) and a PEGylated Epo cysteine analog	mPEG-mal (5 and 20 kDa)	1:15 and 1:20	8.0, RT, 3 hrs		15- to 20-fold molar excess of TCEP	Long et al. 2006
9	Trichosanthin	mPEG-mal (5 kDa)	1:20	7.5, 25°C and 12 hrs	-		An et al, 2007
10	Glutathione, Somatostatin and L-asparaginase	PEG Bis(sulfone) 3	Glutathione 1:8 (mg)	8, Ambient, 18 hrs	10 mM	TCEP-HCl	Balan et al. 2007
11	Somatostatin	PEG bis(sulfone) 3	1:2.8	pH 6.2 , 4°C, overnight		1 equal of TCEP-HCl	Balan et al. 2007

Sl no	Protein	Polymer and Mol Wt	Protein: PEG Ratio	pH, time and Temperature	EDTA Conc	Reductant	Reference
12	Asparaginase	PEG monosulfone (5, 10, and 20 kDa)	1:105	4°C, 16 hrs	10 mM	DTT	Balan et al. 2007
13		PEG monosulfone (10 kDa)	interferon α-2b	7.8, 4°C, 16 hrs	10 mM	DTT (100 mM)	Balan et al. 2007
14	Antigen-binding fragments	mPEG-mal (40-kDa) and linear mPEG-mal (20- and 30-kDa)		6.0,	2 mM		Humphreys et al. 2007
15	Interleukin-1 receptor antagonist	mPEG-mal (10 kDa)		4°C, 4 hrs			Yu et al. 2007
16	Human serum albumin	galactosyl-glucono maleimide and maltosyl-glucono maleimide	1:10	6.5, RT, 2 hrs	10 mM		Salmaso et al., 2008
17	Exenatide	mPEG-mal (5, 20, 30 and 40 kDa)					Gong et al. 2010
18	Glucagon-like peptide-1	mPEG-mal (10 kDa)	1:5	pH 8.0, 4°C, 3 hrs			Gao et al. 2010

Sl no	Protein	Polymer and Mol Wt	Protein: PEG Ratio	pH, time and Temperature	EDTA Conc	Reductant	Reference
19	Recombinant human erythropoietin	mPEG-mal (30 kDa)	1:30	8, 3 hrs		TCEP	Cohan et al. 2011
20	Bacteriophage endolysins	mPEG-mal (10 kDa) or Y-shaped mPEG-mal (40 kDa)	1:25 and 1:10	pH 7.4, 15 minutes		30 min at RT with 10mM DTT and desalted on PD-10 columns equilibrated with buffer	Resch et al. 2011
21	Keratinocyte growth factor 1	mPEG-mal (40 kDa)	1:5, 1:10,:20 1:15 and				Huang et al. 2012
22	Staphylokinase	mPEG-mal (5 and 20 kDa)	4:1	7.2, 4°C, overnight		TCEP at the Sak/TCEP molar ratio of 1:10	Mu et al. 2013
23	Staphylokinase variant with 12 amino acid substitutions	mPEG-mal (5, 10 and 20 kDa)	1:3	7.9, overnight		DTT	Collen et al. 2013

Sl no	Protein	Polymer and Mol Wt	Protein: PEG Ratio	pH, time and Temperature	EDTA Conc	Reductant	Reference
24	TNF-apoptosis inducing ligand	mPEG-mal (5 kDa)		6.0, 4°C, overnight		TCEP	Pan et al. 2013
25	Exendin-4	mPEG-mal (50 kDa)	1:2	RT, 1 hr			Sun et al. 2015
26	Plasminogen activator inhibitor type-2	mPEG-mal (12, 20 and 30 kDa)	1:20-40	7.5, 2-4 hrs		DTT	Vine et al. 2015
27	Uricase	mPEG-mal (750 Da, 5 and 10 kDa)	1:5, 1:10, 1:15	7.2, 4°C, overnight	5 mM		Nanda et al. 2015

Various PEGylating reagents have been used as reagents for thiol PEGylation namely (1) PEG-maleimide (PEG-mal), (2) PEG-vinyl sulfone (PEG-VS), (3) PEG-iodoacetamide (PEG-IA) and (4) PEG-ortho pyridyl disulfide (PEG-OPDS). In comparison to PEG-OPDS, PEG-Mal results in more stable conjugates. PEG-IA and PEG-VS are both less reactive and infrequently used, whereas PEG-mal and PEG-OPDS yield quantitative protein modification and are hence preferred (Mero et al. 2011).

Figure 2.1 represents thiol PEGylation reaction catalyzed by PEG-mal. PEG-maleimide is the most popular PEGylating agents which forms a thioether bond through Michael's addition between the thiol group of cysteine entity and the double bond of the maleimide ring. The terminal maleimide group undergoes alkylation with the sulfhydryl groups to form a stable thioether bond and exhibits absolutely no reactivity with lysine or histidine residues (Yang et al. 2003).

The pH of thiol PEGylation reaction is the most important parameter to be controlled (in the range of 7.0-7.2) since at high values of pH the lysine residues can react with the maleimide moiety and result in random PEGylation (Mero et al. 2011). The main limitation of this PEG reagent is the instability of the maleimide ring, wherein the imido group present in maleimide moiety has a tendency to undergo spontaneous hydrolysis as observed by ^{1}H NMR (Khan, 1984; Kalia and Raines. 2007). But the problem does not exist after PEGylation and the maleimide ring remains stable.

Figure 2.1: Thiol PEGylation reaction using PEG-mal

A new strategy to achieve monoPEGylation was performed by Balan et al. (2007), to perform PEGylation to protein disulfide bridges, where the disulfide link was first reduced. The resulting free thiols was reacted with a special PEG-mono sulfone reagent to give a stable three-carbon PEGylated bridge. Disulfide bridging was initially proposed by Brocchini and coworkers (2007), using a specific cross-functionalized monosulfone PEG. These bonds are suitable for protein conformation and therefore any modification at this level might be potentially harmful and requires preservation of the native distance between the sulfur atoms (Pasut and Veronese. 2007)

2.7 Reports on site-specific PEGylation of few therapeutic proteins using N-terminal PEGylation strategy:

The N-terminal PEGylation uses the ε-amino residue of lysine residue present in a protein having different pKa values (about 9.3–9.5), in comparison to the α-amino group of the protein N-terminus (7.6–8) (Wong et al. 1991). Site-specific PEGylation

is commonly carried out in mild acidic conditions, for example in buffers (of the pH range 5.5–6.5) which match the physiological conditions. Under such conditions, the lysine residues will be protonated and become non-reactive with PEGylating agents, while the free α- amino groups will be available for coupling with the protonated form (Kinstler et al. 1993). This approach brings about the attachment of a single PEG strand at the N-terminus of the protein molecule which ensures retention of biological activity. N-terminal PEGylation strategy has been applied to a wide range of therapeutic proteins like Interleukin-8, GCSF, interleukin-1ra etc. Neulasta[®] (PEGylated G-CSF with 20 kDa PEG-ALD) is the best-known example of N-terminal PEGylation in the market.

2.8 Optimization of PEGylation reaction conditions:

Various reaction parameters involved in PEGylation reactions namely, the concentration ratio of protein and polymer used, polymer molecular weight and chain length, pH and temperature of the PEGylation reaction, concentration of additives, can influence the biological activity and the yield of the resulting site-specific PEGylated conjugate (Mero et al. 2010). Additionally during the progress of PEGylation reaction the dearth of conjugation selectivity is a serious problem as the change in conjugate structure may erratically influence its pharmacokinetic properties (Jevsevar et al. 2010). Hence, monitoring the production and reproducibility of uniform site-specific PEGylated conjugates is inevitable. Considering the strict regulatory requirements in the current pharmaceutical industry, the reproducibility and product yield site-specific PEGylation ought to be extensively reconnoitered.

The determination of the PEGylation reaction conditions which result in a high yield of the desired final product is a challenging activity during subsequent scaling, maintenance of biological activity of the starting preparation and minimizing side effects when used in medical applications (Puchkova et al. 2012). The PEGylation reaction can be optimized to maximize the desired product while limiting undesired byproducts (under- and over-PEGylated species) by adjusting the reaction conditions. **Table 2.5** represents few reports on the optimization of PEGylated reaction conditions using RP-HPLC and the conditions employed.

Table 2.5: Reports on optimization of PEGylated reaction conditions using HPLC

Sl no	Protein	Polymer	Stationary Phase HPLC	Mobile Phase-HPLC	References
1	Unknown Protein	mPEG- SC, 5 kDa	Jupiter, 300 Å, C-18, 5 µm Column	65% TFA (0.1 v/v) 35 5 ACN, flowrate of 0.1 mL per min. Then changed to 40 % TFA and 60 % ACN	Van Arnum et al. 2013
2	Epidermal growth factor	mPEG-COO-NHS	Shodex protein KW-800 column	Isocratic mobile has with 50 mM NaCl and 50 mM phosphate buffer, pH 6	Lee and Park, 2002
3	Unknown Protein		Spherisorb C-18 Column	Solvent A (0.1 % TFA in water) Solvent B (0.1 % TFA in ACN)	Lee and Park., 2002
4	PEGylated protein	mPEG-43 kDa	Protein KW 803, 5 µm and KW 804, 5 µm	20 mM, HEPES, pH 6.5	Li et al. 2008
5	CPT-11 in human serum	mPEG-40 kDa	Jupiter C 18 reverse phase, 5µm	Ammonium acetate/ ACN gradient	Sapra et al. 2008

Sl no	Protein	Polymer	Stationary Phase HPLC	Mobile Phase-HPLC	References
6	Rh GCSF	mPEG-prop (5,10,20 and 30 kDa)	Superdex 200 Column and Vydac C4 column	50 % Buffer A (Water with 0.1 % TFA) and 50 % Buffer B (HPLC grade ACN with 0.1 % TFA)	Zhai et al. 2009
7	GCSF	mPEG-prop	Symmetry 300 Å C4 RP Column Jupiter C4 300 Å.	Buffer A (0.1 % TFA in water) and Buffer B (20 % ACN) with 0.1 % TFA	Puchkova et al. 2012
8	Morcharin	mPEG-2-Lys- NHS (20 kDa)	TOSOH TSK-Gel G2000SW×1 Column	50 mM, pH 7.3, Tris/HCl with 0.2 M Sodium sulphate	Bian et al. 2010
9	Glucagon-like peptide 1 antagonist	mPEG-mal (22 and 43 kDa)	C 18 HPLC Column	Buffer A (0.1 % TFA /Water), 30 mins of gradient to 100 % of Buffer B (0.1 % TFA/ACN)	Tom et al. 2007
10	Native Uricase	mPEG-NHS	Chromosil C-18 Column (5μm)	0.08 % TFA in water and eluted using 0.08 % TFA in solvent with acetonitrile /water	Sankari et al. 2014

2.8.1 pH of the PEGylation reaction mixture:

pH of the buffer solution used for PEGylation reaction mixture plays a vital role in regulating the binding of PEG-protein, stability and enzymatic function of the protein moiety. Reaction conditions such as buffer pH and concentration of PEG can have a major influence on the parameters like the reaction velocity, PEGamer (aggregated conjugates) formation and isoform distribution (Veronese, 2001). Wylie et al. in 2001 reported that pH can significantly affect the PEGylation site. In case of Thiol PEGylation, the reactions are generally carried out at a neutral pH (Mero et al. 2011), whereas mPEG-mal as a PEGylating reagent for site-specific PEGylation of proteins. pH conditions of above 7.5 ought to be avoided, since at higher pH conditions the reaction with primary amine groups like lysine may also occur. In such conditions, the reaction may occur at a much slower rate than the free thiol groups (Mero et al. 2011). There are several reports on the optimization of pH for Thiol PEGylation reaction which are as represented in **Table 2.6. Table 2.6** represents few reports on the effects of molarity and pH of the buffers on Thiol PEGylation reactions.

Table 2.6: Buffer molarity and pH conditions employed for Thiol PEGylation reactions

Sl no	Protein	Buffer solution Molarity and pH	Polymer used	Reference
1	Antigen-binding fragments	0.1 M SP buffer, pH 6.0	mPEG– mal (5-folds)	Humphreys et al. 2007
2	Erythropoietin and its cysteine analogs	pH 8.0	15-20 fold molar excess mPEG-mal (5 -20 kDa)	Long et al. 2006
3	Glutathione	50 mM SP buffer buffer, pH 7.8,	8 fold PEG bis(sulfone)	Balan et al. 2007
4	Peptide hormone somatostatin	100 mM SP buffer, pH 7.2	PEG-mono sulfone	Balan et al. 2007
5	Disulfides in interferonR-2b	50 mM SP buffer, pH 7.8,	10 kDa PEG monosulfone 4or 10 kDa PEG bis(sulfone)3	Shaunak et al. 2006
6	Single-chain Fv proteins	100 mM SP buffer, pH 6.0	mPEG- mal (10 folds of 5, 20 and 40 kDa)	Yang et al. 2003

2.8.2 EDTA Concentration in the thiol PEGylation reaction mixture:

In thiol PEGylation strategy, EDTA sodium salt is frequently used, as it chelates the divalent metal ions, which can otherwise oxidize the sulfhydryl groups (from –SH to –SHO) (Riddles. 1979). Thus, EDTA prevents the oxidation of sulfhydryl groups of the free cysteine molecules of proteins. In general, the formation of disulfide bonds between two cysteine molecules can also be inhibited by metal complexion agents (i.e., EDTA), or removing metal ions (present in reactants, raw material and buffer solutions). The unoxidized sulfhydryl groups (-SH) can be then made available for reaction with the maleimide moiety of the mPEG-mal molecule. This reaction results in the formation of a very stable thioether bond between the mPEG-mal and the protein molecule (Yang et al. 2003). Therefore, the addition of EDTA in the reaction buffer is imperative for Thiol PEGylation. EDTA concentration should be such that it does not have a negative influence on the enzymatic activity of proteins. Few reports on the effect of EDTA concentrations on the residual activities of proteins during Thiol PEGylation reactions is as represented in **Table 2.7**.

Table 2.7: Reports on the EDTA concentration used in Thiol PEGylation studies

Sl no	Protein molecule	Buffer solution	Polymer used	EDTA Conc	Reference
1	Antigen-binding fragments	0.1 M SP buffer, pH 6.0	5 fold mPEG–mal	2 mM	Humphreys et al, 2007
2	Erythropoietin and its cysteine analogs	0.1 M SP buffer, pH 8.0	15-20 fold molar excess of mPEG-mal (5 kDa/20 kDa)	7 mM	Long et al, 2006
3	Glutathione	50 mM SP buffer, pH 7.8	8 fold PEG bis(sulfone)	10 mM	Balan et al, 2007
4	Peptide hormone somatostatin	100 mM SP buffer, pH 7.2	PEG-mono sulfone	10 mM	Balan et al, 2007
5	InterferonR-2b	50 mM SP buffer, pH 7.8,	10 kDa PEG monosulfone 4/10 kDa PEG bis(sulfone)3	10 mM	Shaunak et al, 2006
6	Single-chain Fv proteins	100 mM SP buffer, pH 6.0	10 folds of mPEG-mal (5, 20 and 40kDa)	2 mM	Yang et al, 2003
7	Subunit of a voltage-gated potassium channel	100 mM SP buffer saline	20 mM of PEG mPEG-mal (40kDa)	2 mM	Lu et al, 2001

2.9 PEG-Conjugate Purification:

Purification of PEGylated proteins is a very perplexing and dependent process, since the PEGylation reagent moiety is a hydrophilic, inert and neutral molecule (Fee and Van Alstine. 2011). The removal of unreacted protein, PEGylation reagents, reaction byproducts, buffer salts etc become essential for the recovery of the final PEGylated conjugate in its pure form. **Table 2.8** represents the classification of contents of a PEGylation reaction mixture based on their molecular weights obtained after the completion of reaction.

Table 2.8: Classification of PEGylation products by molecular weight

Low Molecular Weight	High Molecular Weight
By-products from hydrolysis of functionalized PEG	Unreacted functionalized PEG
By-products of PEGylation reactions	Inactive PEG from hydrolysis of PEG reagents
Buffer components	Native protein
Reducing agents and other chemicals additives for stabilizing the protein conjugate	Protein aggregates, under-PEGylated protein and Over-PEGylated protein

The selection of the purification method depends on the properties of both protein and the polymer used. The difference in properties of the native and PEGylated protein like molecular weight, size, electrostatic charge and partitioning coefficient can be utilized as a basis for separation of PEGylated protein from its native counterpart. Dialysis/ultrafiltration can be employed for the removal of low molecular weight components or to exchange the buffer solvents. The elimination of the above mention impurities can be accomplished by using more specific chromatographic techniques coupled with online detector (wherein proteins and their conjugates can be monitored using UV and fluorescence detectors). The unreacted PEGs being transparent in the

UV spectrum can be monitored using refractive index detector or light scattering techniques (Li et al. 2008). Most commonly applied chromatographic methods for purification of PEGylated conjugates include dialysis, dia/ultrafiltration, size exclusion chromatography (SEC), size exclusion-high performance liquid chromatography (SE-HPLC), fast protein liquid chromatography (FPLC) and ion-exchange chromatography (IEC).

Random PEGylation involves the formation of heterogeneous PEGylation products and thus it requires multiple steps of purification. In the case of site-specific PEGylation reaction, though the proteins are homogenous, purification is still required for the elimination of unreacted proteins and PEGylating agents, reaction byproducts, buffer salts etc. However, only a few steps of purification are required to attain homogenous conjugates of uniform size. **Table 2.9** represents few reports on purification methods used for site-specific PEGylated proteins. Few frequently applied PEGylated conjugate purification methods are as described below:

i) Ion-Exchange Chromatography (IEC):

The isoelectric point of PEGylated and native proteins are different. The main effect of PEGylation on ion-exchange separations is to shield the electrostatic charges on the protein surface and to reduce the strength of interactions with higher molecular weight PEG reagents. Thus, ion exchange can be used very effectively to resolve a PEGylation mixture on the basis of PEGylation extent. Particularly for low extents but as the PEGylation extent increases, the effectiveness of separation rapidly diminishes (Fee and Van Alstine. 2011). Usually, cation exchange chromatography remains the method of choice as it exploits differences in charge at the protein surface (Seely and Richey, 2001). In the case of ion-exchange chromatography, highly PEGylated molecules tend to elute first followed by moderately PEGylated isomers and unreacted protein. The unreacted PEG elutes that does not possess any positive charge elutes in column void volume (Wang et al. 2000). PEGylated proteins can also be purified by anion exchange chromatography, wherein the conjugate binds to the column at a higher pH value more than its isoelectric point, and results in a net negative charge on the molecule. Cation exchange columns with functional groups

like –S (methyl sulfonate), -SP (sulfopropyl), –CM (carboxymethyl) and anion exchange columns with functional groups like –DEPE (diethyl aminopropyl) and – DEAE (dimethylamino ethyl) have already been applied for separation of PEGylated and native proteins.

ii) Size Exclusion chromatography:

PEGylation imparts significant changes in molecular weight wherein each chain added to a protein correspondingly increases its molecular size. Therefore SEC and ultrafiltration (and dialysis) proved to be effective methods for separating native and PEGylated proteins (Fee and Van Alstine. 2011). The size of the PEGylated protein will be approximately 5-20 folds more than that of its native counterpart. SEC separates molecules based on the differences in their hydrodynamic volumes, however, it is associated with certain shortcomings which are as follows: a) offers broad peaks with poor resolution of PEGylated conjugates; b) it is a low throughput technique, c) expensive process and d) provides limited applicability for scale-up of the process and e) cannot separate and distinguish between positional isomers of the PEGylated conjugates (Mero et al. 2011). Conversely, SEC is a useful method for removing low molecular weight impurities like by-products formed by the hydrolysis of the functional PEGs, buffer salts, solvents and other low molecular mass reagents (Mero et al. 2011). SEC is an effective tool for evaluation of the degree of protein modification and presence of aggregates. Typically, dextran and agarose based SEC columns are used in conjugation with FPLC systems.

iii) Size-Exclusion Reversed Phase Liquid Chromatography:

Achieving an SEC-based separation for the quantitation of PEGylated protein and free PEG is a challenging task due to factors like the polydispersity of the PEG as well as the PEG-protein conjugate. This will result in a broadening of the elution profile. Narrow differences of molecular weight in native and PEGylated proteins also makes the separation process very challenging. The nature of the interaction between the bound PEG and the surface of the protein may greatly limit the utility of a size-based

separation. PEGylation can also have a profound effect on the protein hydrophobicity and hence RP-HPLC in combination with SEC (SE-HPLC) using a C-4 bonded stationary phase can be employed as a purification strategy. SE-HPLC is often used for characterization of the PEGylated species due to its high resolution and the possibility of coupling the technique with an online mass-spectrometer detector (Park et al. 2008). Though this method is simple, only little amount of sample can be loaded at once.

iv) Ultrafiltration / Diafiltration:

The ability of membrane-based technologies for separating PEGylated proteins is limited due to the following parameters; increased size, greater hydrophobicity and lower electrostatic interactions of PEG conjugates in comparison to a native protein which leads to increased fouling (Kwon et al. 2008). Filtration techniques are suitable for concentration and buffer exchange between chromatographic steps, but may not be really preferred for separation of PEGylated and non-PEGylated proteins. Regenerated cellulose and polyethersulfone membranes have low protein binding retention characteristics, because of this high recovery is possible and hence it is frequently used in purification (Molek and Zydney, 2006).

Table 2.9: Reports on purification methods used for production of site-specific PEGylated proteins

	Protein	Polymer	Reaction conditions	Purification technique	Reference
1	Voltage-gated potassium channel	mPEG-mal	4°C for 15 min or 3, 6, 16-19 h	SEC	Lu and Deutsch. 2001
2	Granulocyt Macrophage Colony-Stimulating Factor	mPEG-mal (5 kDa)	Reduction with 15-fold molar excess of TCEP	1 mL Q-Sepharose HiTrap column	Doherty et al. 2005
3	Recombinant interferon α-2	mPEG-mal (5, 10, 20 and 40 kDA)	pH 8.5, 60 min	S-Sepharose column	Rosendahl et al. 2005
4	Antigen-binding fragments	mPEG-mal (40-kDa) and mPEG-mal (30-kDa)	0.1 M SPB, pH 6.0, with 2 mM EDTA	SE-HPLC on analytical Zorbax GF-450 and GF-250 columns in series	Humphreys et al. 2007
5	Trichosanthin (ribosome-inactivating protein)	20-fold molar excess of PEG-mal (5 kDa)	8°C for 12 h in 10 mM Tris–HCI, pH 7.5	column equilibrated with Tris–HCI, pH 7.5	An et al. 2007

	Protein	Polymer	Reaction conditions	Purification technique	Reference
6	Interleukin	mPEG-mal	4 h at 4°C	SP Sepharose Fast Flow resins	Yu et al. 2007
7	Human serum albumin	mPEG-mal (10 kDa)	1:10 ratio, 50 mMphosphate,10 mM EDTA pH 6.5	SEC-HPLC	Salmaso et al. 2008
8	Glucagon-like peptide	5 fold-mPEG-mal (10 kDa)	2 h at 4 °C in a pH 8.0 solution With 20 mM Tris–HCl	Q-Sepharose Fast Flow resin column	Gao et al. 2010
9	Staphylokinase	mPEG mal (5 kDa and 10 kDa)	4°C overnight	SP Sepharose HP column equilibrated with 20 mM NaAc- HAc buffer, pH 5.0	Mu et al. 2013
10	Exenatide	mPEG-mal (5, 20, 30 and 40 kDa)	-	-	Gong et al. 2010

	Protein	Polymer	Reaction conditions	Purification technique	Reference
11	Staphylokinase variant with 12 amino acid substitution	mPEG-mal (5, 10 and 20 kDa)	reduction with DTT,3 X PEG-mal in 10mmol/L PBS	SEC Superdex G75	Collen et al. 2013

2.10 PEG-Conjugate Characterization:

The demand of PEGylated drugs has been increasing in recent years; however, their rapid proliferation poses significant challenges owing to difficult characterization (Abzalimov et al. 2012). PEGylated proteins have structural heterogeneity, which has a direct influence on the functioning of the protein molecule and ought to be analyzed at different levels. The PEGylated proteins and their isoforms differ in the following aspects: i) the number of PEG chains attached, ii) location of the conjugation sites and iii) PEG chain length (Abzalimov et al. 2012). The factors that have to be monitored while characterizing the PEGylated conjugates are a) biological activity, b) extent of modification; c) molecular weight of the conjugate, d) PEGylation induced conformational changes, e) quantity of PEG attached, d) site of PEGylation has occurred at the protein surface, e) protein concentration and f) storage stability in various buffers.

The simplest and most rapid methods for the determination of extent of PEGylation using colorimetric assays are listed below:

i) TNBS assay (Trinitrobenzene sulfonic acid assay): For amine PEGylated products, the TNBS assay can be used for the determination of free amine groups (Habeeb, 1966). TNBS readily reacts with primary amino groups of amino acid residues in aqueous form at pH 8 to form a yellow colored complex which can be

detected at 335nm. It is mainly used for random and N-terminal PEGylated conjugates.

ii) Ellman/5,5-dithio-bis-(2-nitrobenzoic acid) assay: Ellman's reagent is used to estimate sulfhydryl groups in a molecule and compared with a standard curve created by a sulfhydryl-containing compound such as cysteine. This assay can be used to determine the presence of any remaining free cysteines following PEG-thiol conjugation (Riddles et al. 1983) and to determine the degree of thiol modification.

iii) Iodine assay: Iodine assay is based on the non-covalent interaction of barium iodide with the PEG backbone. It can be used to obtained both qualitative and quantitative information about the PEGylating reagent used before and after PEGylation.

iv) Bicinchoninic Acid Assay: This assay is used for the quantification of protein content in PEGylated reaction mixtures.

The detailed mechanism of all the colorimetric experiments used as PEG-conjugate characterization methods is explained in Appendix.

Detailed characterization of the PEGylated protein conjugates involves the usage of complex biophysical techniques. A few of these techniques applied have been explained in detail as described below:

i) Ion-exchange Chromatography:

IEX is the most widely used technique for the fractionation and purification of PEGylated proteins on a preparative scale and it is also very useful for analytical purposes as it supports efficient separation of positional isomers (Kusterle et al. 2008).

ii) Reversed-Phase high-performance liquid chromatography:

RP-HPLC is an efficient technique for the determination of the yield and purity of the final PEGylated product and impurities present in the reaction mixture. The working principle is based on the differences in hydrophobicity of the native and PEGylated proteins. PEGylated proteins often exhibit higher retention times on RP-

HPLC columns compared to its non-PEGylated counterparts, as PEG is an amphiphilic molecule. It may not be a robust tool for the fractionation of PEGylated species and their native counterparts, but it is also a useful method to detect protein oxidation, aggregation, deamidation, or cleavage of the protein backbone (Mero et al. 2011).

iii) Size-Exclusion Chromatography:

SEC can be employed for the molecular weight estimation of native and PEGylated proteins using a standard calibration curve. The PEGylated conjugates possess a larger hydrodynamic volume in comparison to their native counterparts. SEC methodology cannot provide an accurate determination of the exact molecular weight of PEG-proteins conjugates, but can only be used to monitor the contents of the PEGylation reaction mixture and determine the homogeneity of the conjugate product (Mero et al. 2011).

iv) SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide electrophoresis):

SDS-PAGE is used for determining aggregate formation, purity and apparent molecular weights of the PEGylated species, but cannot be employed as a direct method for the evaluation of exact molecular weight. During electrophoresis, the migration rate of the PEGylated conjugates reduces due to long and profoundly hydrated PEG chains. In SDS-PAGE, the relative migration rate of a protein coated with SDS is inversely proportional to its molecular weight. The separation conditions are the key parameters to accurately determine the molecular weights of protein aggregates. A linear relationship between the logarithmic value of molecular weight and migration rates of the molecules can be generated.

Usually, random PEGylation gives several bands on an SDS-PAGE gel due to product heterogeneity of the PEGylated sample. If the product is monoPEGylated only a single band is generated on the gel (Mero et al. 2011).

v) Storage conditions for PEGylated uricase

Commercially available random PEGylated form of uricase "Krystexxa" (8 mg/mL of PEGylated uricase concentrate) is a suspension in a sodium phosphate buffer solution with sodium chloride dissolved in WFI (water for injection) (pH 7.3±0.3). Physical and chemical stability of this formulation [diluted in 250 ml sodium chloride solution (0.45- 0.9%)] has been demonstrated for 4 hours at 2-8°C and at 20-25°C.

The present study establishes the development of site-specific PEGylation technology for a therapeutic enzyme "uricase" which is used for the treatment of hyperuricemia and gout. The literature related to uricase and its application as a therapeutic enzyme for the treatment of hyperuricemia, various forms of drug delivery used and its chronological history as a PEGylated drug has been discussed.

2.11 Hyperuricemia and Gout:

Purines are a heterocyclic aromatic organic nitrogenous compound comprising of two of the five bases in nucleic acids. Purines can be formed in the body due to various reasons namely: a) breakdown of nucleic acids as a result of cellular turnover, b) dietary intake of nucleic acid, c) cytotoxic chemotherapy as a result of rapid cell death, d) aggressive cancer chemotherapy regimens for treating tumour lysis syndrome (TLS). In tumor lysis syndrome, malignant cells burst and abruptly releases nucleic acids, proteins, and other metabolites into the bloodstream which leads to hyperuricemia, hypercalcemia, hyperphosphatemia etc (Cammalleri and Malaguarnera., 2007). These excess purine nucleotides and deoxynucleotides undergo a series of catabolic reactions to form uric acid. Uric acid remains in the blood plasma as an end product of purine metabolism. Hyperuricemia is a result of uric acid precipitation, which can lead to gout, painful arthritis, disfiguring urate deposits (tophi) and renal failure (Lotfy, 2008). Elevated uric acid has been further associated with chronic kidney disease, ischemic stroke, blood pressure elevation, and lipid abnormalities, although the direct toxic effects of hyperuricemia remain controversial. Gout, the advanced phase of hyperuricemia, is characterized by recurrent attacks of acute inflammatory arthritis, wherein the uric acid crystallizes and deposits in joints

and tendons. The prevalence of gout has been increasing with the increasing incidence in population varying from less than 1 to 15.3% (Mikuls and Saag., 2006). Various elucidations have been put forth for the occurrence of gout, including aging and extensive use of thiazide diuretics and medications like aspirin (Caspi et al. 2000) which trigger hyperuricemia.

2.11.1 Current medications available for gout and their side effects:

Therapies for hyperuricemia associated diseases include agents that do not allow the precipitation of plasma urate and also eliminate the extra urate crystals (Yang et al. 2012). The treatment of hyperuricemia and gout is generally supported with drugs that induce forced diuresis, urinary calcination and inhibition of the xanthine-oxidase enzyme.

Few examples of uricosuric agents are discussed in the following paragraphs: Probenecid and sulfinpyrazone reverse the uric acid excretion by the kidneys. Probenecid is anti-hyperuricemia drug acting on the renal urate transporter and sulfinpyrazone competitively inhibits uric acid reabsorption in the proximal tubule of the kidney. They are not extensively used as they trigger associated kidney and liver toxicity. Formation of excess uric acid can also be controlled by xanthine oxidase inhibitors including Allopurinol, Benzbromarone, and Febuxostat. Allopurinol (Zyloprim®) is orally administered and primarily employed to treat chronic gout by reducing the excess plasma urate concentration (Pacher et al. 2006). But refractory gout was found to reappear when patients suffered from hypersensitivity/non-responsiveness to allopurinol and displaying intolerance to allopurinol toxicity (Chohan and Becker, 2009). The solubility of uric acid in physiological solutions is about 402- 420 µM and allopurinol mediated reduction of uric acid in serum is below 360µM, which requires more than a few months to decrease flares and several years for complete dissolution of tophi (Zhang et al. 2006). It was reported that the tophi persisted even after 10 years of treatment with allopurinol, regardless of having maintained lower serum uric acid levels of 360µM (Doherty, 2009).

Benzbromarone a uricosuric agent and non-competitive inhibitor of xanthine oxidase is known to induce intolerable adverse immunological effects (Sinclair and

Fox, 1975). Febuxostat is also a xanthine oxidase inhibitor used to treat gout, but its efficacy in treating refractory gout has not been established (Terkeltaub, 2010).

Few drugs enhance the rate of renal clearances, like, Fenofibrate, Losartan, and Amlodipine, but have been identified to induce side-effects. Colchicine [Colcrys] is a plant derived metabolite employed to treat gout, but it is extremely toxic and causes gastrointestinal upset at high doses (Chen and Schumacher, 2008). RDEA806, a xanthine oxidase inhibitor is known for its uricosuric effects. In phase 1 clinical study of more than 70 normal healthy volunteers, RDEA806 increased urinary excretion of uric acid in the first 24 h after dosing. Statistically, significant decreases of 35% to 50% in uric acid levels were observed within 14 days (Schlesinger 2010).

2.11.2 Uricase as gout therapeutic:

Uricase (EC 1.7.3.3) is a therapeutic enzyme, catalyzes the oxidative opening of the purine ring of urate to yield allantoin, carbon dioxide, and hydrogen peroxide (Wu et al, 2004). Native uricase is a globular tetramer with a monomer mass of approximately 32 kDa /subunit and a molecular mass in a range of 130-150 kDa (Pitts et al. 1974). The most primeval innovations of employing uricase to reduce the serum uric acid levels were brought about by Oppenheimer (1941). Uricase is effective in removing pre-existing urate crystals in joints (Chohan and Becker, 2009) and displays negligible drug-drug interactions. Uricolytic therapy for gout is rapid and fast acting. Uricases are considered supreme and most effective in the treatment of refractory gout, but existing uricase formulations are incongruous for continuous treatment of gout (Yang et al. 2012).

However, uricase in its native form has discernable immunogenicity and diminutive effective half-life (Edwards 2008). It induced immunogenicity, undergoes proteolytic decay, possesses low solubility and is very expensive (Roberts et al. 2002; Yoshioka et al 2011). In February 2001, the European Medicines Agency (EMEA) approved Rasburicase (Fasturtec®/ Elitek®), a recombinant uricase from *Aspergillus flavus*, for the prevention and treatment of hyperuricemia induced by chemotherapy in subjects with hematologic malignancies. It had a short half-life (21 hours) and a transient urate lowering activity (Richette and Bardin, 2006). It induced repeated gout flares and

hypersensitivity reactions (despite corticosteroid treatment) and hence has reduced interest for its practice in treating chronic gout. Richette et al. 2006 used off-label Rasburicase for the treatment of patients suffering from moderate to severe renal failure with tophaceous gout, intolerant to allopurinol. But acute flares recurring at each Rasburicase infusion led to discontinuation of treatment in an elderly woman with tophaceous gout (Hill et al. 2008).

Routinely, natural uricases are unacceptable as therapeutic formulations for treating refractory gout, as they possess low activities are immunogenic at physiological pH and are sensitive to xanthine inhibition. Eukaryote uricases usually have lower thermostability, lower solubility and higher sensitivity to xanthine (Fridovich, 1965; Liao et al. 2005; Liu et al. 2009). Hence the native uricase is incompatible for administration into the human body.

A variety of drug delivery carriers has been explored in order to deliver uricase, such as alginate microencapsulated uricase (O'Loughlin et al. 2004), dextran-PEG-coupled uricase and erythrocyte encapsulated uricase (Magnani et al. 1992) etc. In a stimulating approach, Tan et al. (2012) have reported studies on the competence of applying novel alkaline uricase enzymosomes (functional lipid vesicles encapsulating uricase from *Candida utilis*) as carriers. They conducted studies on uricase enzyme kinetics, pharmacokinetics, pharmacodynamics, immunogenicity and preliminary safety of enzymozymes employed. They concluded that enzymosomes distinctly improved the biological properties and enhanced the hypouricemic effects of uricase. But the applicability of liposomes as drug delivery carriers can be expensive; liposomes are mostly taken up by macrophages and display organ accumulation in liver and spleen. Tiwari et al. (2015) developed polymeric nanoparticles of uricase and aceclofenac and incorporated them into a gel, for delivering drugs to synovial joints, for effective treatment of gout. Topical application of a gel containing polymeric uricase nanoparticles alone and in combination with aceclofenac nanoparticles in rabbit model test groups has been reported. In the same study this resulted in complete removal of urate crystals and inflammation within 40 days of treatment respectively. **Table 2.10** represents few examples of liposomal and polymeric drug delivery systems developed for uricase.

Table 2.10: A few examples of liposomal and polymeric drug delivery systems developed for uricase

Sl no	Source of Uricase	Conjugating polymer/ carrier	Size (nm) of native uricase	Size (nm) of uricase carrier	PDI	Reference
1	*Candida utilis*	Nanosized alkaline enzymosomes with uricase	-	260-330 nm	0.214-0.362	Zhou et al. 2016
2	Recombinant uricase from *Candida sp*	mPEG succinimidyl carbonate	10.9 nm	30.6 nm	0.21	Zhang et al. 2015
3	Recombinant uricase from *Candida sp*	Encapsulated in super-hydrophilic zwitterionic gel		31.3 nm	0.2	Zhang et al. 2015
4	Uricase	Uricase-multi vesicular liposomes		22. 56 µm		Deng et al. 2015
5	Uricase from the *Candida utilis*	Functional lipid vesicles encapsulating uricase		201.54 nm	0.14	Tan et al. 2012
6	Uricase from *Candida sp.*	poly(methyl acrylic acid-b-sulfobetaine methacrylate)	9 nm	12 nm		Lin et al. 2013

2.11.3 Bioconjugation of Uricase:

Bioconjugation of proteins with various polymers is of increasing concern in pharmaceutical chemistry for delivering small drugs and complex compounds such as peptides, enzymes, and oligonucleotides (Hermanson, 1996). Similarly, considering the case of uricase there are several reports on bioconjugation of uricase with natural and synthetic polymers like dextran (Fujita et al. 1991), polysialic acid (Punnappuzha et al. 2014), polyoxazoline (Viegas et al. 2011) etc. **Table 2.11** represents a review of various polymers employed previously for the bioconjugation of uricase enzyme.

Table 2.11: A review of various polymers employed for the bioconjugation of uricase

Sl no	Source	Conjugating polymer	Residual activity	Immunogenicity	References
1	*Candida utilis*	poly(*N*-vinylpyrrolidone) (6 kDa); poly(*N*-acryloilmorpholine) (6 kDa)	-	Organ accumulation of the polymers was observed	Caliceti et al. 1999
2	*Candida utilis*	poly(*N*-vinylpyrrolidone) 6 kDa; poly(*N*-acriloylmorpholine) 6 kDa; branched mPEG 10 kDa		Anti-poly(*N*-vinylpyrrolidone) and anti-poly(*N*-acryloyl morpholine) antibodies were generated after immunization	Caliceti et al. 2001

Sl no	Source	Conjugating polymer	Residual activity	Immunogenicity	References
3	Microbial Uricase	Polyoxazoline polymers with methyl (PMOZ), ethyl (PEOZ), and propyl (PPOZ) side chains	depended on extent of modification		Viegas et al. 2011
4	*Arthrobacter globiformis*	polysialic acid (colominic acid) from *Escherichia coli* KI	41-63 %	Conjugates showed a the decline in the affinity by 35% and also have retained double the catalytic activity than that of the native Uc after exposure to antiserum.	Punnappuzha et al. 2014

2.11.4 PEGylated Uricase and its commercial availability:

PEGylation, the process of attaching PEG to proteins and peptides, is an example of a highly successful strategy for improving the pharmacological properties of drugs. The most relevant changes of the protein molecule succeeding PEG conjugation are size enlargement, protein surface and glycosylation function masking, charge modification, and epitope shielding. In particular, size enlargement slows down kidney ultrafiltration and promotes the accumulation into permeable tissues by passive enhanced permeation and retention mechanism (Pasut and Veronese. 2007). PEG molecules possess unique properties such as (i) negligible toxicity and biocompatibility, (ii) high solubility in water and many other organic solvents, (iii)

high hydration and flexibility of polymer chain and (iv) FDA approval for human usage.

PEGylated uricases with low immunogenicity and long circulation half-life, have been under clinical investigation since the 1980s (Davis et al. 1981). There are several reports on the application of PEGylation strategy to uricase in order to enhance its pharmacological properties. The first report on PEGylation was published by Nishimura et al. in 1979, wherein Uricase from *Candida utilis* was modified with activated PEG-(2-O-methoxypolyethylene glycol-4,6-dichloro-s-triazine), where the conjugate displayed only 15% residual activity with a complete loss of the binding ability towards anti-uricase serum from rabbit. In 1981, Chen et al. covalently attached PEG to uricase derived from porcine liver and *Candida utilis*. Many researchers have attempted to synthesize PEGylated uricase conjugates. The chronological review for the development of PEGylated uricase is as indicated in **Table 2.12**.

Table 2.12: Chronological review for the development of PEGylated uricase

Source	PEGylating agent	Residual activity	Inference	References
Candida utilis	Activated PEG(2-O-mPEG-4,6-dichloro-s-triazine)	5-31 %	Complete loss of the binding ability towards anti-uricase serum from rabbit	Nishimura et al. 1979
Candida utilis	PEG, 5 kDa	27-87 %		Nishimaru et al. 1980

Source	PEGylating agent	Residual activity	Inference	References
Candida utilis	PEG		20 U of PEGylated uricase reduced urate to near zero on initial injection. After injection with four weekly doses of uricase. Unmodified uricase, was ineffective in lowering plasma urate levels. PEGylated uricase was as effective in the first injection	Abuchowski et al. 1981
Candida utilis	PEG, 5 kDa		PEGylation reduced immunogenicity in the body	Savoca et al. 1984
Candida utilis	PEG, 5, 7.5 and 10 kDa	3-32 %	Immunogenicity reduced	Tsuji et al. 1985
Arthrobacter protoformiae	mPEG		After the injection of PEG-uricase, uricase activity appeared in plasma rapidly, peaking within 24 hrs and persisting for 5 days. No antibody to either PEG-uricase or unmodified uricase developed over a 3-week period and PEG-uricase exhibited a long plasma half-life.	Chua et al. 1988

Source	PEGylating agent	Residual activity	Inference	References
Candida utilis	PEG 5 kDa		PEG increased the half-life from 1-8 hrs.	Fujita et al. 1991
Bacillus fastidious	PEG poly(*N*-acryloyl morpholine) (PAcM)	0-10 %	Increased residence time in blood for all the conjugates as compared with native uricase. Native uricase was rapidly removed from circulation.	Schiavon et al. 2000
Mice liver	PEG		Treatment of uricase-deficient mice with PEG-uricase markedly reduced uric acid levels and preserved the renal architecture. PEG-uricase was less immunogenic than native uricase.	Kelly et al. 2001
Candida utilis	poly(N-vinylpyrrolidone) poly(N-acriloylmorpholine) PEG		Both uricase antigenicity and immunogenicity were altered by PEG conjugation that depended on upon the polymer composition. In Balb/c mice, the native uricase elicited rapid and intense immune response, whereas all the conjugates induced a lower production of anti-native uricase antibodies.	Caliceti et al. 2001

Source	PEGylating agent	Residual activity	Inference	References
Candida utilis	PEG		PEG-20 kDa reduced antigenicity and increased circulating half-life as compared native uricase	Bomalaski et al. 2002
Candida species	PEG 5KDa	75 %	PEG-uricase conjugates were non-immunogenic and retain at least 75% of the uricolytic activity of the native uricase	Williams et al. 2003
Variety of sources	PEG		Uricase from *Candida utilis* had more favorable enzymatic properties and PEG 20 kDa had greatly reduced antigenicity and increased circulating half-life	Bomalaski et al. 2002
Recombinant mammalian uricase	mPEG 10 kDa		PEG immunogenicity needs a lot of investigation	Ganson et al. 2006
Recombinant mammalian uricase	PEG		PEG-uricase were greater than the bioavailability, efficacy and tolerability observed in a previous phase I trial of subcutaneous PEG-uricase.	Sundy et al. 2007

Source	PEGylating agent	Residual activity	Inference	References
Recombinant porcine-like uricase	PEG		Persistent reduction of plasma urate concentrations was in Phase 2 clinical trial.	Sherman et al. 2008
Recombinant uricase from *Candida sp.*	mPEG-*p*-nitrophenyl-carbonate and mPEG-4,6-dichloro-s-triazine		In rabbit and Balb/c mice, the native UC-r elicited an intense immune response being highly immunogenic. PEGylated UC-r, when injected chronically in mice, did not induce any detectable antibody response.	Freitas et al. 2009
Uricase	PEG		8 mg of Pegloticase every 2 weeks induced a lytic decrease of urate concentrations, leading to the dissolution of tophi in 40% of patients.	Reinders and Jansen. 2010
	mPEG 5 kDa		Modeled pharmacodynamics supported that the half-life of uricase and its susceptibility to xanthine are crucial for the pharmacological significance of uricase.	Feng et al. 2010

Source	PEGylating agent	Residual activity	Inference	References
Canine uricase	mPEG-SPA		Removal of the uricase aggregates and the PEG diol contaminant and modifying with small PEG reagents enabled accelerated blood clearance.	Zhang et al. 2010
Candida utilis	PEG		The uricase released from the carrier lasted over 12 hours and their circulation half-life was about sevenfold longer than that of the free uricase.	Tan et al. 2012
Uricase	polysialic acid (PSA) 10 kDa	46-80 %	The conjugates (when probed against anti-uricase antibodies generated in rabbit), showed a clean decline in the affinity by 35 %.	Punnappuzha et al. 2014
Bacillus fastidious	mPEG (5 kDa and 350 Da)	65 %	PEGylated Uricase showed a thermo-inactivation half-life greater than 85 hours and a circulating half-life of about 20 hours in rats *in vivo*	Zhang et al. 2010

Source	PEGylating agent	Residual activity	Inference	References
Recombinant porcine uricase	PEG		Every 3-week dosing is effective and may enhance the utility of Pegloticase for treating refractory gout.	Hershfield et al. 2014
Mammalian recombinant uricase	mPEG		Infusion-related reactions occurred in 94 (45%) of 208 patients receiving Pegloticase.	Baraf et al. 2014

Till date uricase has been PEGylated via the first generation PEGylation, involving random attachment of PEG chains to the abundantly available surface accessible lysine residues.

The most straightforward strategy for the covalent attachment of PEG chains on proteins employs attachment of the PEG chains to naturally occurring nucleophiles (i.e. amine groups (-NH$_2$), to the side chains of the amino acid, lysine) (Veronese, 2001). Due of the existence of several nucleophiles in a protein and the often observed requirement to use an excess of the PEGylation reagent for reasonable stoichiometric conversions, numerous lysine residues in a protein are modified. This results in the formation of a heterogeneous mixture of protein−PEG conjugates (PEGamers).

The first generation PEGylation exhibits the following disadvantages: a) lack of selectivity and applicability only to PEG chains of low molar mass (Roberts et al. 2002), loss of biological activity and unpredictable *invivo* behavior (Pasut and Veronese, 2007), c) heterogeneity of the conjugation site resulting in the production of mono, di and multi-PEGylated conjugates which possess divergent specific activities and hence different immunological implications *invivo* and d) cumbersome

purification process and thus impeded reproducibility of uniform conjugates at the synthesis level (Wang et al. 2002).

In October 2010, FDA approved Pegloticase (Krystexxa[®], formerly Puricase), a PEGylated recombinant porcine uricase, for patients with chronic gout refractory or intolerant to conventional therapies. Given the high cost of therapeutic uricase production and the additional cost engendered by PEGylation, high selectivity and yield are necessary for the development of new PEG–protein conjugates (Pfister and Morbidelli., 2014). Currently, PEGylated forms of uricase commercially available are PEGsitacase (Uricase-PEG 20) [EnzymeRx], Krystexxa™ (Pegloticase) [Crealta Pharmaceuticals LLC], Puricase[®] [Savient Pharmaceuticals], PEG 40-Uricase [Mountain View Pharmaceuticals]. Pegsiticase, a PEGylated recombinant uricase derived from *Candida utilis* is modified by the attachment of multiple 20 kDa strands of PEG from 3SBio, China. Phase 1 studies are completed in the United States with a single dose of Pegsiticase confirmed safety and efficacy in reducing plasma uric acid levels in refractory gout patients. The price of Rasburicase (Fasturtec[®]) is $ 1919.31 for a single 4.5 mg dose regimen and Pegloticase (Krystexxa[®]) (US $2,760 per bottle containing 8mg/mL in the USA).

2.11.5 Shortcomings of current commercially available PEGylated Uricase:

Repeated administration of PEGylated drugs also results in detectable antibodies specifically against mPEG moiety along with few antibodies being developed against the drug itself resulted in accelerated clearance (Cheng et al. 2000; Ganson et al. 2006). PEGylation of uricase did accomplish the aims of increasing the half-life and reducing the frequent dosage, (even monthly doses are appreciably better than placebo) but with a high frequency of antibodies against Pegloticase and hence poor clinical outcomes (Mcdonnell et al. 2014). PEG antibodies and intolerance to the infusion were also reported in some patients with gout treated with Pegloticase (Garay and Labaune, 2011). Animal studies clearly showed that PEG-uricases and some other PEGylated proteins might elicit antibody formation against PEG, which can accelerate the clearance of PEGylated proteins. However, 92% of patients developed antibodies and 58% of patients showed decreased urate-lowering efficacy after

repeated administration during clinical trials of Pegloticase (Krystexxa website and FDA, 2010). In patients with acute lymphoblastic leukemia treated with PEG-Asparaginase (PEG-Asnase), the presence of anti-PEG was very closely associated with rapid clearance of the conjugate (Garratty et al. 2008).

Pegloticase is a chimera of porcine and baboon liver uricases and is designed for the continuous treatment of refractory gout (Chohan and Becker, 2009). A study was reported on the antigenicity of PEG-Uricase by Ganson et al. in 2006. Single subcutaneous injections of PEG-uricase (4 to 24 mg) were administered to 13 subjects with hyperuricemia in 5 subjects. Appearance of relatively low-titer IgM and IgG antibodies against PEG-uricase was observed. These antibodies were directed against PEG itself rather than the uricase protein. During the initial intravenous infusion, Pegloticase has a circulation half-life of 3-7 days. In a similar study, Yang et al. in 2012 reported that Pegloticase, after continued treatment for three months biweekly at a therapeutic dose of 0.14 mg/kg body weight, elicited an immune response against mPEG in nearly 20% of patients. However, after 6 administrations at the required biweekly therapeutic dose of approximately 8 mg, an immune response developed against the mPEG moiety of Pegloticase in nearly 20% patients (Sundy et al. 2007; Yue et al. 2008).

In a report by Baraf et al. 2014, infusion-related reactions were studied with respect to the Pegloticase therapy. In their study, infusions of Pegloticase (8 mg) were administered biweekly or monthly, wherein all patients received prophylaxis and were tested for urate levels prior to each infusion. It was observed that infusion-related reactions occurred in 94 (45%) of 208 patients who received Pegloticase. 10 patients reported infusion reactions at first infusion and 84 during subsequent infusions. Most infusion reactions were rated mild or moderate; 7% were rated severe and 91% of all infusion reactions occurred in patients with pre-infusion serum uric acid concentrations greater than 6 mg/dL. It was concluded that there is a need to optimize the safety of using Pegloticase in clinical practice.

Because of the high prevalence of gout and the less relative success of Pegloticase, research efforts should be dedicated to investigate the immunogenicity of other PEG-uricases in development, such as Pegsiticase. Freitas et al. (2009) developed a recombinant uricase from *Candida sp.* using mPEG-npc or mPEG-CN.

When injected repeatedly in mice for 21 days, the uricase did not induce a detectable antibody response.

In a study conducted by Caliceti et al. in 1999, comparative pharmacokinetic and biodistribution investigation of uricase conjugated with different amphiphilic polymers was carried out. It was observed that the branched PEG derivative accumulated in preferentially in liver and spleen.

2.11.6 Improved approaches of Site-specificity and molecular manipulation:

New strategies relying on the exploitation of less abundant and chemoselective anchors are explored and reported (Kochendoerfer, 2005). This is known as 'Second generation approach', which involves site-specific incorporation of PEG polymers to proteins which target a particular type of amino acid. Frequently used reactive groups applied for PEGylation include N-terminal amino groups and C-terminal carboxylic acid, as well as residues of lysine, cysteine, serine, threonine, histidine, arginine, glutamic acid and aspartic acid (Pasut and Veronese, 2012).

A PEGylated uricase for chronic treatment of refractory gout under physiological conditions should have the high uricolytic activity. A minute quantity of PEG chains attached, along thermo-inactivation half-life and high residual activity after PEGylation. A reduction in immunogenicity is a key objective for the development of improved uricases (Garay et al. 2011). For continued use in the treatment of refractory gout, any PEGylated Uricase must have a therapeutic dose low enough to avoid eliciting an immune response after repeated administrations. Considering safe and continued administrations of a site-specific PEGylated human interferon-α- A for several years, a PEGylated uricase at a monthly therapeutic dose below 4μg/kg body weight may have promise for continued treatment of refractory gout (Yang et al. 2012). In clinical practice, the recommended bi-weekly intravenous dose of Pegloticase is approximately 0.14 mg/kg body weight (Schlesinger, 2011), which is 70 times of the threshold of the safe monthly dose with more PEG chains in PEGylated uricase than in a PEGylated interferon-α-a molecule. **Table 2.13** represents data on the dosage requirement of Rasburicase and Pegloticase (both randomly PEGylated). **Table 2.14** gives a comparison with the dosage levels of single

site-specific PEGylated Human Interferon α-A and Pegloticase. **Table 2.14** clearly indicates the merits of employing site specific PEGylation strategy to reduce dosage levels.

The complications encountered by the present commercially available uricase formulations like high dosage levels, undesirable immunogenicity caused by mPEG upon continuous administration and loss of uricolytic activity even after PEGylation can be overcome by site-specific PEGylation of Uricase. Only a few advances in the design of new PEG derivatives with lower immunogenicity than mPEG have been reported (Suo et al. 2010). Any PEGylated uricase for continued use in the treatment of refractory gout must have a therapeutic dose low enough to avoid eliciting an adverse immune response following repeated administrations.

Table 2.13: Dosage requirement of Rasburicase and Pegloticase (both randomly PEGylated)

Rasburicase (Elitek[®])	Pegloticase (Krystexxa[®])
Monthly intravenous infusions of 10 mg ↓ Can elicit an immune response (Vogt, 2005)	1) Biweekly therapeutic dose of 8 mg (Sundy et al. 2007) ↓ Immune response against m PEG. (Yue et al. 2008) 2) Biweekly IV dose of 0.14 mg/kg body weight. (Schlesinger, 2011)

Table 2.14: Dosage levels of single site-specific PEGylated Human Interferon α-A and Pegloticase

Pegloticase	PEG-Interferon α-a
Biweekly Dosage: 0.14 mg/kg body weight	Weekly Dosage: 4 µg
140 µg $\times$ 2	
In 1 week: 280 µg	In 1 week: 4 µg

2.11.7 Molecular Engineering and the future scope for development of uricase formulations:

In order to produce robust uricase mutants, molecular engineering should change with a highly active uricase with superior thermostability. They should possess a sufficient number of accessible amino acid residues capable of reaction with activated PEG derivatives, optimized via site-specific PEGylation (Yang et al. 2012). By manipulating the amino acid sequence, proteins can be customized to obtain site specific PEGylation at the desired site. Data of uricase structure and location of active sites on the surface of the molecule can help in designing and choosing PEGylating reagents in a way that the uricolytic activity is maintained and immunogenicity reduced due to lower degrees of PEGylation. Immunogenic sites on uricases can be predicted using bioinformatics tools.

The first technique for molecular engineering of an enzyme is rational design based on the detailed understanding of three-dimensional structure, the catalytic mechanism, and structure-activity and thermostability correlation of the enzyme (Lonsdale et al. 2010). In a patent filed by Braxton (1998), it has been reported that the knowledge of three-dimensional structure of the protein may not be mandatory for determining a suitable site for PEGylation. Asparagine residues (Asn) are glycosylated (n-glycosylation) and replacement of these Asn residues by cysteines residues, followed by cysteine-specific PEGylation produced proteins with significantly increased serum half-life (Braxton, 1998).

Site-directed mutagenesis has also been used to introduce free cysteine suitable for thiol coupling with PEG-maleimide. Yang et al. 2003, introduced free cysteine in human Fv fragment and further PEGylated it using PEG-maleimide. He et al. (1999) incorporated a cysteine group in trichosanthin by site-directed mutagenesis.

Up until now, there are very few reports available on the site-specific PEGylation of uricase and none using thiol group modification. Although, a research group led by Chen et al. 2008 worked on using genetic engineering techniques to incorporate unnatural amino acids in the uricase molecule for site-specifically modifying uricase with homogeneous glycosyl and PEG derivatives. Chen et al. (2008) developed a method for genetically incorporating p-azido-L-phenylalanine into

target protein in *Escherichia coli* in a site-specific manner utilizing a tyrosyl suppressor tRNA/aminoacyl-tRNA synthetase system. They substituted *p*-azido-L-phenylalanine for Phe170 or Phe281 in uricase. The products were purified and their enzyme activities were analyzed. In their further studies, they have reported the outcomes on the high-level production of uricase containing keto functional groups for site-specific PEGylation. In this report, an *E. coli*-based optimized system for the production of uricase with keto functional groups were incorporated efficiently and site-specifically. The p15A-based single-plasmid system compatible with most *E. coli* expression vectors and strains efficiently produced a high yield of uricase with pAcF incorporated site-specifically. Under optimized expression conditions, 24 mg/L mutant uricase was produced; corresponding to 40% of the yield of wild-type uricase. The mutant uricase was modified with methoxy-PEG-oxyamine (mPEG5K-ONH$_2$) in a site-specific manner. This method imparts new insights into structure-function relationship research and provides a proof that site-specific PEGylation can improve the pharmacological properties of uricase.

In a patent filed by Fan et al. (2013), the production of humanized uricase has been demonstrated, wherein the humanized recombinant uricase is a chimeric protein comprising of amino acids of non-human mammal uricase and amino acids of human uricase. The humanized uricase comprised the first 240 amino acids at the N-terminal from dog uricase sequence and the 241-304 from the human uricase amino acid sequence. Kratzer et al. (2013) developed a "human-like" uricase to exploit the possibility that a functional uricase with a high sequence identity to the human pseudogene would be recognized as self and would not elicit an immune response.

Sherman et al (2004 and 2011), have described the usage of a mutein of porcine uricase free of large aggregates of uricase which can be rendered substantially non-immunogenic by conjugation with a sufficiently small number of PEG strands for the retention of uricolytic activity. According to their studies, the conjugates formed by aggregate free uricase molecules were less likely to induce antibody formation and clearance in comparison to the PEGylated conjugates prepared by uricase preparations containing large aggregates. PEG molecules of various chain lengths and structures can be explored for PEGylation and their influence on the immunogenicity can be evaluated.

SCOPE AND OBJECTIVES

In order to overcome the shortcomings of commercially available PEG-Uricase therapeutics, we aim to synthesize, purify and characterize uniform site-specific PEGylated Uricase-mPEG-mal and Uricase-mPEG-prop conjugates. These conjugates are expected to retain most of their original uricolytic activity, possess enhanced stability and increased plasma half-life, exhibit reduced immunogenicity and less PEG organ accumulation.

Objectives:

1) Characterization of methoxy (polyethyleneglycol)-maleimide (mPEG-mal) and methoxy (polyethyleneglycol)-propionaldehyde (mPEG-prop) PEGylating agents.

2) Synthesis of site-specific PEGylated conjugates of Uricase from *Bacillus fastidious* using mPEG-mal and mPEG-prop via thiol and N-terminal PEGylation strategies.

3) Optimization of important PEGylation reaction parameters like Uricase to PEGylating agent concentration ratio, PEG molecular weight, pH of the reaction mixture, additive concentration to check their influence on the yield and uricolytic activities of the conjugates.

4) Purification of the Uricase-mPEG-mal and Uricase-mPEG-prop conjugates.

5) Characterization of the purified conjugates for the determination of their residual uricolytic activity, degree of modification, conjugate molecular weight, post-PEGylation conformational changes in the uricase structure and conjugate size.

6) Studies for determining the storage stability of Uricase-mPEG-mal and Uricase-mPEG-prop conjugates at various storage temperatures.

7) Determination of kinetic parameters of the Uricase-mPEG-mal and Uricase-mPEG-prop conjugates to test their affinity towards uric acid.

8) Immunogenicity analysis of the Uricase-mPEG-mal and Uricase-mPEG-prop conjugates to evaluate their therapeutic efficiency

3.1 Material, equipment and animals used:

Uricase from *Bacillus fastidious* (average molecular weight: 35 kDa; specific activity: 9 U/mg)*,* Bradford reagent, uric acid, uric acid sodium salt, L-Cysteine hydrochloride monohydrate, tris-hydrochloride, glycine, Ellman's reagent (5,5-dithiobis-2-nitrobenzoic acid, DTNB), phosphate buffered saline (PBS), methoxy(polyethylene glycol)-*p*-nitrophenyl carbonate (5 kDa) and methoxy(polyethylene glycol)-maleimide (mPEG-mal: 750 Da, 5 KDa and 10 kDa), Sephadex G-100, Sephacryl S-200, were procured from Sigma-Aldrich (India) and methoxy(polyethylene glycol)-propionaldehyde (mPEG-prop 10 and 20 kDa) were obtained from Jenkem Technology (China). EDTA sodium salt, boric acid, sodium hydroxide, acrylamide, bisacrylamide powder, β-mercaptoethanol, barium chloride, potassium iodide salt, TEMED (Tetramethylethylenediamine), perchloric acid, resublimed iodine, DTT and hydrochloric acid, bovine serum albumin, sodium dihydrogen phosphate, disodium hydrogen phosphate, vitamin B12 and blue dextran (2000 KDa) were procured from HiMedia Chemicals (India). Sodium cyanoborohydride and 2,4,6-trinitrobenzene sulfonic acid (TNBS) were obtained from Loba chemicals (India). HPLC grade acetonitrile and trifluoroacetic acid were obtained from Rankem Chemicals (India). Sodium dodecyl sulfate and glycerol were purchased from Merck India. Bromophenol blue dye, coomassie brilliant blue R-250 dye, methanol and acetic acid were obtained from Nice Chemicals, India. Ready-to-load medium range protein marker with a molecular weight range of 14 kDa-110 kDa was obtained from Bangalore Genei, India. Rabbit uricase ELISA (Enzyme linked immune sorbate assay) kit was procured from Genxbio Health Sciences Pvt. Ltd, India. Millipore grade deionized water was used for all the trials.

0.5 mL capacity Amicon protein concentrators with 10 kDa molecular weight cutoff (MWCO) were obtained from Merck Millipore (UK). 150 kDa MWCO (molecular weight cutoff) protein concentrators were procured from Thermo Fisher Scientific Company. 25, 40, and 60 ml glass burettes were purchased from Vensil Glass Company (India). A Zorbax Eclipse XDB-C18 Analytical Column (4.6X150 mm, 5µm) for RP-HPLC analysis was obtained from Agilent Technologies (USA). A Waters 2695 Separation module RP-HPLC system was used to resolve the

PEGylation reaction mixtures. ÄKTA pure Fast protein liquid chromatography system equipped with HI PREP 16/60 Sephacryl S-200 and AKTA AVANT FPLC system equipped with a Superdex 200 10/30 GL was used for purifying the PEGylation reaction mixture. Ultrasonication bath was obtained from Labman scientific instruments (India), HeidolphReax control vortex meter (Heidolph Instruments, Germany) and UV-Visible spectrophotometer (Hitachi, Japan) was employed for measuring the absorbance values of protein and uric acid quantification. A Mini-PROTEAN® SDS-PAGE System (Biorad, India) was used for gel electrophoresis. AlphaImager Mini® gel documentation system (Bioscreen Instruments, India) equipped with dual wavelength trans-illuminator and Alphaview analysis software was employed for molecular weight determination of native and PEGylated uricase. A JASCO-18 spectropolarimeter was used to generate the circular dichroism spectra of native and PEGylated uricase. The size of native uricase and PEGylated uricase conjugates were determined by dynamic light scattering experiments using a Malvern Zetasizer Nano ZS particle size analyzer (UK).

Around nine, New Zealand white rabbits (*Oryctolagus cuniculus*) were used to conduct the immunological study. Rabbits were procured from Central Animal House, Kasturba Medical College, Bejai and standard care was provided as per CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) guidelines. An ELx800 Absorbance ELISA Reader (BioTek, USA) was used to determine the antibody titer in ELISA experiments.

3.2 Methods:

3.2.1 Characterization of the PEGylating reagents:

3.2.1.1 Determination of degree of activation of mPEG-mal:

100 mL of 0.1 M sodium phosphate buffer (pH 8.0; 1mM EDTA sodium salt) was deoxygenated in an inert atmosphere by bubbling nitrogen gas for 30 minutes and subjected to ultra-sonication for 10 minutes. It was then used as the reaction medium for conducting PEGylation reactions. To 1 mL of 2 mM cysteine solution (prepared in the above buffer), 1 equivalent of mPEG-mal (10.2 mg) was added to 1 mL of 2 mM Cysteine) (Mero et al. 2011). The reaction mixtures were incubated at 4°C overnight for 14 hours. 1 mL of only 2 mM cysteine was also incubated under the same conditions as a control.

Thiol/free sulfhydryl concentration determination was performed using Ellman's assay, which is as described in **Appendix III**. Briefly, in the test solution 1, 50 µL of Ellman's reagent was added to 970 µL of the buffer solution and 30 µL of PEGylated cysteine sample and for test solution 2, 30 µL of cysteine sample was added instead of its PEGylated counterpart along with the other reaction components. In the blank solution, 30 µL of buffer solution was added instead of PEGylated cysteine solution as shown in **Table 3.1.**

The reaction mixtures were incubated for 15 minutes at room temperature and the % activation was calculated according to the formula:

$$\% \text{ Activation} = [1 - \frac{(\text{Abs412nm}) \text{PEGylated Cysteine Rxn Mix} - (\text{Abs412nm}) \text{Blank}}{(\text{Abs412nm}) \text{Cysteine Rxn Mix} - (\text{Abs412nm}) \text{Blank}}] \times 100 \%$$

Table 3.1 Preparation of test solutions for the Ellman's assay

Blank	Cysteine Standard Solution	Glycine Standard Solution
50 µL of Ellman's reagent	50 µL of Ellman's reagent	50 µL of Ellman's reagent
1000 µL of SP buffer	970 µL of SP buffer	970 µL of SP buffer
	30 µL of Cysteine std solution	30 µL of PEG-Cysteine solution

Also, the procedure for the calculation of the sulfhydryl and cysteine concentration determination is as described in detail in the **Appendix III**.

3.2.1.2 Determination of degree of activation of mPEG-prop:

For the preparation of PEG-Reaction mixture, one equivalent (2 µM) of mPEG-propionaldehyde was added (21.6 mg of mPEG-prop to 1 mL of glycine solution) to 1 mL of 2 mM glycine solution. The reaction mixture was incubated for 30 minutes at room temperature with continuous agitation.1 mL of 2 mM glycine solution was prepared as control and incubated at the same conditions as shown in **Table 3.2**. The reaction mixtures were incubated for 30 minutes after which their absorbance was read at 420 nm.

Trinitrobenzene sulfonic acid assay was performed in duplicates as described in detail in **Appendix II** section. The degree of activation (%) was calculated according to the formula:

$$\% \text{ Activation: } \left[1 - \frac{(\text{Abs of PEGylation rxn mix} - \text{Abs of Blank})}{(\text{Abs of glycine standard} - \text{Abs of Blank})}\right] \times 100\%$$

Table 3.2 Preparation of test solutions for the TNBS assay

Blank	PEG reaction mixture	Glycine Standard Solution
20 µL of TNBS solution	20 µL of TNBS solution	20 µL of TNBS solution
980µL of borate buffer	955 µL of borate buffer	955 µL of borate buffer
	25 µL of PEG-glycine reaction mixture	25 µL of Glycine std solution

3.2.2 Effect of EDTA concentration on the uricase activity and sulfhydryl concentration:

1 mg/mL of uricase (in 0.1 M deoxygenated sodium phosphate buffer pH 7.2) was incubated in aliquots of the above buffer with different concentrations of EDTA sodium salt (ranging from 0-10 mM) for 1 hour and 18 hours duration at 4°C. After the completion of incubation time, residual activities and the sulfhydryl concentrations of all the samples, were determined by uricase activity assay and Ellman's method respectively. The detailed procedures of uricase enzyme assay and Ellman's assay are described in **Appendix III**.

3.2.3 Synthesis for randomly PEGylated uricase conjugates:

Uricase from *Bacillus fastidious* (1mg/mL) was allowed to react with methoxy(polyethylene glycol)-*p*-nitrophenyl carbonate (mPEG-np) at different protein to polymer concentration ratios (1:5, 1:10, 1:15 and 1:20) (Uc:mPEG-np) in 0.1 M sodium borate buffer solution (pH 9.0). The reaction mixtures were incubated for 2 hours at 30°C with slight agitation. After incubation, the reaction mixtures were dialyzed overnight at 4ºC against 100mM sodium borate buffer (pH 9.0), using a pre-treated dialysis membrane (20 KDa-cutoff) [Pre-treatment of the dialysis membrane was carried out by incubating the membranes for 10 minutes in boiling water with 1mM EDTA]. The dialyzed sample was used as a source of PEGylated uricase.

The PEGylated conjugates were subjected to different colorimetric assay procedures for the confirmation of PEGylation namely: 1) Uricase enzymatic assay for determining the residual activity of the PEGylated conjugates (with the activity of native uricase was considered as 100%), 2) Bradford's assay for the determination of uricase concentration, 3) TNBS Assay for determining the degree of amine substitution at the amino acids (lysine) with free amino groups and 4) Iodine assay for the quantification of free and unreacted mPEG-np. The methodologies of all the colorimetric assays are described in detail in the **Appendix.**

PART I

3.2.4 Synthesis of Uc-mPEG-mal conjugates using thiol PEGylation strategy:

Two different methodologies were implemented for synthesis of Uc-mPEG-mal conjugates

i) With the reduction of disulfide bonds in uricase using DTT as a reducing agent [for the exposure of hidden free thiol groups] followed by PEGylation

ii) Without the reduction of uricase (using native uricase as a whole tetramer molecule) followed by PEGylation.

Uricase from *Bacillus fastidious* (1mg/mL) was allowed to react with mPEG-mal at different Uc to mPEG-mal concentration ratios (1:5, 1:10, 1:15 and 1:20) (Uc:mPEG-mal) at pH 7.2 according to the method of Mero et al. (2011).

Another set of reactions were carried out in deoxygenated buffer solutions of 0.1 M and 1 M buffer strength (with 5 mM of EDTA sodium salt), using 1:10 concentration of Uc:mPEG-mal. Sodium citrate (for pH 6.0), sodium phosphate (for pH 6.5-7.5), and HEPES buffer (for pH 8.0) were used. The reactions were carried out at 4ºC for 12-18 hours. After incubation, the reaction mixtures were dialyzed using a pre-treated dialysis membrane (20 kDa MWCO) against 100mM SP buffer, pH 7.2 at 4ºC. The dialyzed samples were used as a source of PEGylated uricase conjugates and the residual activities were determined. PEGylation was confirmed using SDS-PAGE method.

i) With reduction of uricase (using DTT as reducing agent)

The reduction of uricase was carried out according to the method described by Yang et al (2003). DTT was used as a reducing agent, in order to reduce the disulfide bonds connecting the four subunits of the uricase tetramer and expose the free thiol groups hidden in the uricase cleft.

The reduction solution containing 3 mg/ml of uricase was added with 2 mM DTT and 2 mM EDTA in 0.1 M deoxygenated SP buffer. The pH of the reduction mixture was adjusted to 7.8. The reduction was conducted at 37°C for 2 hours. After the completion of incubation, the free DTT was removed using centrifugal filtration using 0.5 mL capacity Amicon protein concentrators (10 kDa MWCO) at 13,000 rpm for 4 minutes. The concentrated and reduced uricase was diluted appropriately to a concentration of 1mg/mL and was allowed to react with mPEG-mal in different ratios of Uc:mPEG-mal [1:5, 1:10, 1:15 and 1:20] in 0.1M deoxygenated sodium phosphate EDTA buffer (pH 7.2). The reaction was conducted at 4°C for 12-18 hours with gentle stirring. After the completion of the reaction, the PEGylated reaction mixtures were concentrated by subjecting them to centrifugal filtration using 0.5 mL capacity Amicon protein concentrators (50 kDa MWCO) at 13,000 RPM for 4 minutes to aid the removal of unreacted mPEG-prop, reaction byproducts and for desalting of reaction mixtures. The concentrated samples were subjected to gel filtration chromatography using Sephadex G-100 and used as a source of PEGylated Uricase. The PEGylated enzyme was subjected to three different colorimetric assay procedures namely: 1) Uricase enzymatic assay for determining the residual activity of the PEGylated uricase, wherein the activity of native uricase was considered as 100%, 2) Bradford's assay for uricase quantification and 3) SDS-PAGE to confirm PEGylation.

ii) Without reduction of Uricase from *Bacillus fastidious*

Uricase from *Bacillus fastidious* (1 mg/mL) was allowed to react with mPEG-mal in various ratios of Uc:mPEG-mal [1:5, 1:10, 1:15 and 1:20] in 0.1 M deoxygenated SP EDTA buffer at pH 7.2 at 4°C for 8-16 hours with gentle stirring. After the completion of the reaction, the PEGylated reaction mixtures were concentrated using

centrifugal filtration. 0.5 mL capacity Amicon protein concentrators (50 kDa MWCO) were used at 13,000 rpm for 4 minutes to aid the removal of unreacted mPEG-prop, reaction byproducts and for desalting of reaction mixtures. The concentrated samples were subjected to gel filtration chromatography and used as a source of PEGylated Uricase. The PEGylated enzyme was subjected to three different colorimetric assay procedures namely: 1) Uricase enzymatic assay for determining the residual activity of the PEGylated Uricase, wherein the activity of native Uricase was considered as 100%, 2) Bradford's assay for uricase quantification and 3) SDS-PAGE to confirm PEGylation.

3.2.5 Optimization of thiol PEGylation reaction conditions:

Buffer media pH optimization: Uricase from *Bacillus fastidious* (1mg/mL) was allowed to react with mPEG-mal in the ratio of 1:15. The reactions were carried out in deoxygenated buffer solutions of 0.1 M and 1 M buffer strength. Sodium citrate (for pH 6.0), sodium phosphate (for pH 6.5-7.5), and HEPES buffer (for pH 8.0) were used. 5 mM of EDTA sodium salt was maintained in all the reaction buffers. The reaction mixture was adjusted to a final volume of 1 mL.

Optimization using Response surface methodology: The reactions were carried out at 4ºC for 12-16 hours. After incubation, the reaction mixtures were dialyzed using a pretreated dialysis membrane (20 kDa-cutoff) against 100mM sodium phosphate buffer, pH 7.2 at 4ºC. Uricase to mPEG-mal concentration ratio (wt/wt), the molecular weight of mPEG-mal (kDa) and the EDTA concentration (mM) in the reaction mixture were established to be significant parameters. These parameters which influenced the yield of the PEGylated uricase conjugate were predicted from preliminary one variable at a time (OVAT) experiments. **Table 3.3** represents the levels of variables for the Box–Behnken optimization design.

      For response surface methodology experiments, the values of the levels of all these factors were chosen according to several previous reports and preliminary experiments. Three levels were coded as -1, 0 and +1 which represented low, medium and high values. These three variables were optimized to maximize the product yield using a three-level and three-variable Box-Behnken statistical design using Design

expert®. The selected ranges of these variables were selected based on initial experiments and literature review. The optimum values of the parameters were determined by the response optimizer of the Design Expert® 8.0 software. **Table 3.4** represents design matrix for the optimization of reaction conditions

Table 3.3 The levels of variables for the Box–Behnken optimization design

Sl no	Variable	Variable Code	-1	0	+1
1	Uricase to mPEG-mal Ratio	A	1:5	1:10	1:15
2	Mol wt of mPEG-mal (Da)	B	750 Da	5 kDa	10kDa
3	EDTA concentration (mM)	C	0	5	10

Table 3.4 Design matrix for the optimization of reaction conditions

Run no	Uc:mPEG-mal ratio	mPEG-mal Mol wt	EDTA Conc
1	-1	-1	0
2	1	-1	0
3	-1	1	0
4	1	1	0
5	-1	0	-1
6	1	0	-1
7	-1	0	1
8	1	0	1
9	0	-1	-1
10	0	1	-1
11	0	-1	1
12	0	1	1
13	0	0	0
14	0	0	0
15	0	0	0
16	0	0	0
17	0	0	0

3.2.6 Preparation of Uc-mPEG-mal conjugates for optimization studies:

Aliquots of 100 mM SP buffer (pH 7.2) containing 0, 5 and 10 mM of dissolved EDTA sodium salt were deoxygenated by bubbling nitrogen gas (in an inert atmosphere) for 30 minutes and subjected to ultrasonication for 10 minutes. The resulting buffer solutions were used as the reaction medium for carrying out PEGylation reaction. Uc (1 mg/mL) in SP buffer was made to react with the mPEG-mal polymer of various molecular weights 750 Da, 5 kDa and 10 kDa separately. The reaction was carried out for 12-16 hours at 4°C with constant agitation. The PEGylated reaction mixtures were concentrated after the completion of the reaction using centrifugal filtration with 0.5 mL capacity Amicon protein concentrators (20 and 50 kDa MWCO). The reaction mixtures were subjected to centrifugal filtration at 13,000 RPM for four minutes to aid desalting, removal of unreacted mPEG-mal and byproducts.

3.2.7 Yield determination by Reverse phase High-performance Liquid Chromatography:

The yield of the Uc-mPEG-mal conjugates was determined using RP-HPLC according to the method described by Park and Na (2008). The PEGylation reaction mixture was resolved using a Waters 2695 Separation module RP-HPLC system. A mobile phase consisting of phase A (0.1% [v/v] TFA in water) and phase B (0.1% [v/v] TFA in ACN) was used for each run at a flow rate of 1 mL/min in an isocratic mode. The mobile phase was subjected to sonication for 5 minutes in an ultrasonication bath prior to analysis. The concentrated PEGylation reaction mixtures were appropriately diluted using 100 mM SP buffer (pH 7.2) to bring down the protein concentration to 250 µg/mL (as determined by Bradford method). 10 µL of the diluted PEGylation mixture was injected onto a Zorbax Eclipse XDB-C18 Analytical column (4.6X150 mm, 5µm pore size) maintained at 25°C. Chromatographic separation was carried out with a run time of 10 minutes. The proteins were detected using a UV detection system at 280 nm. Each trial was carried out in triplicates. The fractions containing purified Uc-mPEG-mal conjugates were

concentrated and freeze-dried after the evaporation of ACN using an Eppendorf SpeedVac$^®$ system.

The production yields (%) and relative amounts of Uc-mPEG-mal conjugates were obtained according to the method of Park and Na (2008), by dividing the peak area of each product by the peak area of native uricase at 250 µg/mL concentration. The purity of the Uc-mPEG-mal conjugates was assessed by SDS-PAGE.

3.2.8 Statistical analysis:

For predicting the optimal point, a second order polynomial function was fitted to correlate a relationship between independent variables (Uc:mPEG-mal concentration ratio, mPEG-mal molecular weight and EDTA concentration) and its response (conjugate yield). This **Eq 1** also gave the interactive effects of the variables, which is as follows,

$$Y= \beta o+ \beta_1 A+\beta_2 B+\beta_3 C+\beta_{12} AB+\beta_{13} AC+\beta_{23} BC+\beta_{11} A^2+\beta_{22} B^2+\beta_{33} C^2 \dots\dots\dots \textbf{Eq 1}$$

where Y is the predicted response, βo model constant, A, B, and C are independent variables, $\beta_1, \beta_2, \beta_3$ are linear coefficients, β_{12}, β_{22} and β_{33} are cross product coefficients and β_{11}, β_{22} and β_{33} are quadratic coefficients. The quality of the fit of the polynomial model equation is expressed by the coefficient of determination R^2.

The data of the yield of monoPEGylated uricase conjugate was subjected to analysis using Design Expert$^®$ 8.0 software for the Box–Behnken experimental design. Design Expert$^®$ 8.0 uses the least square regression analysis to fit a model equation to the given data set. For the response surface, a simplex search algorithm was used to determine the optimal points of the factors.

3.2.9 Purification of Uc-mPEG-mal conjugates using SE-FPLC:

After optimization of reaction conditions, the optimized values of the variables were considered to carry out the PEGylation reaction again with mPEG-mal (5 kDa). The reaction mixture was concentrated to 3-4 mg/mL using centrifugal filtration as

described previously. The reaction mixture was subjected to fractionation using SE-HPLC and further purified with a ÄKTA pure FPLC system equipped with an HI PREP 16/60 Sephacryl S-200 for purification of the uricase conjugates.

The column was equilibrated with 100 mM SP buffer (pH 7.2) and 100 µL of PEGylation reaction mixture was injected onto the column and the reaction components were eluted in an isocratic mode using the same buffer at a flow rate of 0.8 mL/min. The eluted fractions were detected at 280 nm. Unicorn$^{®}$ 6.4 software was employed for the chromatogram analysis and purified conjugates were collected and subjected to SDS-PAGE analysis for molecular weight determination.

3.2.10 Characterization of Uc-mPEG-mal conjugates:

3.2.10.1 Determination of residual activity by uricase enzymatic assay:

The comparison of residual activities of Uc-mPEG-mal conjugates with native uricase was done by uricase enzymatic assay according to the method of Mahler et al. (1955) described in **Appendix I**.

3.2.10.2 Protein quantification using Bradford reagent:

Bradford method of protein estimation was employed to quantify uricase for determining the specific activities of native and Uc-mPEG-mal conjugates. Briefly, to 0.1 mL of the native and Uc-mPEG-mal conjugate samples, 3 mL of Bradford reagent was added separately and the reaction mixtures were incubated for 10 minutes at room temperature. In the blank solution, 0.1 mL of 0.1 M SP buffer was used instead of the uricase sample. The absorbencies of the complex formed in the reaction mixtures were measured at 595 nm. The cuvettes were cleaned methodically after measurement of each sample with ethyl alcohol followed by a thorough rinse with deionized water and air dried.

3.2.10.3 Determination of degree of modification of Uc-mPEG-mal conjugates using Ellman's assay:

Ellman's assay was employed for the determination of the extent of modification and the % of free thiols present after PEGylation. Briefly, to 970 µL of 0.1 M sodium phosphate buffer (pH 8.0, 1 mM EDTA), 50 µL of Ellman's reagent (4mg Ellman's reagent dissolved in 1mL of reaction buffer) and 30 µL of the native and Uc-mPEG-mal samples were added to make various test solutions. For the blank solution, 30 µL of buffer solution was added instead of uricase solution. The reaction mixtures were incubated at room temperature for 15 minutes and their absorbencies were recorded at 412 nm. The percentage free thiol groups was calculated using the formula given below:

The percentage of free thiol groups $= \frac{\text{(Abs of PEGylated protein)} - \text{(Abs Blank)}}{\text{(Abs of native protein)} - \text{(Abs Blank)}} \times 100\ \%$

3.2.10.4 Determination of molecular weights of the Uc-mPEG-mal conjugates using polyacrylamide gel electrophoresis:

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method described by Laemmli (1970). Acrylamide-bisacrylamide mixtures were used for the preparation of gels. The percentage of gel was set as 12 % and 5 % for separating and stacking gels respectively. The procedure has been described in detail in **Appendix V**. The gels were stained with comaasie blue and viewed under a gel documentation system. The molecular weights of native and Uc-mPEG-mal conjugates were determined using a standard curve (protein molecular weight versus relative mobility of protein molecules present in the protein marker solution) provided by Bangalore Genei Company as shown in **Appendix V.**

For the determination and visual quantification of native and PEGylated uricase on the electrophoresis gel, the gel was stained using the barium iodide staining technique. This technique was adopted from the report of Kurfurst (1992). The procedure has been described in detail in **Appendix IV**.

3.2.10.5 Determination of conformational changes in uricase molecule after PEGylation using circular dichroism spectrometry:

Native uricase and Uc-mPEG-mal conjugates (1 mg/mL uricase concentration) dissolved in 10 mM SP buffer (pH 7.4) were used for analysis. Circular dichroism spectra were recorded between 190 and 260 nm using a JASCO J-810 spectropolarimeter, having a 0.1cm path length quartz cell (with a data pitch of 0.1 nm) at 20 $^\circ$C, 50 $^\circ$C and 70 $^\circ$C. The spectra of buffer blanks were measured and subtracted from the sample CD spectra. The far and near-UV CD spectra were analyzed in terms of α-helix content in the range 190-260 nm.

3.2.10.6 Size analysis of native and Uc-mPEG-mal conjugates:

The apparent size (the intensity weighted mean diameter) of the native and Uc-mPEG-mal conjugates was measured using Malvern particle size analyzer (UK) at a temperature of 25°C.

3.2.10.7 Storage stability of native uricase and Uc-mPEG-mal conjugates:

The storage stability of native uricase and Uc-mPEG-mal conjugates was evaluated by incubating them separately in phosphate buffered saline (PBS) (pH 7.5) at different temperature conditions (4°C, 25°C and 37°C) for several days. The enzyme activity of all the samples was determined every alternate day till the samples exhibited negligible/zero enzymatic activity.

3.2.10.8 Determination of kinetic parameters:

The K_m values of native uricase and Uc-mPEG-mal conjugates were estimated by the double reciprocal plot method. Using different substrate (uric acid) concentrations (0–5 mM), the uricase activity was assayed as described in the **Appendix I**. The K_m and V_{max} values were calculated by the Lineweaver–Burk plot.

3.2.10.9 Immunogenicity analysis of the native uricase and Uc-mPEG-mal conjugates to evaluate their therapeutic efficiency:

Nine New Zealand white rabbits (*Oryctolagus cuniculus*) were used in the present study. Permission for conducting the animal study was obtained from the Institutional animal ethics committee at Kasturba Medical College (KMC), Mangaluru. Rabbits were procured from Central Animal House, KMC, Bejai, Mangaluru and standard care was provided to animals as per CPCSEA guidelines.

Rabbits were randomly assigned to 3 groups consisting of 2 rabbits each: Group 1 (NC) was administered with 100 µg uricase in 0.5 mL of 100 mM PBS (phosphate buffered saline) subcutaneously. Group 2 (Nat-Uri) was administered with 0.5 mL of native uricase (in 100 mM PBS) subcutaneously and Group 3 (Uc-mPEG-mal) was administered with 0.5 mL of PEGylated uricase (in 100 mM PBS) subcutaneously on day 0. Subcutaneous boosters were administered on 7, 14, 21 and 28th day (100 µg uricase in 0.5 mL of PBS and the control group received an equivalent volume of PBS as placebo). Blood samples were collected from a marginal vein on day 35 from all the animals. The blood was collected in EDTA (anticoagulant) coated sealed tubes and centrifuged at 2000-3000 RPM for 20 minutes. The serum was collected, stored at -20°C and later used for determining the antibody titer using ELISA technique. At the end of the study, animals were rehabilitated and returned to Central Animal House, Bejai, Mangaluru.

For *invitro* ELISA experiments, the antibody coated ELISA plate and all the reagents from the kit were held at room temperature for 30 minutes once removed from 4°C environment. The standard solution (anti-uricase antibody generated in rabbits-320ng/ml) was subjected to serial dilution. 48-well, microplates were used for conducting the ELISA experiment for the determination of anti-uricase antibody titer. The blank well consisted of only chromogen reagent A & B, wherein the stop solution was added without the sample along with an anti-uricase antibody labeled with biotin and streptavidin-HRP. To the standard solution wells, 50µl standard solutions and 50µl streptomycin-HRP were added. To the sample wells, 40µl sample, 10µl uricase antibodies and 50µl streptavidin-HRP were added. The plate was covered with a seal plate membrane, incubated at 37°C for 60 minutes and gently shaken.

Each well was filled with a washing solution and drained after 30 seconds. For color development, 50µl chromogen reagent A was added to each well followed by the addition of 50µl chromogen reagent B to all the wells. The plate was incubated with slight agitation for 10 minutes at 37°C in dark (away from light for color development). 50µl of stop solution was added to each well to stop the reaction. The measurement of absorbance (OD) of each well was carried out one by one at 450 nm wavelength within 10 minutes after having added stop solution. The absorbance value of blank well was set to zero for the assay. All the corresponding OD values were calculated and the linear regression equation of the standard curve was generated using Microsoft Excel. All the data was expressed as mean ± SEM (Standard error of mean). The antibody titer (ng/mL) of all the samples were calculated according to the calibration curve generated using the standards.

PART II

3.2.11 Synthesis of Uc-mPEG-prop conjugates using N-Terminal PEGylation strategy:

Uricase from *Bacillus fastidious* (Uc) (1mg/mL) was allowed to react with mPEG-prop (10 and 20 kDa) at concentration ratios (wt/wt) of 1:5, 1:10, 1:15 and 1:20 in SP buffer solution (pH 5.0). After an hour of incubation at 4°C, 20 mg of sodium cyanoborohydride powder (reducing agent) was added to each vial and the reaction was continued at 4°C overnight with slight agitation. The PEGylated reaction mixtures were concentrated using 0.5 mL capacity Amicon protein concentrators (50 kDa MWCO) and centrifuged at 13,000 rpm for 4 minutes. After the concentration, the reaction mixture was immediately diluted using SP buffer of pH 7.4.

The degree of amine substitution in the uricase molecule after N-Terminal PEGylation was estimated using trinitrobenzene sulfonic acid (TNBS) method as described in **Appendix II** according to the method of Habeeb (1966). Briefly, 250 µL the native uricase and Uc-mPEG-prop conjugate samples made in SP buffer (pH 7.2) were added with 250 µL of 0.01% TNBSA and mixed well. The reaction mixtures were incubated at 37°C for two hours. After incubation, 250 µL of 10% SDS and 125

µL of 1 N HCl were added to each sample. The absorbance of the resulting mixture was measured at 335 nm. In the blank solution, 250 µL of phosphate buffer (pH 7.2) was added instead of the protein sample. The percentage substitution of the uricase was calculated using the formula given below:

$$\text{Degree of amine substitution (\%)} = [1 - \frac{(\text{Abs of PEGylated protein}) - (\text{Abs Blank})}{(\text{Abs of native protein}) - (\text{Abs Blank})}] \times 100\%$$

3.2.12 Optimization of reaction conditions to maximize the yield of Uc-mPEG-prop conjugates:

Uricase and mPEG-prop conjugates were allowed to react at different molar ratios of 1:1, 1:5, 1:10 and 1:15 (wt/wt) in 0.1 M SP buffer (pH 5.0). The conjugation reaction was carried out for 16 hours at 4 °C.

In order to find the optimum reaction pH, the reactions were carried out (using 1:10 Uc: mPEG-prop concentration ratio) at different pH values in the range of 4-7. The conjugate yield was determined using the RP-HPLC method described by Park and Na (2007) described in Section **3.2.7**. Briefly, the concentrated PEGylation reaction mixtures were resolved using a Waters 2695 Separation module RP–HPLC system. 10 µL of PEGylation mixture was injected onto a Zorbax Eclipse XDB-C18 Analytical column (4.6*150 mm, 5 µm pore size) maintained at 25 °C. The proteins were detected using a UV detection system at 280 nm, wherein all the trials were carried out in triplicates. The yields were obtained by dividing the peak area of each product by the peak area of native uricase.

3.2.13 Purification of Uc-mPEG-prop conjugates:

Preliminary purification using centrifugal ultrafiltration and SE-HPLC was carried out using the methodologies described in section **3.2.9.** Briefly, AKTA AVANT FPLC system equipped with a Superdex 200 (10/30) column (maintained at 4°C) was used for purification of PEGylation reaction mixture. The column was equilibrated with SP buffer (100 mM; pH 7.4) as a mobile phase at a flow rate of 0.5 mL/min. 500 µL of

PEGylation reaction mixture was injected onto the column along with the mobile phase in an isocratic mode and the eluted fractions were detected at 280 and 214 nm. Unicorn® 6.4 software was employed for the chromatogram generation and analysis. The eluted fractions containing Uc-mPEG-prop conjugates were concentrated using Eppendorf SpeedVac® system and stored at -20°C for further used. The eluted fractions were also subjected to SDS-PAGE analysis for molecular weight determination.

3.2.14 Characterization of Uc-mPEG-prop conjugates:

3.2.14.1 Determination of residual uricolytic activity:

The comparison of residual activities of Uc-mPEG-prop conjugates with native uricase was carried out by uricase enzymatic assay according to the method of Mahler et al. (1955).

3.2.14.2 Molecular weight determination and Size analysis of native uricase and Uc-mPEG-prop conjugates:

Molecular weight was determined using SDS-PAGE techniques as described in **Appendix.** The size/hydrodynamic radii of native uricase and Uc-mPEG-prop conjugates were determined using Malvern particle size analyzer (UK).

3.2.14.3 Determination of conformational changes in uricase molecule after PEGylation using circular dichroism spectrometry:

The procedure was carried out as described in section **3.2.10.5**. Briefly, native uricase and Uc-mPEG-prop conjugates (1 mg/mL) in 10 mM SP buffer (pH 7.4) were used for CD spectrometer analysis. Circular dichroism spectra were recorded between 190 and 260 nm using a JASCO J-810 spectropolarimeter with a data pitch of 0.1 nm (0.1cm path length quartz cells) at 20 °C, 50 °C and 70 °C.

3.2.14.4 Determination of kinetic parameters:

The K_m values of native uricase and Uc-mPEG-prop conjugates were estimated by the double reciprocal plot method using various concentrations of uric acid (0–5 mM). The K_M and V_{max} values were calculated by the Lineweaver–Burk plot.

3.2.14.5 Evaluation of storage stability of native uricase and Uc-mPEG-prop conjugates:

The storage stability of native uricase and Uc-mPEG-prop conjugates were evaluated by incubating the conjugate in SP buffer (pH 7.5) at different temperatures (4°C, 25°C and 37°C) for 18 days. The enzyme activity of all the samples was determined periodically till the samples exhibited zero activity.

3.2.14.6 Immunological evaluation of native uricase and Uc-mPEG-prop conjugates:

Generation of antibodies in rabbits and measurement of antibody titer using ELISA was carried out using the methods described in Section **3.2.10.9**.

All the experiments were performed as per the methodologies described in **Chapter 3** for accomplishing the objectives of the present research work. This chapter presents the results of the experiments including determination of degree of activation of the PEGylating agents used (mPEG-mal and mPEG-prop), synthesis of site-specific PEGylated uricase conjugates using thiol and N-terminal PEGylation, optimization of the reaction conditions for enhancing the yields of PEGylated uricase conjugates, purification of the synthesized conjugates, their characterization, conjugate storage stability analysis and immunogenicity analysis. Results were presented in the form of tables and figures wherever necessary. Detailed discussion on the results with proper justification and literature support are also presented in this chapter.

4.1 Characterization of PEGylating agents:

In this research work, mPEG-maleimide (Mol Wt: 750 Da, 5 kDa, and 10 kDa) and mPEG-propionaldehyde (Mol Wt: 10 and 20 kDa) were used as PEGylating agents. The quality of PEGylating reagents used (with desirable properties) is a crucial parameter to achieve reliable quality of the final PEGylated drug conjugate and its consistent reproducibility. Consistent methods for characterizing PEGylating reagents and PEGylated drug conjugates at various stages of PEGylation process are crucial for the development of an efficient and successful process. The following properties were considered to assess the quality and characterize PEGylating agents.

4.1.1 Polydispersity Index:

The polydispersity index (PDI), is a measure of the distribution of molecular mass in a given polymer sample. Biological systems are very sensitive to polydispersity and molar mass of the polymers used in drug delivery carriers (Dhal et al. 2009; Barz et al. 2011). Hence, monodispersed/PEGylation reagents (with very low PDI values) are preferred as PEGylating agents for biologics. A PDI value of less than 1.1, ensures acceptable homogeneity, better the plasma half-life with easily reproducible immunogenicity (Pasut and Veronese 2007).

4.1.2 Molecular weight of PEGylating reagents:

As PEG is highly hydrophilic, the molecular weight of PEGylated proteins drastically increases due to binding of water molecules, followed by a sharp increase in hydrodynamic radius (Kontermann 2012). For a large molecule like uricase, evaluation with PEGylation reagents of a wide variety of molecular weight can help in analyzing and controlling conjugate size.

Considering the case of PEGylated uricase, Williams et al. (2010) demonstrated that PEGylation of uricase from various microbial sources with only a few strands of 5 kDa PEG led to size enlargement and marked reduction in the catalytic activity. The number of 5 kDa PEG strands which inactivated uricase by

50%, was smaller than the number needed to confer a long half-life as studied in rodents. Similar results have been reported in the literature dealing with preparation of 5 kDa PEGylated conjugates of uricases from various sources (Nishimura et al. 1981; Abuchowski et al. 1981; Chen et al. 1981; Davis et al. 1981; Tsuji et al. 1985; Schiavon et al. 2000).

Taking these factors into account, PEGylating reagents namely mPEG-mal (Mol Wt: 750 Da, 5 and 10 kDa) and mPEG-prop (Mol Wt: 10 and 20 kDa) with PDI was in the range of 1.02 to 1.05 were used.

4.1.3 Determination of degree of activation of PEGylating reagents:

The degree of activation of the PEGylating agent is one of the most important parameters decides the exact amount of PEG required for the PEGylation reaction (Mero et al. 2011). In the present study, the degree of activation of a PEGylating agent is defined as the percentage of PEG units with an active maleimide/propionaldehyde functional group in a given amount of PEGylation reagents.

As per the literature, the preferred degree of activation of the PEGylating agent should be in the range of 70-90% for obtaining maximum yield of the PEGylated conjugate (Mero et al. 2011). **Table 4.1** represents the degree of activation (in percentage) of the thiol-reactive mPEG-mal polymer (determined using Ellman's assay) and N-terminal amino group reactive mPEG-prop (determined using TNBS assay).

It can be concluded that all the PEGylating reagents employed in the present study possessed desired values of the degree of activation. The degree of activation also indicates the ratio of uricase to a mPEG concentration to be considered for site-specific PEGylation studies of uricase.

Table 4.1: Determination of degree of activation of mPEG derivatives

Sl no	PEGylating agent	Molecular weight	Strategy of PEGylation	% Degree of activation
1	mPEG-mal	750 Da	Thiol	82.34
2	mPEG-mal	5 kDa	Thiol	78.34
3	mPEG-mal	10 kDa	Thiol	88.78
4	mPEG-prop	10 kDa	N-Terminal	70.58
5	mPEG-prop	20 kDa	N-Terminal	73.53

4.2 Effect of EDTA concentration on the uricase activity and thiol group/ sulfhydryl concentration:

EDTA sodium salt is frequently used in thiol PEGylation reactions as it chelates the divalent metal ions. These metal ions can otherwise oxidize the sulfhydryl groups on the cysteine residues of the protein molecule (from −SH to −SHO) (Riddles 1979). After the addition of EDTA in thiol PEGylation reactions, the unoxidized sulfhydryl groups (-SH) are then available for reaction with the maleimide moiety of mPEG-mal molecule. This reaction results in the formation of a very stable thioether bond between the maleimide moiety of mPEG-mal and the cysteine residue of the protein molecule (Yang et al. 2003). In a spectrophotometric method reported by Alexander (1958) in a solution of cysteine (0.003 M; pH 6.8; without EDTA), 30 % of the sulfhydryl content was oxidized within 45 minutes. But there was no decrease in the sulfhydryl content during the first 60 minutes in the presence of EDTA. Hence, it is essential to maintain 1-5mM of EDTA concentration in the buffer system while quantifying the amount of cysteine in protein solutions (Riddles et al. 1979; Trivedi et al. 2009).

Figure 4.1 shows the effect of EDTA concentration on the thiol group/sulfhydryl concentration of uricase from *Bacillus fastidious*. In the present study, upon incubation of uricase in 0.1 M SP buffer solution (with 7 mM EDTA), the uricase molecules were found to possess maximum amount of sulfhydryl groups at the 0'th

hour of incubation. After 18 hours of incubation, the thiol concentration was observed to be less than that observed at the 0th hour irrespective of the EDTA concentrations. This considerable decrease may be due to the oxidation of the uricase, which possibly occurred during the incubation period. For 1 mg/mL of uricase considered for the present study, lower concentrations of EDTA (up to 7 mM) were found to be ineffective in maintaining the thiol group concentration of the cysteine molecules. **Table 2.8** represents a few reports on the EDTA concentration used in thiol PEGylation reactions, which directly indicates that an EDTA concentration in a range of 7-10 mM is imperative for the maintenance of free thiol groups (Long et al. 2006; Balan et al. 2007; Shaunak et al. 2006). Hence, all the further experiments were carried out in a buffer solution with 7 mM EDTA concentration for the maintenance of free thiol content and uricolytic activity.
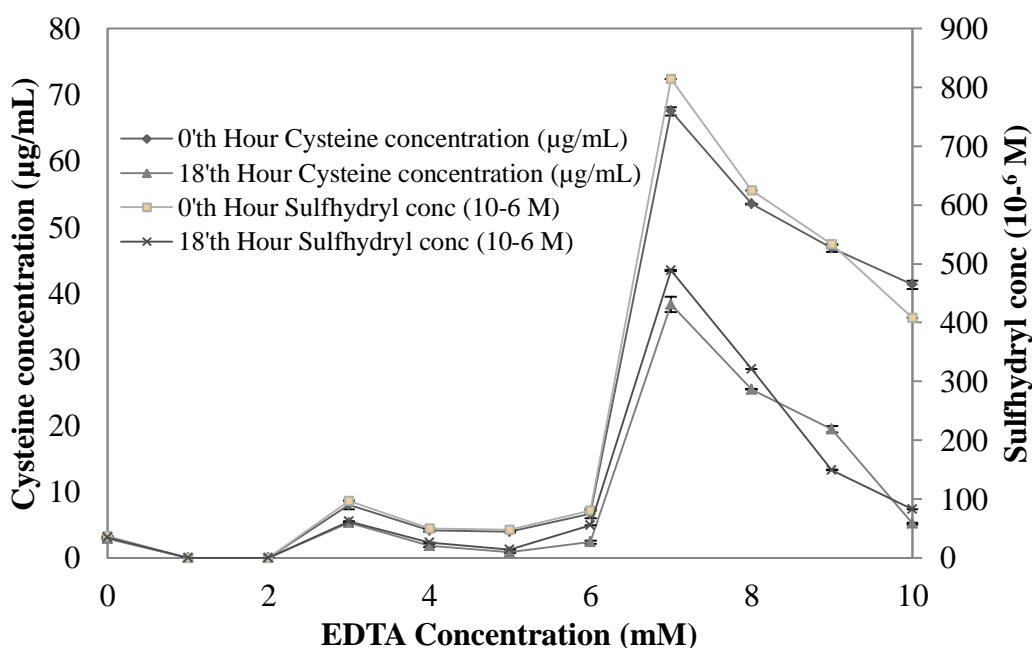
Figure 4.1: Effect of EDTA concentration on thiol group content of uricase

According to the literature **(Table 2.3)**, thiol PEGylation reactions are generally carried out for a time period of 3-18 hours at 4°C. **Figure 4.2** indicates the effect of incubation time of uricase in SP buffer with different EDTA concentrations on the uricolytic activity of uricase. The effect was studied at $0^{,th}$ hour and $18^{,th}$ hour of incubation in the SP buffer. It can be observed that at the $0^{,th}$ hour of incubation (in

the absence of EDTA), uricase possessed its maximum activity but with progression in time and increasing EDTA concentration, there was a gradual decrease in the uricolytic activity. Hence in the present study, 7 mM EDTA concentration was used for all further studies to maintain uricolytic activity as well the thiol group concentration. In a report by Aly et al. (2013), uricase from *Streptomyces exfoliates* retained only 50% of its uricolytic activity upon incubation in a buffer solution with 20 mM EDTA. In another report by Atraqchi et al. (2012), uricase from *Pseudomonas aeruginos*a retained only 85.2 % residual activity upon incubation in buffer containing 5 mM EDTA salt. EDTA can have a negative influence on the residual activity, but its presence is important to function as a chelating agent and maintain free thiol groups.
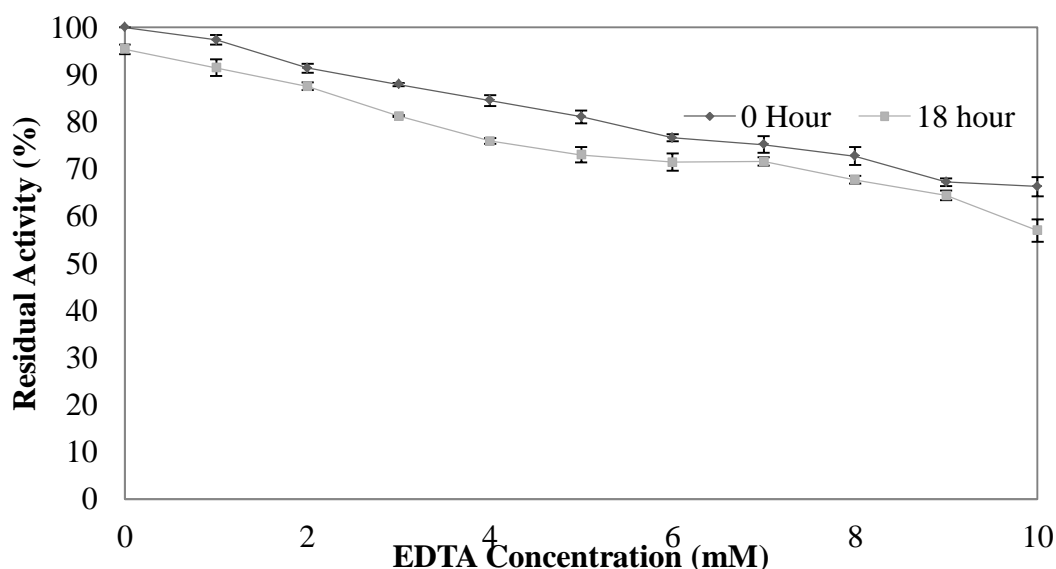
Figure 4.2: Effect of incubation time and EDTA concentration on uricase activity

4.3 Synthesis of random PEGylated uricase conjugates:

Uricase from *Bacillus fastidious* was PEGylated in a random manner/first generation PEGylation using methoxyPEG-*p*-nitrophenyl carbonate (mPEG-npc, 5 kDa) as a PEGylating agent. During random PEGylation, a urethane linkage was formed between the amine group of a surface accessible lysine residue of the uricase molecule and the terminal 4-nitrophenyl carbonate group (npc) derivative of PEG

molecule (Williams et al. 2010). The reaction releases a *p*-nitrophenol moiety as a byproduct, which results in the development of pale yellow color in the reaction mixture. The intensity of this colored byproduct increases with the extent of conjugation (Veronese 1985) and is measured using spectrophotometry.

Figure 4.3 (a) represents the reaction mechanism of urethane link formation between the amine group of the surface accessible lysine residue and the *p*-nitrophenol moiety of the mPEG-np molecule. **Figure 4.3 (b)** indicates the release of the yellow color of *p*-nitrophenol as a byproduct during the electrophoretic migration of the randomly PEGylated conjugates.

The uricolytic activities of the random conjugates obtained after fractionation of reaction mixture through a Sephadex S-200 column are as shown in **Table 4.2.** The uricolytic activities of the conjugates were compared to that of the native uricase, by considering the activity relative to that of the native uricase (considered as 100%).
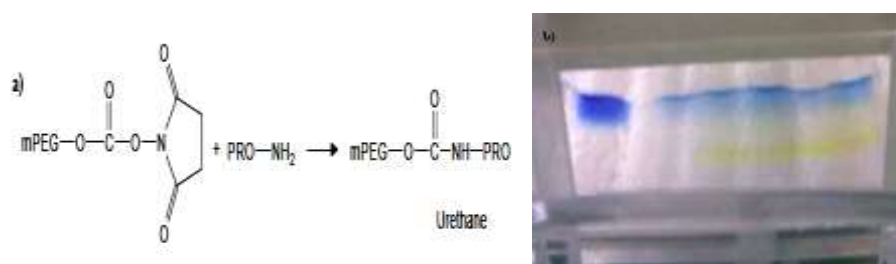
Figure 4.3: (a) Urethane bond formation during random PEGylation and (b) *p*-nitrophenol (yellow) released as a reaction byproduct of random PEGylation reaction

The residual uricolytic activities of the randomly conjugated uricase were reduced by almost 90% when uricase and mPEG-prop were reacted at a concentration ratio of 1:10. In this ratio, the conjugates were observed to retain only 10.2% of uricolytic activity. In various PEGylation reaction mixtures with different ratios of uricase to mPEG-npc concentrations ratios tested, the conjugates were found to retain very less residual activities (ranging from 62.7-10.2 %) as represented in **Table 4.2.**

Table 2.13 in the literature review section gives a review of reports on the loss of residual activities encountered by randomly PEGylated uricase obtained from various

microbial sources. PEGylation at multiple sites of the uricase molecules results in masking of active sites and consequently reduces their residual activity. Also, when the surface accessible amino acid residues are in close proximity, attachment of mPEG to a single lysine residue would produce steric hindrance for the neighboring amino acids, leading to blockage of active sites (Caliceti et al. 2010). In a report by Zhang et al. (2010), random PEGylation of recombinant uricase from *Bacillus fastidious* with NHS esters of mPEG-5000 resulted in the conjugates retaining only 65% of residual activity. Similar results have been reported by Nishimaru et al. (1979;1981) and Abuchowski et al. (1981) wherein severe loss in the uricolytic activities were observed following random PEGylation, as a result of masking of active sites and steric hindrance. Schiavon et al. (2000) have reported a complete loss in uricolytic activity following random PEGylation with linear PEG (5 kDa).

Table 4.2: Residual uricolytic activities for randomly conjugated Uc-mPEG-npc

Uc-mPEG-npc	Trial 1	Trial 2	Residual Activity (%)
Unmodified Uricase	97.65	100	98.825
1:5	64.86	60.57	62.73
1:10	10.84	9.58	10.21
1:15	19.96	25.38	22.67
1:20	16.87	9.85	13.36

The results obtained in the present study indicate that the possession of such low residual activities is not desirable for invivo administration of PEGylated uricase conjugates for therapeutic purposes. Also, due to the attachment of a large number of mPEG strands, there could be a major possibility for induction of PEG-associated immunogenicity upon its *invivo* administration.

4.3.1 Determination of percentage amine substitution of random PEGylated uricase conjugates by TNBS Assay:

The degree of modification of PEGylated uricase defines its overall pharmaceutical efficiency like residual uricolytic activity, plasma half-life, immunological properties and bio-compatibility. Binding of the mPEG at various sites resolves the overall degree of conjugate modification wherein the binding depends on operating variables like temperature, agitation and duration of the process. Also, the concentration of mPEG reagent used for the reaction is a significant operating variable for evaluation of the degree of modification.

In the present study, TNBS assay was used for the determination of percent degree of modification after random PEGylation of the uricase molecule. The percentage of amine substitution for all the conjugates was found to be in the range of 70-90%, which resulted in severe loss of uricolytic activities of the random PEGylated conjugates. The amine content displayed by the native uricase was considered as 100 %. The degree of modification was independent of the concentration ratio of uricase to mPEG-npc used for PEGylation. As the native uricase molecule possessed many surface-accessible lysine residues, PEGylation created a steric interruption between the adjacent PEG molecules attached to the surface.

The number of amino groups substituted as a result of PEGylation can be directly related to the residual uricolytic activity of the conjugates **(Table 4.3).** Also, a report by Freitas et al. (2010) advises that the structure of uricase endorsed a steric hindrance between PEG molecules on the surface of uricase. **Table 4.3** represents the percentage amine substitution in randomly PEGylated conjugates.

In a study reported on random PEGylation of recombinant uricase from *Candida sp* with mPEG-npc and mPEG-dichloro-s-triazine, the percentage degree of modification (determined using TNBS colorimetric assay) was found to be 20 % after conjugation (Freitas et al. 2010). Punnappuzha et al. (2014) have reported random conjugation of uricase from *Arthrobacter globiformis* with polysialic acid of 10 kDa molecular weight. In their report, four different uricase to PSA ratios (1:50, 1:100, 1:150 and 1:200) were tested, wherein the extent of modification was reported as 46.29 %, 66.03

%, 78.54 % and 80.02 % respectively which was directly proportional to the concentration of polymer used.

Table 4.3: Percentage amine substitution in randomly PEGylated conjugates

Uc:mPEG-npc (wt /wt)	Average % amine substitution
1:5	69.99
1:10	91.0
1:15	87.88
1:20	88.83

PART I

4.4 Synthesis of Uc-mPEG-mal conjugates using thiol PEGylation strategy:

In this section, site-specific PEGylation of uricase from *Bacillus fastidious* using mPEG-mal as a PEGylating reagent has been described. **Figure 4.4** represents the reaction scheme for thiol PEGylation of protein molecules at the free thiol group of a cysteine residue using mPEG-mal. Cysteine residues are valued targets for site-specific modification of proteins, as they are available in their free form at a relatively lower profusion in comparison to the oxidized cysteine species (Fodje and Al-Karadaghi 2002). They offer an excellent scope for the synthesis of homogeneous and uniform PEGylated conjugates. Based on the studies on the structures and primary sequence of uricase from various strains of *Bacillus fastidious* (PDB Id: 4R8X and 4R99) the number of cysteine molecules with free –SH group/thiol group ranged from 1-2 groups per subunit in a single uricase molecule (Feng et al. 2015).
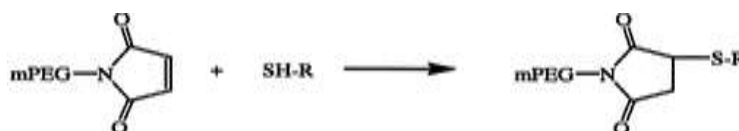
Figure 4.4: **Reaction scheme for thiol PEGylation at the free thiol group of a cysteine molecule**

In the present work, Uc-mPEG-mal conjugates were synthesized using two methods. In the first method, uricase was directly PEGylated with mPEG-mal and in the second method native uricase was reduced to single subunits using a very strong reducing agent DTT. DTT was used to reduce the uricase homotetramer into single subunits in order to expose the hidden thiol (-SH) groups from the protein cleft and make the free thiols more accessible to mPEG-mal chains for PEGylation reaction to start. The synthesis was confirmed through SDS-PAGE analysis (**Figure 4.5**) taking into account the increased molecular weight.
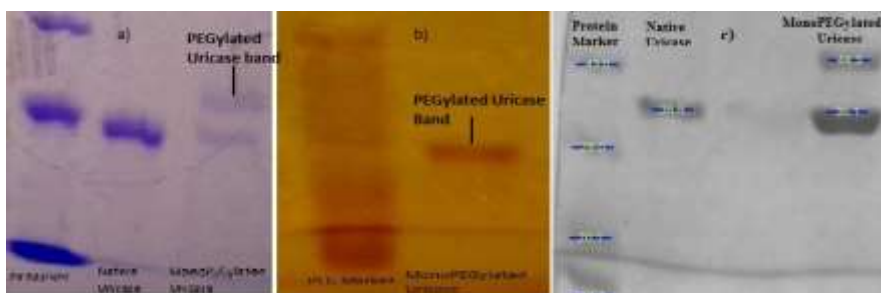
Figure 4.5: SDS-PAGE gel images for native uricase and Uc-mPEG-mal conjugates (a) Coomassie blue stained gel, (b) Iodine stained gel and (c) gel documentation image

Table 4.4: Residual uricolytic activities for Uc-mPEG-mal conjugate a) reduced and b) non-reduced samples

Uc-mPEG-mal (reduced)	Trial 1	Trial 2	Average Residual Activity (%)
Native Uricase Control	100	98.26	99.13
1:5	45.56	48.25	46.91
1:10	46.26	51.74	49.0
1:15	32.29	41.17	36.73
1:20	36.52	32.9	34.71

Uc-mPEG-mal (non-reduced)	Trial 1	Trial 2	Average Residual Activity (%)
Native Uricase (control)	100	100	100
1:5	89.03	83.81	86.42
1:10	86.24	93.68	89.96
1:15	95.86	89.36	92.61
1:20	69.37	75.51	72.44

After reduction of native uricase with DTT into single subunits, it displayed higher uricolytic activity of around 0.602 U/mL in comparison to the non-reduced uricase, whose uricolytic activity was measured to be 0.597 U/mL before PEGylation. The reduced uricase displayed a slightly higher activity, due to the exposure of hidden active sites (located within the homotetrameric molecule). The dialyzed and partially diluted uricase solution was used as the source of uricase for PEGylation with mPEG-mal. **Table 4.4 (a)** and **(b)** indicate the residual activities of the reduced and non-reduced uricase molecules before and after PEGylation. DTT being a very strong reducing agent had a negative influence on the uricolytic activity of the conjugates during the course of PEGylation process and imparted a significant difference in the residual activities of the Uc-mPEG-mal conjugates. The conjugates prepared with the non-reduced uricase possessed around 70-90 % of the native uricase activity, whereas conjugates prepared with the reduced uricase possessed around 35-50 % of the native uricase activity.
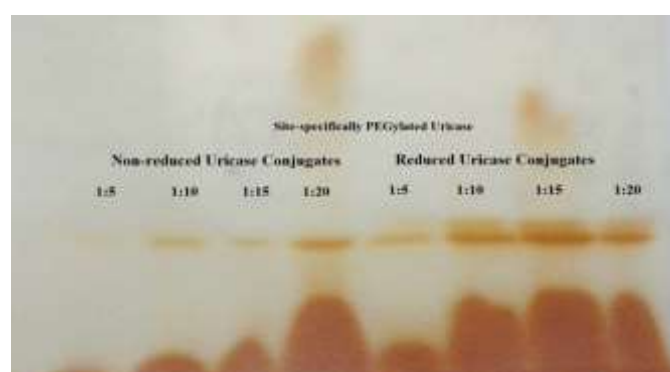
Figure 4.6: Iodine stained SDS-PAGE gel image of the Uc-mPEG-mal conjugates synthesized with reduced and non-reduced uricase

Figure 4.6 represents the iodine stained SDS-PAGE gel image of the Uc-mPEG-mal conjugates synthesized with reduced and non-reduced uricase. The position of the Uc-mPEG-mal conjugates synthesized using various mPEG-mal concentrations can be observed as brown colored bands. The bands confirmed the synthesis of Uc-mPEG-mal conjugates. The brown patches at the bottom portion of **Figure 4.6** represent the unreacted free mPEG present in the crude PEGylation reaction mixture.

The thiol concentrations of the reduced and non-reduced unmodified uricase were quantified using the Ellman's assay for sulfhydryl group concentration quantification. The sulfhydryl concentration of the non-reduced native uricase was found to be 224.4×10^{-6} M. After reduction, due to the exposure of the free thiol groups, the reduced uricase contained a higher concentration of the sulfhydryl groups, (ie approximately 255.5×10^{-6} M) as determined by the Ellman's assay. After site-specific PEGylation with mPEG-mal, there was a further reduction in the concentration of the free sulfhydryl groups as a result of masking of thiol residues due to site-specific PEGylation by mPEG-mal. The decrease in thiol concentration was independent of the concentration of mPEG-maleimide used. Another set of reaction mixtures consisted of unreduced native uricase, which was directly used for PEGylation with mPEG-mal, wherein the conjugates possessed higher uricolytic activity. **Table 4.5** represents the thiol/sulfhydryl concentrations of reduced and non-reduced PEGylated uricase conjugates synthesized using different concentrations of mPEG-mal. Considering the above results, all the further studies were carried out without reducing the uricase molecule and directly PEGylated using mPEG-mal in a site-specific manner.

Table 4.5: Sulfhydryl group concentration (M) for Uc-mPEG-mal (Reduced) and Uc-mPEG-mal (Unreduced)

Uc-mPEG-mal (Reduced)	Sulfhydryl concentration (M)	Uc-mPEG-mal (Unreduced)	Sulfhydryl concentration (M)
Native uricase	224.4×10^{-6}	Native uricase	222.6×10^{-6}
Reduced uricase	255.5×10^{-5}	1:5	84.19×10^{-6}
1:5	158.3×10^{-6}	1:10	116.89×10^{-6}
1:10	128.6×10^{-6}	1:15	133.67×10^{-6}
1:15	163.23×10^{-6}	1:20	118.67×10^{-6}
1:20	153.33×10^{-6}		

Figure 4.7 (a) and **(b)** indicate the coomassie blue and iodine stained SDS-PAGE gel images of random and Uc-mPEG-mal conjugates. It can be observed that the randomly PEGylated conjugates (heavier molecules) migrated slowly and the Uc-mPEG-mal conjugates (with lower molecular weight) migrated faster in a given electrophoresis time. The position and molecular weights of the conjugates confirmed the synthesis and formation of random and Uc-mPEG-mal uricase conjugates. Subject to the relative mobility values of the conjugates, the molecular weights of the randomly PEGylated conjugates were determined to be in the range of 97-66 kDa per subunit.
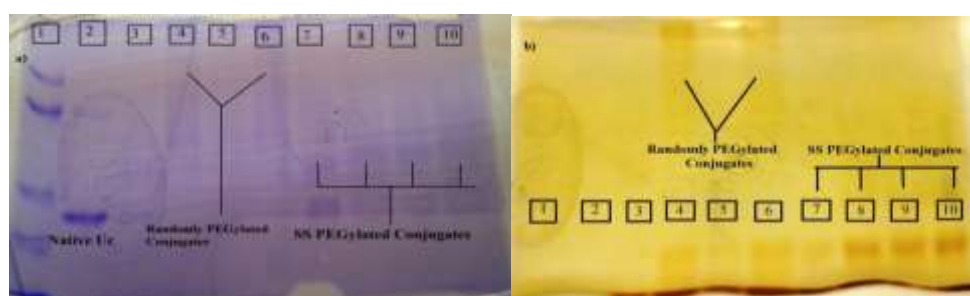
Figure 4.7: SDS-PAGE gel images of native uricase and random PEGylated and Uc-mPEG-mal conjugates (a) coomassie blue stained gel and (b) iodine stained gel

The molecular weights of the Uc-mPEG-mal conjugates were determined to be in the range of 50-60 kDa per subunit.

Broadly, the residual uricolytic activities were higher in the case of Uc-mPEG-mal conjugates than the randomly PEGylated uricase conjugates. Hence, site-specific PEGylation proves to be a better strategy in comparison to random PEGylation.

4.4.1 Preliminary assessment of reaction conditions on the uricolytic activities of Uc-mPEG-mal conjugates:

Thiol PEGylation reactions are generally carried out in a pH range of 6.0-8.0 (Mero et al. 2011). The residual activities possessed by the PEGylated conjugates are as represented in **Table 4.6.** It was observed that Uc-mPEG-mal conjugates synthesized in 0.1 M SP buffer possessed higher residual activities in comparison to the ones

synthesized in 1 M SP buffers. **Figure 4.8** indicates the effect of pH on the uricolytic activities of mPEG-mal. The Uc-mPEG-mal conjugates retained their maximum residual activities (79.24%) when PEGylation was conducted in SP buffer of pH 7.2. **Table 2.7** represents literature reports on the buffer medium, molarity and pH conditions employed for Thiol PEGylation reactions. It can be observed from the **Table 2.7** that the optimum value of pH for thiol PEGylation to occur is in the range of 6.5-7.4. While using mPEG-mal as a PEGylating reagent for site-specific PEGylation of proteins, buffer pH of above 7.5 should be avoided. At higher (alkaline) pH conditions, the reaction of the terminal maleimide can occur with primary amine group-containing amino acids like lysine residues. In such conditions, the reaction may occur at a much deliberate rate than the free thiol groups (Mero et al. 2011).

PEG-vinyl sulfone (PEG-VS) is another PEGylating agent which can be used for thiol PEGylation, which reacts slowly with thiols at marginally basic conditions (pH 7–8) and forms a thioether linkage to the protein. But this reaction can advance faster if the pH is increased and it may react with lysine residues. mPEG-mal is more reactive to thiols in comparison to PEG-VS, even under acidic conditions (pH 6–7) (Roberts et al. 2002).
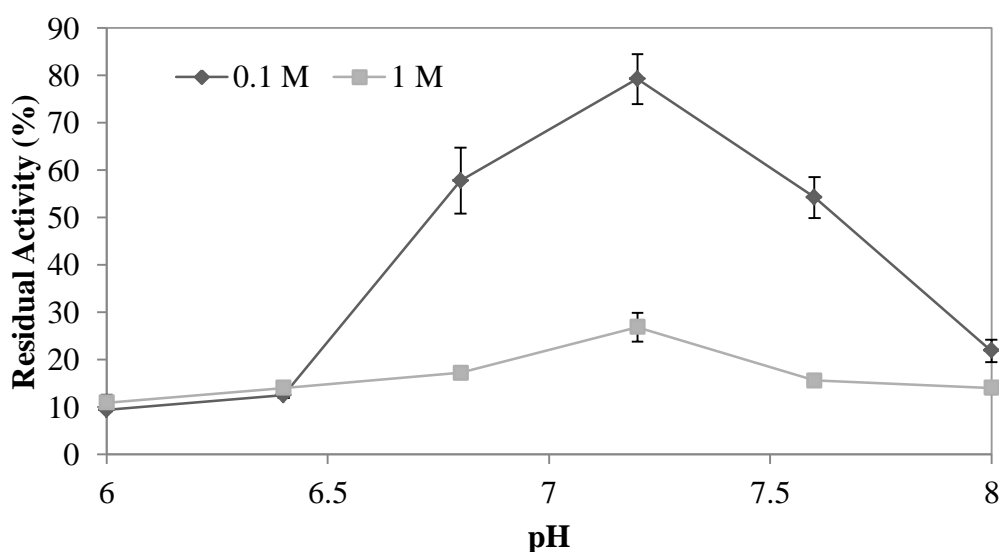
Figure 4.8: Effect of pH and buffer molarity on the uricolytic activities of Uc-mPEG-mal conjugates

Table 4.6: Residual activities of Uc-mPEG-mal conjugates synthesized at various pH conditions

Sl no	pH	Residual activity in 0.1 M Buffer (%)	Residual activity in 1 M Buffer (%)
1	6.0	9.37	10.93
2	6.4	12.46	14.06
3	6.8	57.8	17.19
4	7.2	79.24	26.87
5	7.6	54.25	15.62
6	8.0	21.87	14.06

Figure 4.9 represents the coomassie blue stained SDS-PAGE gel images of Uc-mPEG-mal conjugates synthesized in a buffer solution [**Figure 4.9 (a): 0.1 M SP buffer** and **Figure 4.9 (b) 1.0 M SP buffer]** of pH values ranging between 6.0-8.0. **Figure 4.9 (c)** indicates the iodine stained gel of Uc-mPEG-mal conjugates synthesized in buffer solutions (0.1 M buffer strength). It was observed that higher buffer strength resulted in decreased yield of the Uc-mPEG-mal conjugates. The conjugates also appeared to have undergone denaturation/structural changes and disintegration.
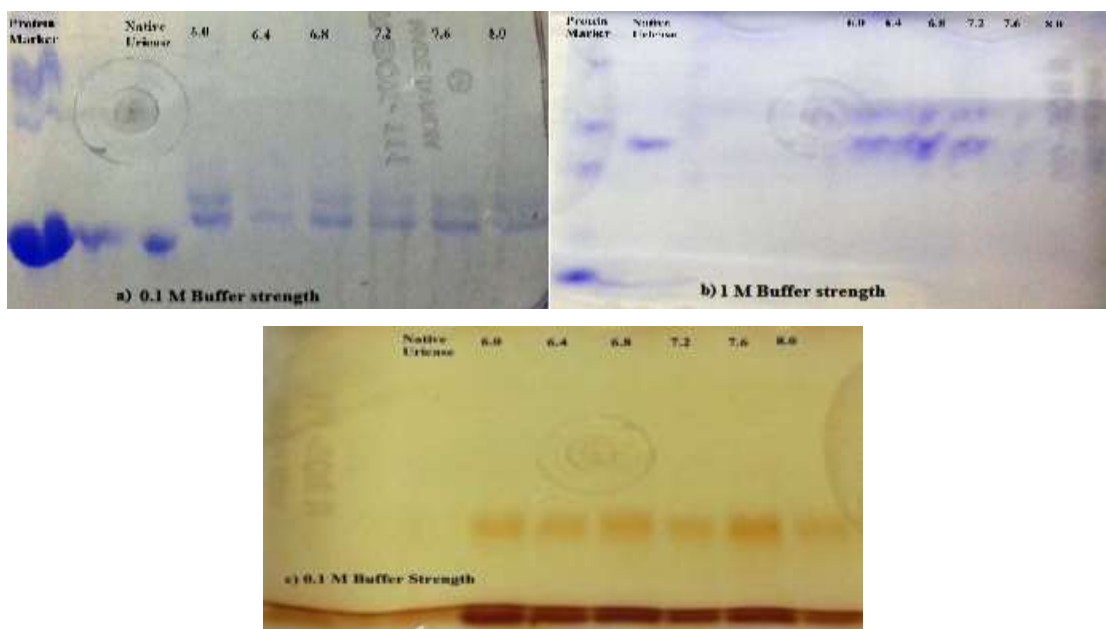
Figure 4.9: SDS-PAGE gel images of native uricase and Uc-mPEG-mal conjugates synthesized in (a) 0.1 M SP Buffer, (b) 1 M SP Buffer and (c) iodine stained gel image of Uc-mPEG-mal conjugates synthesized in buffer solutions of pH values ranging from 6.0-8.0.

4.4.2 Statistical optimization of reaction conditions for thiol PEGylation of uricase:

Previous studies have suggested that along with the pH of reaction buffer and molarity, uricase to mPEG-mal concentration ratio (wt/wt), molecular weights of mPEG-mal (kDa) and the EDTA concentration (mM) are significant parameters which directly influence the yield of Uc-mPEG-mal conjugates. For enhancing their yield, response surface methodology was employed to optimize these parameters and determine the conjugate yield using RP-HPLC methodology.
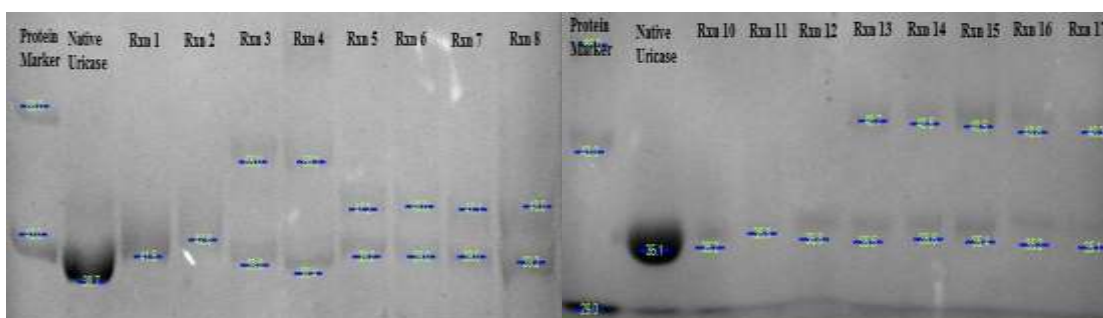
Figure 4.10: Gel documentation image for SDS-PAGE gel for all the reactions used in optimization experiment for the production of Uc-mPEG-mal conjugates

Figure 4.10 represents the gel documentation images of all the reaction mixtures used for optimization study. The molecular weight of the conjugates were determined to be 41.5, 48.0 and 58.3 kDa per subunit, upon PEGylation with mPEG-mal of molecular weights 750 Da, 5kDa and 10 kDa respectively. It was deduced that approximately one to two mPEG-mal molecules were covalently bound per subunit of uricase molecule, as observed by the iodine stained gel. It can be deduced that a single molecule of uricase (a homotetramer) was bound with 4-8 mPEG-mal chains. The most relevant perturbations of a protein molecule following PEG conjugation are size enlargement, protein surface and glycosylation function masking, charge modification, and epitope shielding. In particular, size enlargement of the entire conjugate slows down kidney ultra-filtration rate and is

retained *invivo* for prolonged periods of time (Veronese 2001). Hence molecular weight determination is an important parameter.

RP-HPLC may not be an exceptional and robust tool for the fractionation of PEGylated and non-PEGylated species, but it is also a valuable method to identify protein oxidation, deamidation, or cleavage of the protein backbone (Mero et al. 2011). For the calculation of conjugate yield, RP-HPLC methodology was implemented. PEGylated proteins display higher retention times on HPLC columns in comparison to their non-PEGylated counterparts, as PEG is an amphiphilic molecule. But the conjugate yield can be suitably determined using this method. **Figure 4.11** represents the RP-HPLC chromatograms of a) native uricase, b) Uc-mPEG-mal conjugates (750 Da), c) Uc-mPEG-mal conjugates (5 kDa) and d) Uc-mPEG-mal conjugates (10 kDa) reaction mixtures as detected at 280 nm. The retention time of native uricase and Uc-mPEG-mal conjugates was determined to be 1.267 and 1.485 (for 750 Da), 1.433 (for 5 kDa) and 1.455 (for 10 kDa mPEG-mal) minutes respectively.

It is indispensable to find the optimum PEGylation reaction conditions which can result in a higher yield of the desired PEGylated product during subsequent scaling. In order to preserve the biological activity of the enzyme and minimizing side effects upon usage in therapeutic applications (Puchkova et al. 2012). In the current study, the effect of thiol PEGylation reaction parameters like uricase to mPEG-mal concentration ratio (wt/wt), molecular weights of mPEG-mal chains (kDa) used and EDTA concentration (mM) in the reaction mixture was studied. These variables were analyzed at three levels to determine the most optimal values for obtaining the highest yields of Uc-mPEG-mal conjugates.

Table 4.7 represents the levels of variables used for the Box–Behnken statistical design. **Table 4.8** represents the design matrix used for optimization of reaction parameters using Box-Behnken design. The experimentally obtained and the predicted values of Uc-mPEG-mal conjugate yields have been listed in **Table 4.8.** According to the Box-Behnken design, seventeen combinations were chosen

for optimization and trials were carried out in duplicates at two different detection wavelengths (214 and 280 nm).
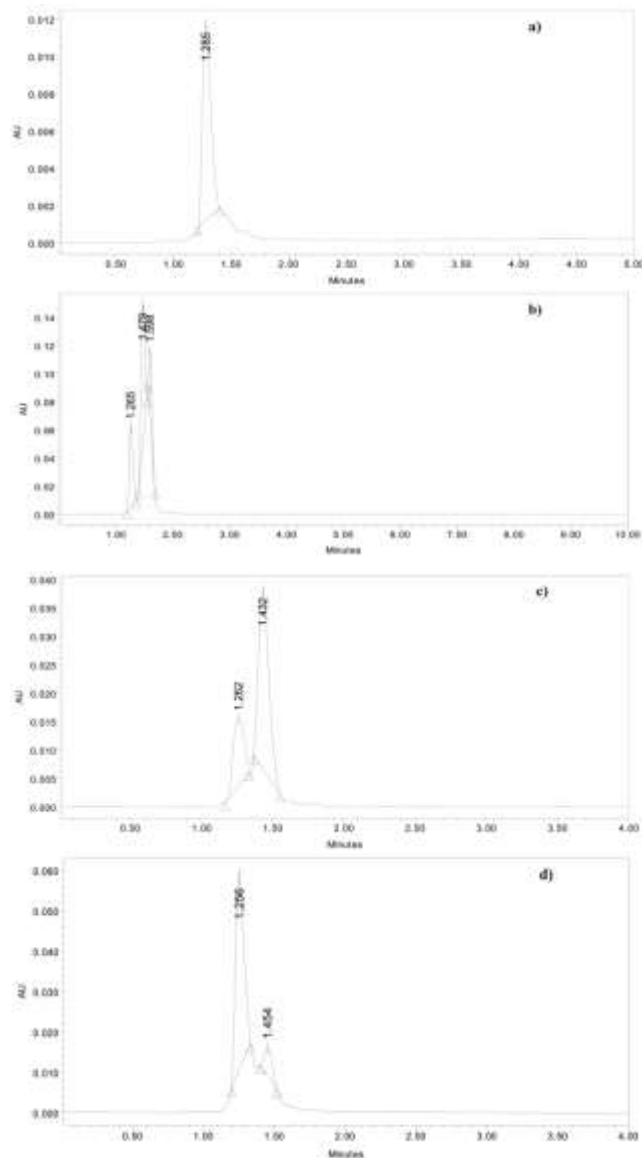
Figure 4.11: RP-HPLC chromatograms of a) native uricase and b) Uc-mPEG-mal conjugates synthesized with mPEG-mal of molecular weights b) 750 Da, c) 5 kDa and d) 10 kDa

Table 4.7: Levels of variables for the Box–Behnken design

Sl no	Variable	Variable Code	-1	0	+1
1	Uricase to mPEG-mal Ratio	A	1:5	1:10	1:15
2	Mol wt of mPEG-mal (Da)	B	750 Da	5 kDa	10kDa
3	EDTA Concentration (mM)	C	0	5	10

For predicting the optimal point, a second order polynomial function was fitted to correlate a relationship between the effect of variables on its response (yield of Uc-mPEG-mal conjugates) as indicated by the **Eq 2** given in methods section.

$$\text{Yield of Uc-mPEG-mal conjugates} = 60.30 + 19.14A - 19.27B - 17.11C - 10.23AB - 1.19AC$$
$$+ 9.06BC + 1.37A^2 - 27.11B^2 - 5.70C^2 \ldots\ldots\ldots\ldots\textbf{Eq 2}$$

As represented in **Table 4.8**, the measured Uc-mPEG-mal conjugate yield values were obtained experimentally, while the predicted values were generated by a second-order polynomial equation and the response optimizer. Variables A, B, and C represent the levels of Uc-mPEG-mal concentration ratio, molecular weights of mPEG-mal and EDTA concentration respectively. At the model level, the correlation terms (R and R^2) were estimated. The correlation between the measured and predicted values was done by estimating correlation coefficient R^2. The value of correlation coefficient R^2 was found to be 0.9109. This indicates that 8.91 % of the total variations were not explained by the model, which may have been caused due to experimental errors during analysis.

　　Table 4.9 represents the analysis of variance (ANOVA) data of the obtained response for the yield of Uc-mPEG-mal conjugates. A model *F*-value of 7.95 implies that it was significant. A *p*-value of 0.0061 (which is less than 0.05) indicated that the model terms were significant. The "Pred R-Squared" of 0.7391 was in reasonable agreement with the "Adj R-Squared" of 0.7963. An "Adeq Precision" ratio of 9.271 indicated an satisfactory signal and this model could be used to traverse the design space. The optimum values (coded variables) for achieving maximum yield of Uc-mPEG-mal conjugates obtained by the software were determined as A=0.8, B= -0.48, C= -0.71.

Table 4.8: Design matrix for Box–Behnken design representing the response of Uc-mPEG-mal conjugate yield as influenced by Uc to mPEG-mal concentration ratio, mPEG-mal molecular weight and EDTA concentration

Run	Uc to mPEG-mal concentration ratio (A)	Mol wt of mPEG-mal (B)	EDTA Concentration (C)	Experimental Yield of Uc-mPEG-mal conjugate	Predicted Yield of Uc-mPEG-mal conjugate
1	-1	-1	0	21.397	24.452
2	1	-1	0	80.77	83.186
3	-1	1	0	8.783	6.366
4	1	1	0	27.25	24.194
5	-1	0	-1	55.638	52.741
6	1	0	-1	95.67	93.412
7	-1	0	1	18.644	20.902
8	1	0	1	53.897	56.793
9	0	-1	-1	73.085	72.926
10	0	1	-1	10.955	7.5262
11	0	-1	1	25.891	20.578
12	0	1	1	0	0.1586
13	0	0	0	58.84	60.295
14	0	0	0	55.751	60.295
15	0	0	0	65.155	60.295
16	0	0	0	83.703	60.295
17	0	0	0	38.029	60.295

Table 4.9: ANOVA table for the yield of Uc-mPEG-mal conjugates

Source	Sum of Square	Degrees of freedom	Mean square	F-value	p-Value	Coefficients
Model	12307.46	9	1367.50	7.95	0.0061	60.30
A	2930.91	1	2930.91	17.04	0.0044	19.14
B	2970.47	1	2970.47	17.27	0.0043	-19.27
C	2343.25	1	2343.25	13.62	0.0077	-17.11
AB	418.33	1	418.33	2.43	0.1628	-10.23
BC	5.71	1	5.71	0.033	0.8606	-1.19
AC	328.32	1	328.32	1.91	0.2096	9.06
A^2	7.87	1	7.87	0.046	0.8367	1.37
B^2	3095.12	1	3095.12	18.00	0.0038	-27.11
C^2	136.81	1	136.81	0.80	0.4021	-5.70

The nature of the 3D surface plots (**Figure 4.12**) indicated that the highest yield was obtained within the chosen ranges of the factors and their optimum values were obtained by extrapolating the highest point on the response surface plot. From F**igure 4.12**, the following optimum values of the significant factors were found to be: Uc to mPEG-mal concentration ratio of 1:12, the mPEG-mal molecular weight of 2.76 kDa and 3.55 mM of EDTA concentration. These values were determined using numerical optimization using the software, with the response goal (Yield of Uc-mPEG-mal conjugates) set as the maximum. The optimal value of the yield of Uc-mPEG-mal conjugates from the regression equation was found to be 96.43 % (**Figure 4.12**). It was observed from all the response curves, that the yield is highly dependent on the chosen variables.

The concentration of mPEG-mal to be added in the thiol PEGylation reaction mixture commonly depends on the availability and quantity of surface accessible cysteine moieties with a free thiol (–SH) group. According to the study on primary and secondary structures of uricase from various strains of *Bacillus fastidious* (PDB Id: 4R8X and 4R99), the number of cysteine molecules with free –SH groups ranged from 1-2 groups

per subunit (Feng et al. 2015). Uricase being a homotetramer probably contains a total of 4-8 free thiol groups on its surface, which were available for reaction with the maleimide moiety of mPEG-mal. In the present study, Uc to mPEG-mal concentration ratios of 1:5, 1:10 and 1:15 were used. It was observed that there was an increase in the yield of Uc-mPEG-mal conjugates with increasing mPEG-mal concentration. A Uc:mPEG-mal concentration ratio of 1:12 resulted in the achievement of maximum yield, as determined by the 3D plots.

PEGs with lower molar masses of 1 kDa to 5 kDa are often used for the conjugation of larger drugs, such as antibodies, larger enzymes, and nano-particulate systems. Williams et al. (2010) demonstrated that coupling a relatively small number of strands of 5 kDa PEG led to a marked reduction of the uricolytic activity of uricases extracted from various sources. Sherman et al. (2008) found that conjugates of porcine-like uricase could be prepared with a sufficient number of strands of 10 kDa mPEG to achieve prolonged half-lives in laboratory animals. This could suppress their antigenicity and immunogenicity while retaining approximately 90% of the uricolytic activity. In the present study, mPEG-mal polymer with lower molecular weight resulted in the production of higher yields of Uc-mPEG-mal conjugates (around 96 %). It was also observed that there was a decrease in yield of the Uc-mPEG-mal conjugates with an increase in the molecular weight of mPEG-mal (10 kDa) used.

In a report by Zhai et al. 2009, the molecular weight of mPEG-propionaldehyde used for the synthesis of PEGylated recombinant human GCSF did not have any direct correlation with a yield of the conjugates formed. But in the present study, optimization of molecular weights of mPEG-mal proved to be very crucial and had a major influence on yield.

In thiol PEGylation reactions, EDTA sodium salt was used to chelate the divalent metal ions in the reaction buffer (derived from reactants used for the reaction, salts, additives and buffer components used) which could otherwise oxidize the sulfhydryl groups (from –SH to –SHO) (Riddles 1979). The unoxidized sulfhydryl groups (-SH) then become available for reaction with the maleimide moiety of mPEG-mal. This

reaction results in the establishment of a very stable thioether bond amongst the mPEG-mal and the protein molecule. Therefore, the addition of EDTA in the reaction buffer is imperative for thiol PEGylation, but its concentration should not have a undesirablen influence on the enzymatic activity of proteins. In the present study, the optimum EDTA concentration was determined to be 3.55 mM, which resulted in a very high yield of the Uc-mPEG-mal conjugates. But, higher concentrations of EDTA sodium salt proved to have a negative undesirable influence the uricolytic activity of uricase. Accordingly, complete removal of excess EDTA from the reaction mixture using centrifugal filtration and subsequent chromatographic purification steps could circumvent the problem of activity loss.
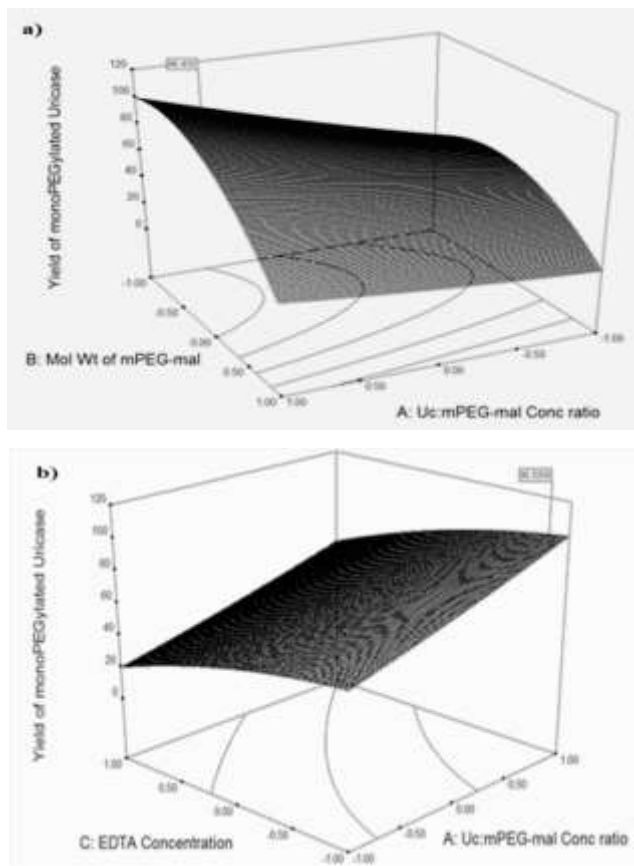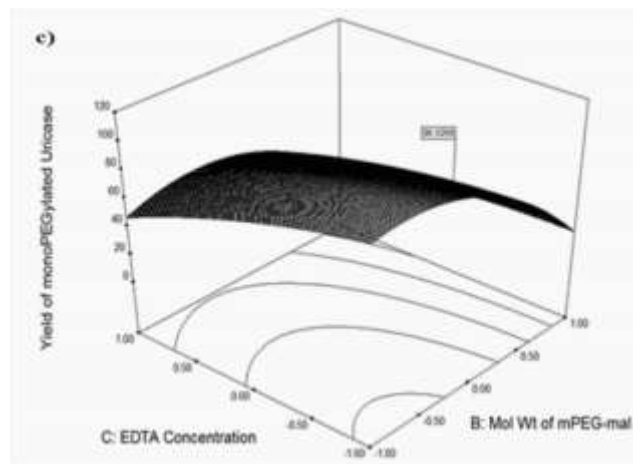
Figure 4.12: Response surfaces (3D plot) for the yield of Uc-mPEG-mal conjugates as influenced by reaction conditions

Optimal values of the variables influencing the yield obtained experimentally were corroborated and related with the predicted data. The PEGylation reaction was carried out with 1:12 concentration ratio of Uc to mPEG-mal and 3.55 mM EDTA concentration using 5kDa mPEG-mal in the reaction mixture. The experimentally determined maximum yield of Uc-mPEG-mal conjugates was 92.16 %, where the predicted value of the polynomial model was 96.43%. The verification exposed a high degree of accuracy of the model indicating model validation under the verified conditions.

4.4.3. Purification of Uc-mPEG-mal conjugates by centrifugal ultrafiltration, SE-HPLC and SE-FPLC:

After optimization of the important PEGylation reaction conditions, PEGylation reaction was conducted again using Uc and mPEG-mal (5 kDa) in 1:12 concentration ratio with 3.55 mM EDTA mixed in the reaction mixture. The experimentally determined yield of Uc-mPEG-mal conjugates was determined to be 91.01 %.

In the next step, the PEGylation reaction mixture was purified. Apart from molecular weight, variances in the properties which can be considered for the fractionation of PEGylated proteins from their native counterparts are very few (Fee and Alstine 2011). In the present study, fractionation and purification of the Uc-mPEG-mal conjugates from the PEGylated uricase reaction mixture was carried out on the basis of differences in molecular weights of the reaction components. As a preliminary step, the PEGylation mixture was subjected to centrifugal ultrafiltration for the removal of unreacted mPEG-mal, buffer components, EDTA sodium salt and reaction byproducts etc. After which the concentrated protein solution was subjected to fractionation using SE-HPLC.

Figure 4.13 indicates the chromatogram of native uricase in which a single peak corresponding to native uricase was eluted with a molecular weight of 161.98 kDa eluted at a retention time of 8.12 minutes. **Table 4.10** and **4.11** indicate the retention time and molecular weight of the fractions eluted after the purification process. The extra peaks indicate broken fragments of uricase and peptides.

Table 4.10: Retention time and molecular weight data for Figure 4.13

Peak number	Time	Height	Width	Symmetry	Mol wt
1	8.12	1.2	0.1698	0.808	161.98
2	11.51	2.80E-01	0.1313	0.47	4.144476
3	12.16	4.90E-01	0.3277	1.131	2.044621
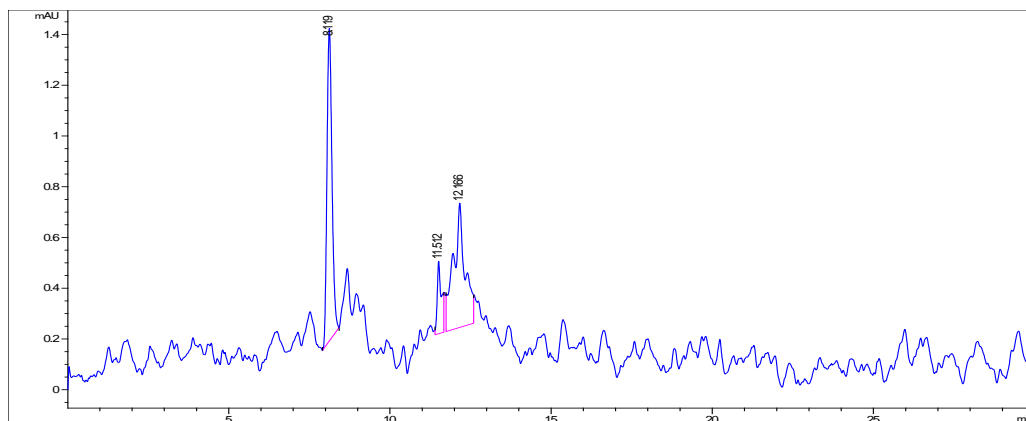
Figure 4.13: SE-HPLC chromatogram of native uricase
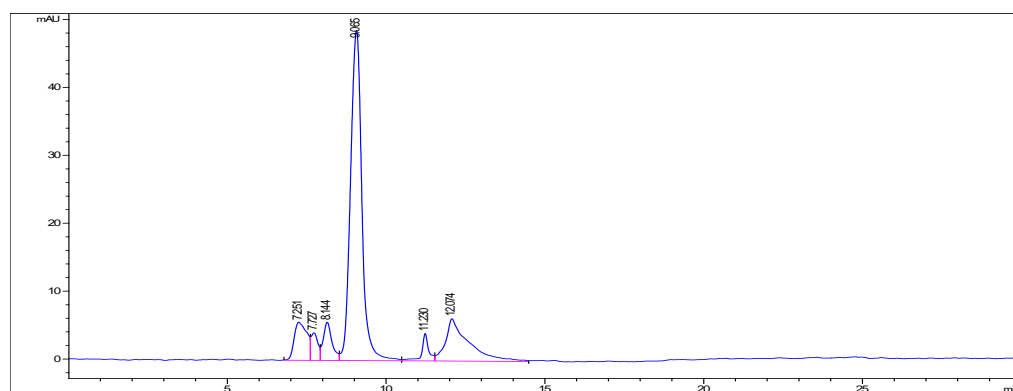
Figure 4.14: SE-HPLC chromatogram of Uc-mPEG-mal conjugate reaction mixture

Table 4.11: Retention time and molecular weight data for Figure 4.14

Peak number	Time	Height	Width	Symmetry	Mol wt
1	7.25	5.6	0.4183	0.577	413.74
2	7.73	4.1	0.2184	0.679	247.39
3	8.14	5.6	0.2851	0.761	157.66
4	9.06	48.6	0.3898	1.012	58.29
5	11.23	4	0.216	0.985	5.62
6	12.07	6.2	0.6459	0.372	2.25

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The chromatogram corresponding to the Uc-mPEG-mal conjugate containing reaction mixture (**Figure 4.14**) indicated the appearance of several peaks corresponding to Uc-mPEG-mal conjugates, aggregated uricase, unreacted native uricase, its broken fragments and mPEG-mal and some reaction byproducts/buffer components. Native uricase (molecular weight of 157.66 kDa) was eluted at a retention time of 8.14 minutes. Uc-mPEG-mal conjugates having a greater molecular size (247.39 kDa) were eluted at a retention time of 7.72 minutes. The presence of trace amounts (8.23 %) of protein aggregates (413.74 kDa) was also observed. The fractions collected were subjected to SDS-PAGE analysis, wherein the separation of native and mPEG-mal conjugates is as represented in **Figure 4.15.**
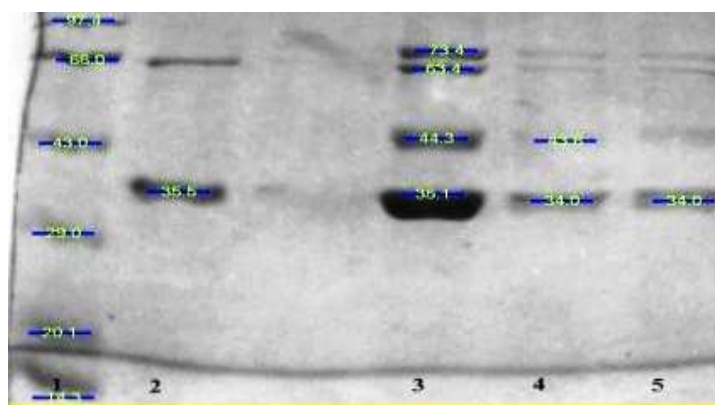
Figure 4.15: SDS-PAGE analysis of the native uricase and PEGylated uricase reaction mixture. Lane 1: protein molecular weight marker, Lane 2: native uricase, Lane 3: PEGylation reaction mixture, Lane 4 and 5: diluted PEGylation reaction mixture after centrifugal ultrafiltration and SE-HPLC

Figure 4.16 (a) represents the chromatogram for native uricase and **Figure 4.16 (b)** represent reaction mixture containing Uc-mPEG-mal conjugates purification using SE-FPLC. Components of the PEGylation reactions such as unconjugated PEG, unmodified uricase, Uc-mPEG-mal conjugates and other buffer components were separated efficiently. **Figure 4.17** represents the gel documentation image of SDS-PAGE gel with native uricase, Uc-mPEG-mal conjugates before and after SE-FPLC. From the SDS-

PAGE gel images, it can be observed that the unmodified uricase was completely eliminated after purification using SE-FPLC and the eluted fraction of purified Uc-mPEG-mal conjugates resulted in a single band on the SDS-PAGE gel.
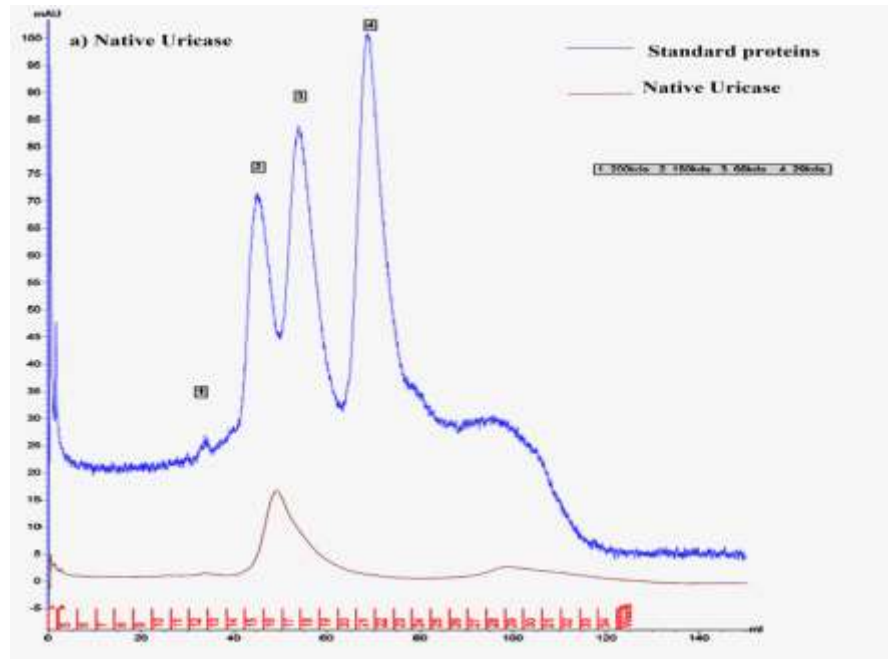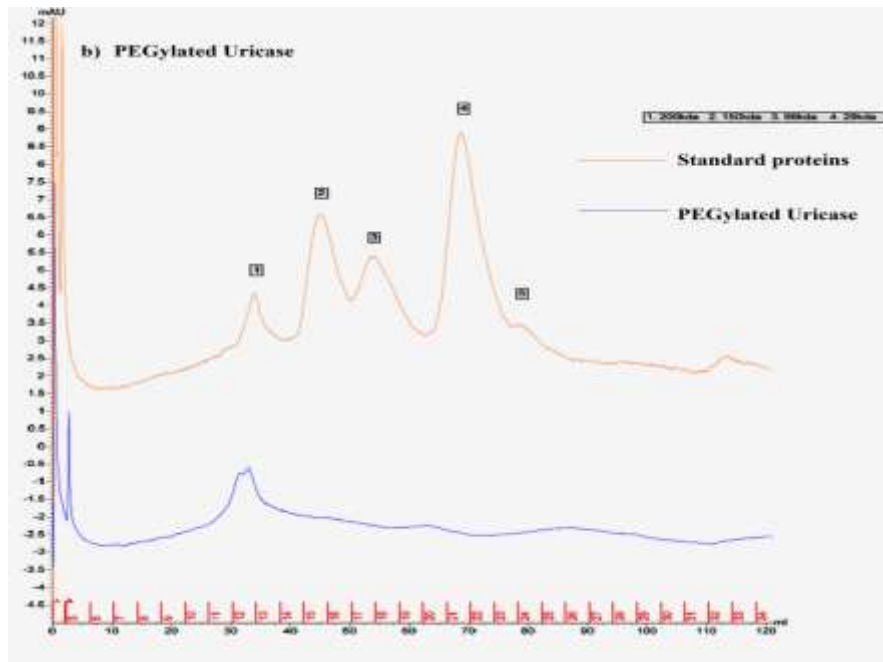
Figure 4.16: SE-FPLC chromatogram for (a) native uricase and (b) Uc-mPEG-mal conjugate
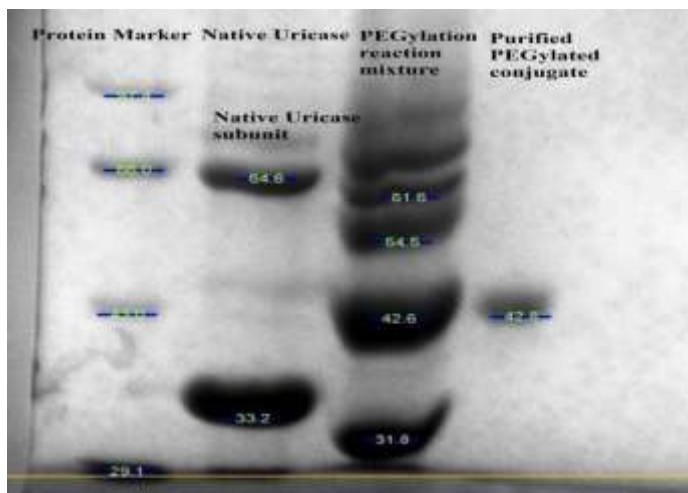
Figure 4.17: Gel documentation image for SDS-PAGE gel for native uricase and Uc-mPEG-mal conjugates before and after purification. Lane 1: Protein molecular weight marker, Lane 2: native uricase, Lane 3: PEGylated uricase reaction mixture before purification, Lane 4: purified Uc-mPEG-mal conjugate

4.4.4. Characterization of Uc-mPEG-mal conjugates:

4.4.4.1. Determination of residual activity by uricase enzyme assay:

In the present study, uricase from *Bacillus fastidious* was conjugated in both random and site-specific manner using mPEG derivatives. It was observed that the Uc-mPEG-mal conjugates (site-specific PEGylated uricase molecules) possessed a higher uricolytic activity (89.96 %) in comparison to random PEGylated uricase conjugates wherein the residual uricolytic activity ranged from 10.27-62.73 %. From **Table 4.2,** it can be concluded that randomly PEGylated uricase possessed less than 70 % residual activity (Zhang et al. 2010; Bomalaski et al. 2002). This was due to excessive and differential masking of the active sites on the surface of uricase, which interfered with uricase-uric acid interaction.

The uricase molecule was PEGylated in a site-specific manner in its reduced (after reduction with DTT) and non-reduced forms. After reduction of uricase followed by its PEGylation, the Uc-mPEG-mal conjugates possessed a residual activity of only 49% (Considering the activity of reduced uricase to be 100%. The reduction of the uricase homotetramer into single subunits due to the breaking of disulfide bridges resulted in rapid loss of uricolytic activity. Hence, the reduction process before PEGylation required for the exposure of free thiol groups did not prove to be a successful step for the synthesis of Uc-mPEG-mal conjugates for better retention of uricolytic activity.

The uricolytic activities of the non-reduced native uricase and its corresponding Uc-mPEG-mal conjugates were determined to be 100% and 89.96% respectively.

The Uc-mPEG-mal conjugate synthesized by the non-reduced uricase molecule possessed significant uricolytic activity following PEGylation, which serves as a very important criterion for a PEGylated protein to be used for therapeutic purposes. Hence, the residual bioactivity of any therapeutic protein can be restored for a prolonged period by implementing site-specific PEGylation strategy. Possession of high uricolytic activity also reduces the need for repeated administration.

4.4.4.2. Determination of degree of modification of Uc-mPEG-mal conjugates using Ellman's assay:

The degree of modification governs the uniformity of the conjugates and provides a measure of any immunological ramifications caused due to conjugate heterogeneity. For the present study, the secondary and tertiary structures of uricase molecule from various strains of *Bacillus fastidious* (PDB Ids: 4R8X and 4R99) were studied. The number of cysteine molecules with a free thiol group (-SH) was found to be in the range of 1-2 groups per subunit in a uricase molecule (Feng et al. 2015), which indicate the presence of 4-8 cysteine molecules in one molecule of uricase.

In the present study, the sulfhydryl concentration of Uc-mPEG-mal conjugates (119.61×10^{-6}) was lesser than native uricase (222.6×10^{-6}), indicating utilization of free thiol groups by the process of PEGylation. These results designated the number of modified free thiols groups present on the accessible cysteine residues determined by Ellman's assay. The percentage of free thiols in the Uc-mPEG-mal conjugate samples was found to be 8.9 %. The modification extent was found to be 68.3% of thiol groups (as determined by Ellman's assay). **Figure 4.18** represents the samples of native uricase and Uc-mPEG-mal conjugates subjected to Ellman's reactions. It was observed that the test-tube C containing Ellman's reaction mixture for native uricase was much darker in color (higher in intensity) in comparison to test-tube A containing Ellman's reaction mixture of Uc-mPEG-mal conjugates. This was due to the presence of higher number of thiol groups in the native uricase molecule.

Figure 4.18: Ellman's reaction performed for native uricase and Uc-mPEG-mal conjugates. (Test-tube B contains blank buffer, Test-tube A contains Ellman's reaction mixture for Uc-mPEG-mal conjugates and Test-tube C contains Ellman's reaction mixture for native uricase)

4.4.4.3. Molecular weight of the Uc-mPEG-mal conjugates using SDS-PAGE:

The molecular weight of the Uc-mPEG-mal conjugates synthesized using mPEG-mal of molecular weights 750 Da, 5 and 10 kDa was estimated using SDS-PAGE analysis. **Figure 4.19** represents the coomassie blue stained gel images of native uricase and all the Uc-mPEG-mal conjugates synthesized using mPEG-mal of different molecular weights. **Table 4.12** represents the molecular weight values of native uricase and Uc-mPEG-mal, from which it was concluded that approximately two mPEG-mal molecules were covalently bound to one subunit of uricase molecule (as observed by the iodine stained gel). Consequently, it was deduced that a single molecule of uricase (a homotetramer) is bound with 4-8 mPEG-mal chains. Size enlargement displayed by the conjugate signifies the possible slowing down of kidney ultra-filtration rate (Veronese, 2001).

The kidney glomerular filtration cut-off of globular proteins is around 70 kDa (Kontermann, 2012). A PEGylated molecule displays approximately 4-10 folds increase in hydrodynamic volume after PEGylation. PEG with a hydrodynamic radius greater than glomerular membrane pore size can be filtered at lower rates, due to its high flexibility (Kontermann, 2012). The pore size of the glomerular membrane ranges from 3 to 5 nm

(Lobo et al. 2004). The hydrodynamic radius of the 20-30 kDa PEG has been determined to be in the range of 5-6.6 nm.

Table 4.12: Molecular weights of native uricase and Uc-mPEG-mal conjugates

Sl no	Name of the Sample	Mol Wt of single subunit of Uc-mPEG-mal (kDa)
1	Native Uricase	35.1
2	Uc-mPEG-mal (750 Da)	39.2
3	Uc-mPEG-mal (5 kDa)	46.7
4	Uc-mPEG-mal (10 kDa)	52.3

In the current study, the average sizes of native uricase and Uc-mPEG-mal (5 kDa) were determined as 8.28 and 11.01 nm respectively (as shown in **Figure 4.20**). The results indicate that the Uc-mPEG-mal conjugates synthesized with 5 and 10 kDa mPEG-mal strands can prove to be a potential uricase formulations having a lower kidney ultrafiltration rate and consequently a prolonged plasma half-life. With these added qualities retention of higher residual activity can be a valuable advantage.
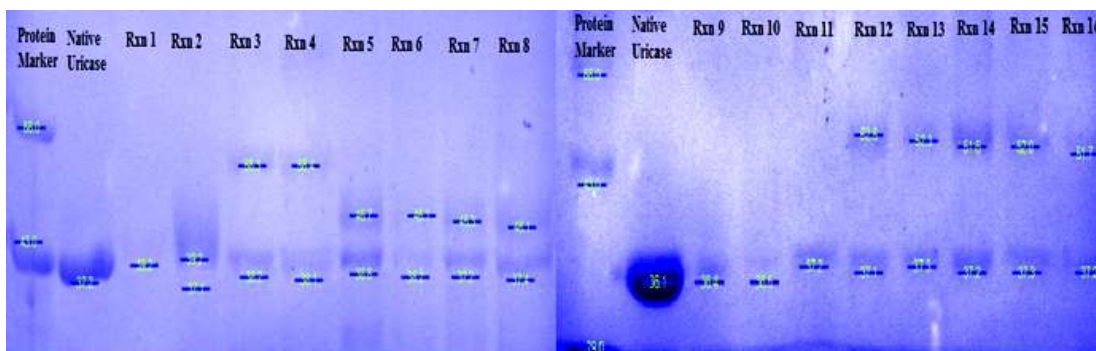
Figure 4.19: Coomassie blue stained gel images of native uricase and Uc-mPEG-mal conjugates

4.4.4.4. Studies on changes in the secondary structure of native and Uc-mPEGmal using far UV-circular dichroism:

Circular dichroism spectroscopic studies were performed to explore the effect of PEGylation induced conformational changes in the secondary structure of uricase. The far UV-CD spectra of the native uricase and Uc-mPEG-mal conjugates at different temperatures (20°C, 50°C and 70°C) are as represented in **Figure 4.20**. It was perceived that there were slight conformational variations induced in the secondary structures of the native uricase after PEGylation which indicated few changes in function. The ellipticity values of native and Uc-mPEG-mal conjugates were found to decrease with increase in CD operational temperature. It was observed that Uc-mPEG-mal conjugates displayed enhanced stability at all the temperatures in comparison to their native counterpart. According to the previous reports dealing with the PEGylation of uricase with mPEG derivatives, it was observed that the secondary structure of uricase remained unaffected significantly following conjugation at room temperatures (Caliceti et al. 2001;Freitas et al. 2010). Uricolytic assay conducted at 25°C did not show any significant change in uricolytic activity of uricase.
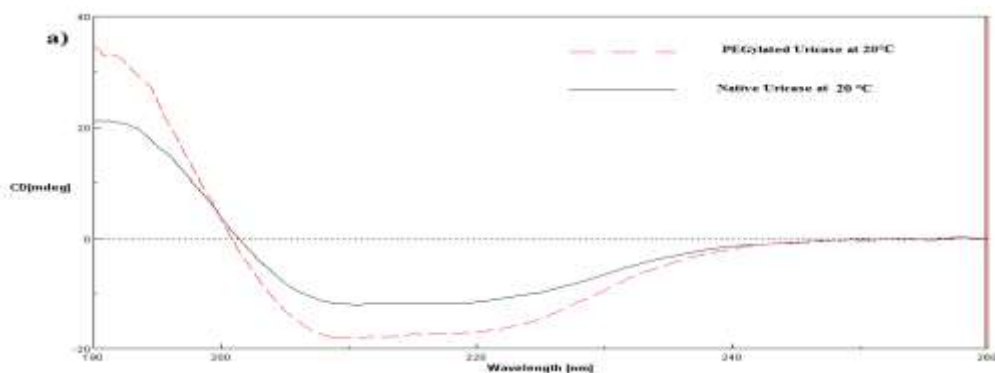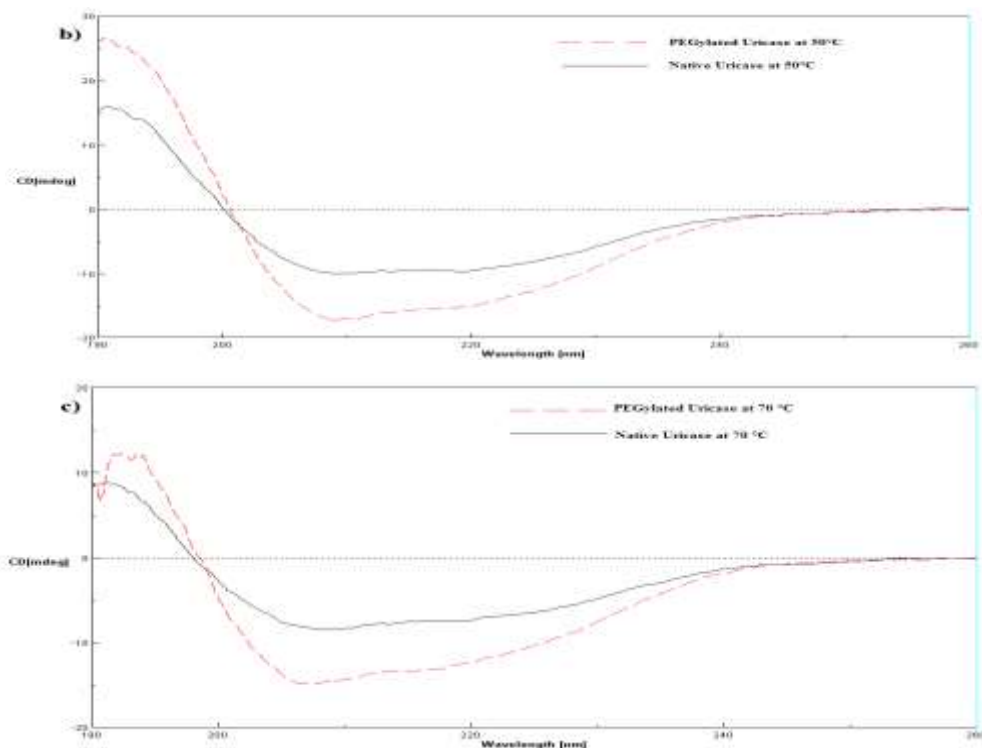
Figure 4.20: Circular dichroism spectra of native uricase and Uc-mPEG-mal conjugates at different temperatures (a) 20 °C, (b) 50 °C and (c) 70 °C

Table 4.13 represents the data of α-helix content (%) obtained after conducting the spectral scan at various temperatures. The data indicated that there was a decrease in the α-helix content of native uricase after PEGylation. At 20°C, the α-helix content of the uricase after PEGylation was decreased by 2 %, indicating the masking of α-helices by mPEG-mal. mPEG-mal strands attached to α-helical motifs might have destabilized the associated helices ensuing in a lower α-helix content. Similar outcomes have been described in the literature for PEGylated uricase from other sources, wherein the secondary structure of the protein molecules was slightly affected after PEGylation (Caliceti et al. 2001; Kinstler et al. 1996; Veronese et al. 2007; Malzert et al. 2003). In a previous report by Freitas et al. (2010), PEGylation induced a fractional resistance to thermal denaturation of uricase, wherein native uricase showed a loss of 58% of the α-helix structure at 70°C while PEGylated uricase had a slight decrease in the α-helix

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structure at 70°C. In a report by Caves et al. (2012), results of CD spectroscopy showed that the main point of uricase thermal inactivation followed an irreversible two-state mechanism wherein a loss of 20 % of the helix content and partial exposure of tryptophan residues was observed with increase in temperature. Native uricase and Uc-mPEG-mal conjugate molecules were prone to denaturation following an increase in temperature. In the current study, there was a decrease in the α-helix content of native uricase after PEGylation at all temperatures, indicating masking of a few residues.

Table 4.13: Percentage of α-helix content of native uricase and Uc-mPEG-mal conjugates at different temperatures

Sl no	Sample	α-helix content at 20°C	α-helix content at 50°C	α-helix content at 70°C
1	Native Uricase	36.7%	30.5%	22.1%
2	Uc-mPEG-mal(5kDa)	34.6%	30.1%	16.8%

It was also observed that the percentage of β-pleated sheets was altered but did not change significantly with an increase in temperature following PEGylation (data not shown).

4.4.4.5. Size analysis of native uricase and Uc-mPEG-mal conjugates:

The size of native uricase after PEGylation is an essential parameter to be determined as it directly influences its kidney ultrafiltration rate and plasma half-life. In the current study, the average size of native uricase and Uc-mPEG-mal conjugates were determined to be 8.28 and 11.01 nm respectively (as shown in **Figure 4.21**). The size of native uricase obtained in the present study was in agreement with the standards reported in the literature (Zhang et al. 2015; Lin et al. 2013). An increase in the hydrodynamic radius in post-PEGylation indicated that the Uc-mPEG-mal conjugates can have a higher plasma

half-life and a slower kidney ultrafiltration rate in comparison to native uricase. In a report by Zhang et al. (2015), recombinant uricase from *Candida utilis* was PEGylated in a random manner with mPEG-succinimidyl carbonate and the apparent size of the final conjugate was found to be 3-folds higher in comparison to native uricase.
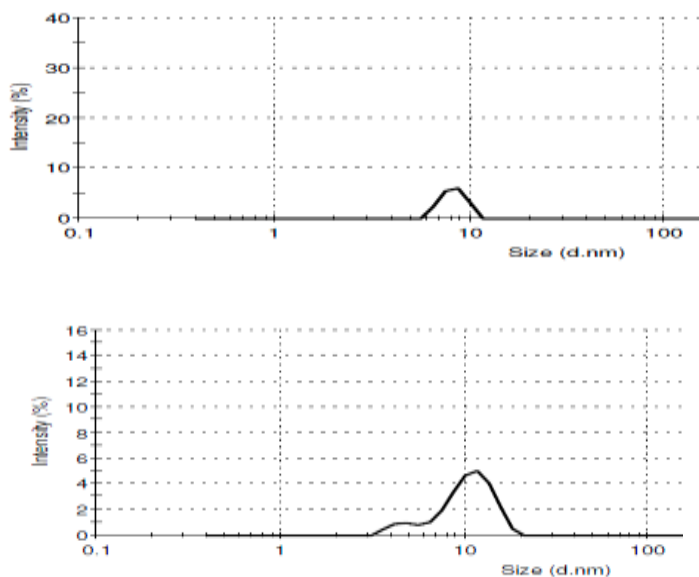
Figure 4.21: Size analysis of (a) Native uricase and (b) Uc-mPEG-mal conjugate

Higher size can significantly increase the *invivo* plasma half-life for prolonged periods and also provides protection from proteolytic enzymes however, if the number of PEG strands attached is high, it can also lead to accumulation of excess PEG in the kidney and liver. But in the present study, the number of mPEG strands attached was lesser compared to randomly PEGylated uricase conjugates. Hence, the problem of organ accumulation cannot occur using Uc-mPEG-mal developed in the present study.

Table 2.11 represents a few examples of liposomal and polymeric drug delivery systems developed for uricase and the corresponding increase in the hydrodynamic radii and zeta potential values. In a report by Lin et al. (2013), Uricase from *Candida sp.* was conjugated with zwitterionic block copolymer poly(methyl acrylic acid-b-sulfobetaine methacrylate). This resulted in a size enlargement of native uricase from 9 nm to 12 nm. Encapsulation of uricase in liposomal vesicles resulted in the development of

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enzymosomes of very large sizes, in the range of 260-330 nm (Zhou et al. 2016), 22.56 μm (Deng et al. 2015) and 201.54 nm (Tan et al. 2012). Enzymosomes are liposomal constructs synthesized to serve as a mini-environment in which the therapeutic enzymes are covalently restrained to the surface of liposomes (Hundekar et al. 2015). However, the concept of incorporating uricase into enzymosomes is debatable. As the enzymosomes are meant for targeted delivery to the tumor cell and may display organ accumulation. But, the main function of uricase is to catalyze the uric acid oxidation (which is distributed throughout the bloodstream). This demerit supports the concept of uricase to be used in its polymer conjugated form rather than liposomal encapsulation.

4.4.5. Evaluation of storage stability of native uricase and Uc-mPEG-mal conjugates:

In the present work, the effects of incubating native uricase and Uc-mPEG-mal conjugates on their residual activity at temperatures of 4°C, 25°C and 37°C for 18 days (as represented in **Figure 4.22)** were investigated. The results indicated that the Uc-mPEG-mal conjugates maintained a higher residual activity and were more stable in comparison to native uricase at all the temperatures.
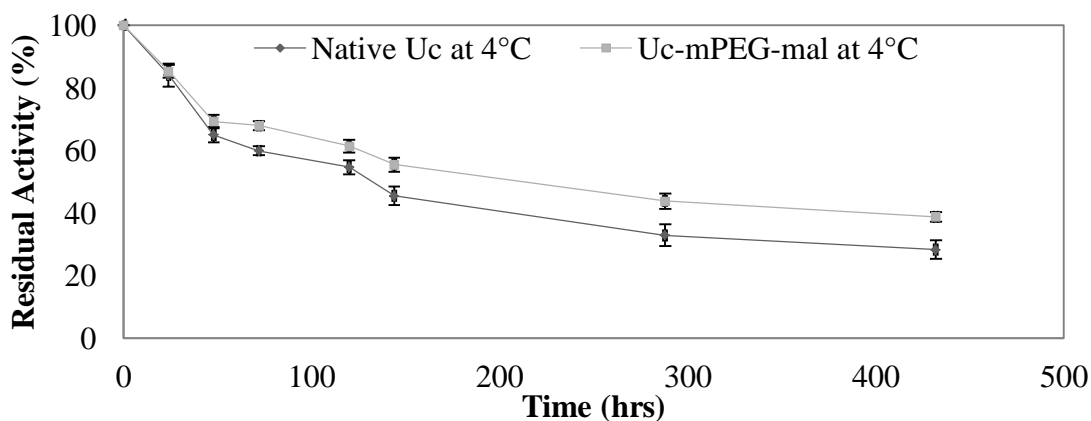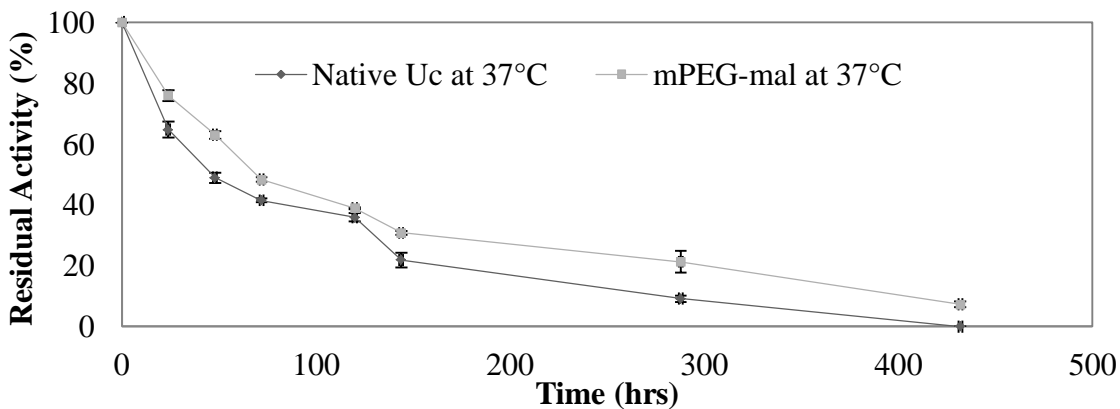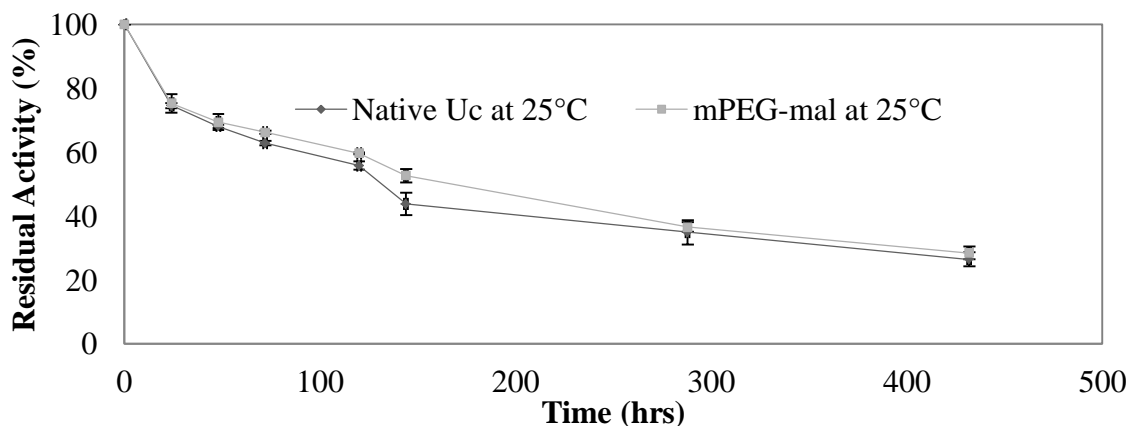
Figure 4.22: Storage stability of native uricase and Uc-mPEG-mal conjugates at various temperatures

Figure 4.22 indicates that the Uc-mPEG-mal conjugates remained more stable and possessed a higher residual activity in comparison to native uricase at physiological pH (7.5). The results displayed that the activity loss was lesser for conjugate and evidently higher for native uricase throughout 18 days of incubation at all temperatures. The Uc-mPEG-mal conjugates retained approximately 40% of their uricolytic activity even after incubation at 4°C for 18 days. The Uc-mPEG-mal conjugates retained around 21.26 % residual uricolytic activity on the 12'th day of incubation at physiological conditions of pH 7.5 and 37°C.

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Commercially available random PEGylated form of uricase "Krystexxa" (8 mg/mL of PEGylated uricase concentrate) is a suspension in a SP buffer solution with sodium chloride dissolved in water for injection (WFI) (pH 7.3±0.3). According to European medical agency the physical and chemical stability of this formulation [diluted in 250 ml sodium chloride solution (0.45- 0.9%)] has been demonstrated for 4 hours at 2-8°C/20-25°C.

In the present study, the optimal storage conditions for the Uc-mPEG-mal conjugates for a longer time (18 days) was found to be 100 mM SP buffer (pH of 7.5) at 4°C [in its undiluted form]. In a study by Freitas et al. (2010), the stability of random PEGylated uricase was tested where the conjugates were more active at close physiological pH and were stable up to 70°C.

A conceivable mechanism to elucidate the stabilization of the enzyme following PEGylation might be the fortification of the active site by the PEG molecules that increase the structural rigidity of the protein (Freitas et al. 2010; Yu et al. 2009). Without the protecting effect of PEG sheath, the structure in the active site of the native enzyme is more susceptible to changes at low pH values, leading the active site to an irreversible change (Soares et al. 2002).

PEGylation increases the hydrodynamic radius of the conjugate (due to the hydrophilic property of the PEG), which might have resulted in steric hindrance and consequently protection of the fragile uricase molecule. In a study by Yu et al. (2009), PEGylation of recombinant human interleukin-1 receptor antagonist with mPEG-aldehyde pointedly amplified the stability of protein in aqueous solution at room temperature. This was described by good homogeneity and high residual bioactivities. Circular dichroism studies conducted in the present study also supported the fact that PEGylation contributes towards maintaining the stability of the conjugates by fortifying rigidity (as represented in **Figure 4.20**).

4.4.6. Determination of kinetic parameters:

Computational and structural studies of PEG molecules forecast that they tend to fold and subjugate a large surface area of the protein, probably interfering with substrate binding (Manjula et al. 2003). To investigate the therapeutic competence, it is imperative to determine the uricolytic activity of the Uc-mPEG-mal conjugates by estimating the K_M values which will indicate the affinity of uricase towards uric acid. This will give be a good estimate *invivo* behavior of PEGylated uricase.

In the present study, K_M was determined from the enzyme kinetic analysis grounded on initial velocity measurements and the results were fitted to a Lineweaver–Burk plot. The K_M value obtained for native uricase from *Bacillus fastidious* was determined to be 4.899 $\times10^{-5}$ M, which is similar to the K_M values for uricase from other microbes previously reported in the literature, like recombinant uricase from *Candida utilis*-5.4$\times10^{-5}$ M (Freitas et al. 2010), a different strain of *Bacillus fastidious*- 5.0$\times10^{-5}$ M (Schiavon et al. 2000), *Aspergillus flavus*- K_M of 6.1$\times10^{-5}$ M and *Candida utilis*- K_M of 2.0$\times10^{-5}$ M (Bomalaski et al. 2002), *Arthrobacter globiformis*- 4.18$\times10^{-5}$ M (Punnappuzha et al. 2014) etc.

After PEGylation of uricase, K_M value of uricase conjugate reduced to 4.347 $\times10^{-5}$ M from 4.899$\times10^{-5}$ M indicating that PEGylation reaction improved the affinity of uricase towards uric acid (**Figure 4.23).** The lower K_M value can be correlated to the higher diffusibility of the substrate, elicited by the hydrophilic character of the PEG that assists the binding of the uricase to poorly soluble uric acid (Freitas et al. 2010). The K_M of Uc-mPEG-mal conjugates has physiological and clinical applicability since they displayed higher affinity towards uric acid. Previous literature also indicated a reduction in K_M values of uricase after its immobilization and hence increased substrate affinity (Arora et al. 2007; Freitas et al. 2010).

In contrast to the present findings, in a study by Punnappuzha et al. 2014, it was found that the conjugation of uricase with alternate polymers like Polysialic acid (PSA) increased the K_M values of the uricase conjugates. The apparent K_M of uricase had

increased slightly from 4.18 x 10^{-5} M to 5.46 x 10^{-5} M. This result indicated that the polysialated uricase displayed a lower affinity towards uric acid. In a study by Freitas et al. (2010), the K_M values were found to be 2.7×10^{-5} M (after PEGylation with mPEG-npc) or 3.0×10^{-5} M (after PEGylation with mPEG-CN) for the conjugates as equated to 5.4×10^{-5} M for the native uricase. This suggested an improvement in the substrate affinity of uricase which may be attributed to the masking of some of the active sites by the PEG strands. Polysialylation decreased the uricase-uric acid affinity due to masking of conjugation sites, whereas PEGylation increased the affinity as PEG is hydrophilic in nature and allows more enzyme-substrate interaction.
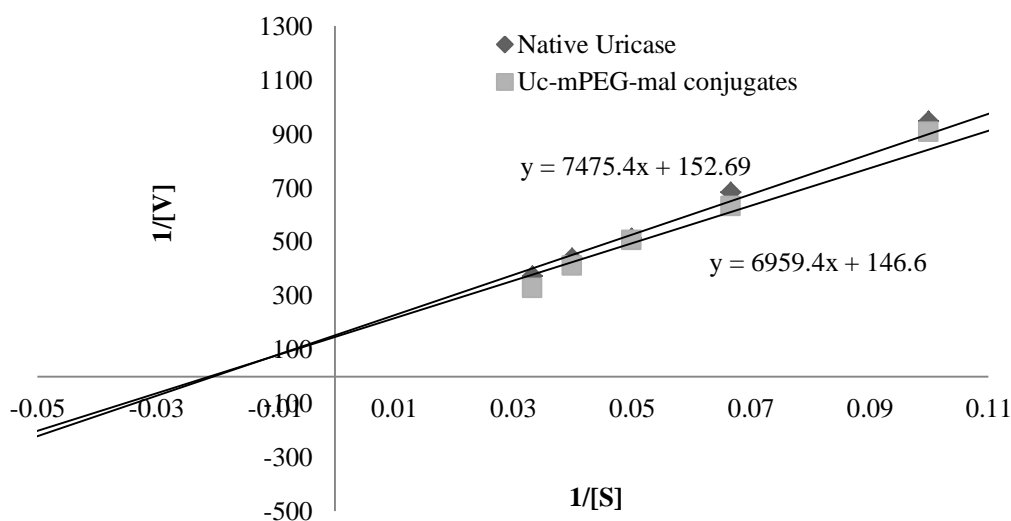
Figure 4.23: Lineweaver-Burk Plot for native uricase and Uc-mPEG-mal conjugates

4.4.7. Immunogenicity studies to evaluate the therapeutic efficacy of native uricase and Uc-mPEG-mal conjugates:

In the present study, antibodies produced in rabbits (used as animal models) against native uricase and Uc-mPEG-mal conjugates were quantified. The antibody titer (as a measure of immunogenicity) was calculated for native uricase and Uc-mPEG-mal

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conjugates using a rabbit uricase ELISA kit. **Figure 4.24** indicates the standard calibration curve constructed using a serially diluted standard solution of anti-uricase antibodies. This calibration curve was used to find the antibody titer of the serum samples containing anti-uricase antibodies produced against native uricase.

Figure 4.25 indicates the antibody titer of serum samples containing anti-uricase antibodies produced against native uricase and Uc-mPEG-mal conjugates. Native uricase induced immunogenicity in rabbits as expected, whereas Uc-mPEG-mal conjugates were weakly immunogenic. It can be observed that the antibody count of native uricase was the highest owing to its microbial origin and recognition as an antigen by the immune system of the rabbit. In contrast to native uricase, the samples containing Uc-mPEG-mal conjugates displayed a lesser antibody count. The optical density (and hence the antibody titer) displayed by the Uc-mPEG-mal conjugates were 3-5 folds lesser than that of native uricase samples. Uc-mPEG-mal conjugates displayed a 73 % reduction in the antibody titer in comparison to native uricase.

The attachment and wrapping of PEG molecules increased the hydrodynamic radius of the Uc-mPEG-mal conjugate which resulted in masking of the immunogenic sites on the uricase molecule. Thus the immunogenicity caused by native uricase was reduced due to its entrapment within the PEG strands, consequently rendering the developed conjugates more favorable to be applied for therapeutic purposes. The thiol PEGylation strategy used in the present study targets 4-8 cysteine residues per uricase molecule, wherein the long PEG strands have a tendency to wrap around the uricase molecule and reduce recognition by the immune system (Nanda et al. 2016).

In a report by Tan et al. (2012), functional lipid vesicles encapsulating uricase prominently decreased the immunogenicity of uricase. In another report by Zhang et al. (2012), immunogenicity induced by canine uricase randomly modified with mPEG-propionic acid (5 kDa) was evaluated, where conjugates stimulated very few IgM and IgG antibodies in comparison to native uricase after four injections, which indicated a reduction in immunogenicity.

In a study conducted by Caliceti et al. (1999) on comparative pharmacokinetic and biodistribution investigation of uricase conjugated with different amphiphilic polymers, it was observed that the branched PEG derivative accumulated specially in liver and spleen. In a report by Ganson et al. (2006), on testing the antigenicity of PEG-uricase, antibodies were directed against PEG, rather than the uricase. In a similar study by Yang et al. (2012), it was established that after continued treatment of Pegloticase, an immune response against PEG in nearly 20% of patients was detected. Uc-mPEG-conjugates synthesized in the present study have a very minute amount of mPEG-mal attached; hence the above-stated demerit of organ accumulation displayed by randomly PEGylated uricase conjugate can be deciphered. This consequently boosts the scope for the development of site-specific PEGylated uricase.

$$y = 0.0063x + 0.15$$
$$R^2 = 0.9848$$
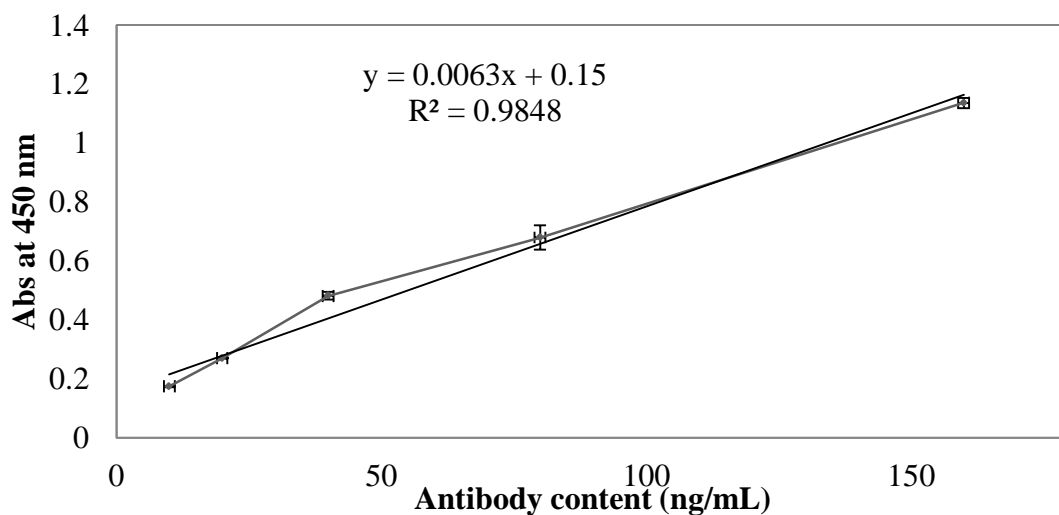
Figure 4.24: Calibration curve for antibody standards
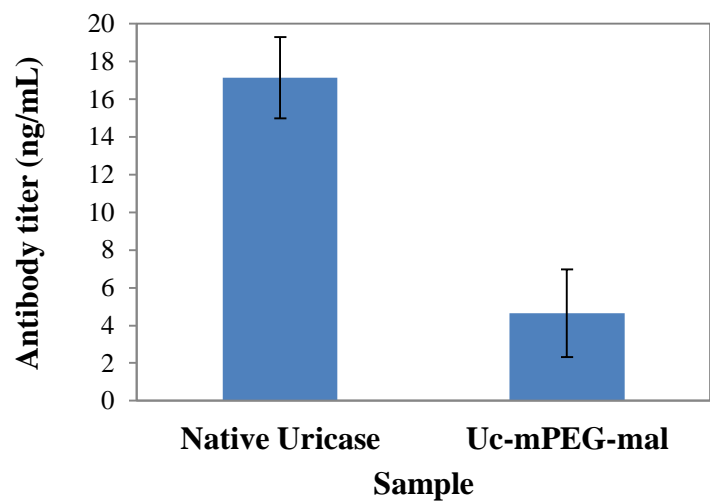
Figure 4.25: Antibody titer for native uricase and Uc-mPEG-mal conjugates subjected to ELISA

4.5 Synthesis of N-terminal PEGylated of Uricase from *Bacillus fastidious:*

In the first reaction of N-terminal PEGylation, the propionaldehyde moiety of mPEG-prop was covalently linked to the amino group on the N-terminus of the protein (**Figure 4.26**). This selectivity was conceivable by captivating advantage of the different pKa values among the ε-amine group of lysine and the α-amine group of lysine situated at the N-terminus.

At a pH in the range of 5–6, all the ε-amines (ε-NH$_2$) in a protein tend to be protonated whereas the amine group (α-NH$_2$) is still moderately present as a free base available for coupling with activated PEG molecules. The main reason for this selective unprotonation is the difference in their pKa values ie: 7–8 for α-amine and 10–11 for ε-amine (Wong et al. 1991). In these reactions, an unstable Schiff base is initially obtained which reduced to a stable secondary amine. The linkage formed in the first step (Schiff's-base linkage) can be reversed by hydrolysis, but it is swiftly stabilized to a stable, non-hydrolysable amine linkage in the second step by sodium cyanoborohydride reduction.
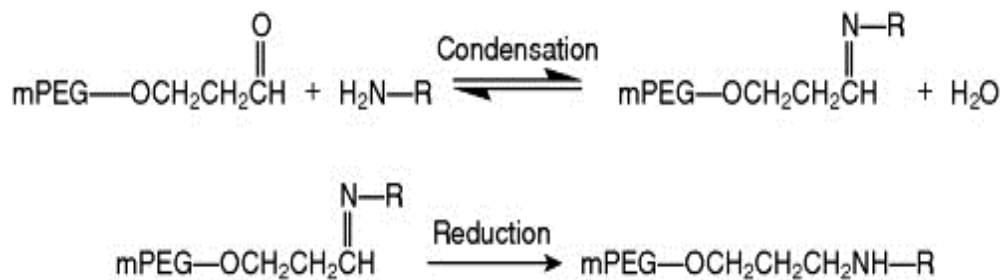
Figure 4.26: Reductive amination of protein using mPEG–propionaldehyde

The synthesis of N-terminal PEGylated uricase from *Bacillus fastidious* (Uc-mPEG-prop) was carried out using mPEG–prop (10 and 20 kDa) as the PEGylating reagent. mPEG-propionaldehyde with a terminal aldehyde group specifically reacted with the N-terminus of the uricase molecule at an acidic pH (pH 5.0), in presence of sodium cyanoborohydride. N-terminal PEGylation with mPEG-prop forms a secondary amine

linkage and conserves the positive charge on the N-terminal amine group (Lee et al. 2003).

Figure 4.27 (a) and (b) represent the gel documentation (Coomassie blue) and iodine stained gel images of SDS-PAGE gels. The molecular weights of the conjugates formed by 10 kDa mPEG- prop (Uc-mPEG-prop-10) and conjugates formed by 20 kDa mPEG- prop (Uc-mPEG-prop-20) conjugates were determined to be 60 kDa and 88.6 kDa per subunit after conjugation respectively. The Uc-mPEG-mal conjugates synthesized using mPEG-mal of molecular weights 750 Da, 5 kDa and 10 kDa possessed molecular weights of 39.2, 46.7 and 52.3 kDa per subunit respectively. These results indicated that the overall molecular weight of the PEGylated uricase conjugates directly depends on the molecular weight of the PEGylating agent used. Consequently, the size and molecular weight of the conjugates directly influences their kidney ultrafiltration rates.
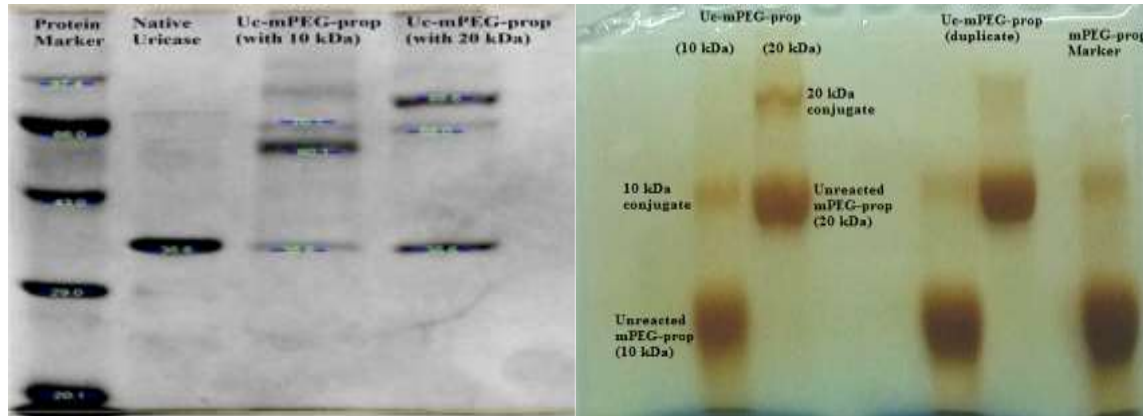
Figure 4.27: (a) Coomassie blue and (b) iodine solution stained gels indicating the positions and apparent molecular weights of Uc-mPEG-prop conjugates

The rate of excretion of PEGylating agents through the kidneys predominantly depends on their molecular weights. PEG with a hydrodynamic radius greater than glomerular membrane pore size can be filtered at lower rates, due to great flexibility (Kontermann, 2012). PEGylating agents of size above 40 kDa have been shown to be slowly eliminated

via renal elimination and by passive filtration (Woodburn et al. 2013). Hence PEGylating agents of size below 40 kDa are further preferred to be used for PEGylation of bulky protein molecules like uricase, which have a greater size.

4.5.1. Optimization of reaction conditions to maximize the yield of Uc-mPEG-prop conjugates:

The concentration of the PEGylating agents in the PEGylation reaction mixture directly influences the extent of PEGylation (Harris et al. 2001). **Figure 4.28 (a)** shows the effects of molar ratios of uricase to mPEG-prop, on the percentage substitution of the N-terminal amine residue and their respective residual uricolytic activity. The highest modification rate of 80.7% was obtained at a molar ratio of 1:15 of uricase to mPEG-prop. However, it was observed that a higher ratio resulted in increased polymorphism of PEGylation (as shown in **Figure 4.28 (b)**) and decreased uricolytic activity (approximately 60%).
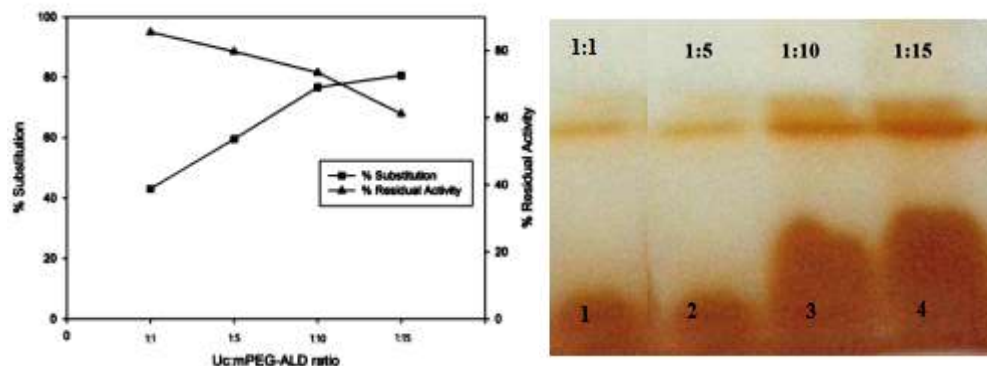
Figure 4.28: (a) Effect of uricase to mPEG-prop concentration ratio (wt/wt) on percentage substitution and residual activity of Uc-mPEG-prop conjugates and (b) iodine stained SDS-PAGE analysis gel image depicting the effect of Uc to mPEG-prop-10 concentration ratio. Lanes 1 – 4 represents conjugates synthesized with 1:1, 1:5, 1:10 and 1:15 molar ratios of uricase to mPEG-prop (10 kDa)

In order to avoid polymorphism and achieve monoPEGylation, a molar ratio of 1:10 was chosen in the subsequent experiments. Also, the conjugate yield, residual activity and the percentage substitution of the N-terminal amine residue were higher in the sample containing 1:10 ratio of uricase to mPEG-prop. In the case of Uc-mPEG-mal conjugates synthesized previously, uricase to the mPEG-mal concentration ratio of 1:12 resulted in maximum conjugate yield. This ratio did not have any negative influence on the uricase activity.

N-terminal PEGylation of proteins is generally carried out at mild acidic conditions (Kinstler et al. 1996). In the present study, PEGylation of uricase was carried out using mPEG-prop (10 kDa) in buffer solutions at different pH values ranging from 4.5-7.5. It was observed that the percentage yield of Uc-mPEG-prop conjugates was highly dependent on the pH of the reaction medium and the maximum yield was obtained at pH 5.0. The yields of Uc-mPEG-prop conjugates (represented in **Table 4.14)** at different pH values were determined using RP-HPLC technique and the chromatograms are as represented in **Figure 4.29**. The uricase to mPEG-prop concentration ratio as well as the molecular weight of mPEG-prop reagent used had a dominant effect on the resolution of Uc-mPEG-prop conjugates as detected by a UV detector.

The maximum yield of Uc-mPEG-prop conjugate (76.88 %) was obtained by conducting the PEGylation reaction in buffer with a pH value of 5.0. But soon after PEGylation reaction, the conjugates were diluted using SP buffer with pH 8.5, to restore the uricolytic activities of the conjugates. The yield of the Uc-mPEG-prop conjugates decreased at neutral conditions as a result of protonation of the N-terminal amine group. Similar results have been reported for different therapeutic proteins like recombinant lidamycin apoprotein (Li et al. 2015), interferon-β-1a (Korzhavin et al. 2015), fibroblast growth factor-21 and staphylokinase dimers (Liu et al. 2012) at the N-terminal amine group.

The yield of Uc-mPEG-prop conjugates (76.88 %) was less in comparison to the yield of Uc-mPEG-mal conjugates (95.16 %) after optimization of reaction conditions, which may be due to inadequacy and singularity of the conjugating group (ie: N-terminal amine group). This reason consequently reduced the prospect of the reaction exclusively at the

N-terminal amine group, but ensured monoPEGylation of uricase exclusively at the N-terminal.
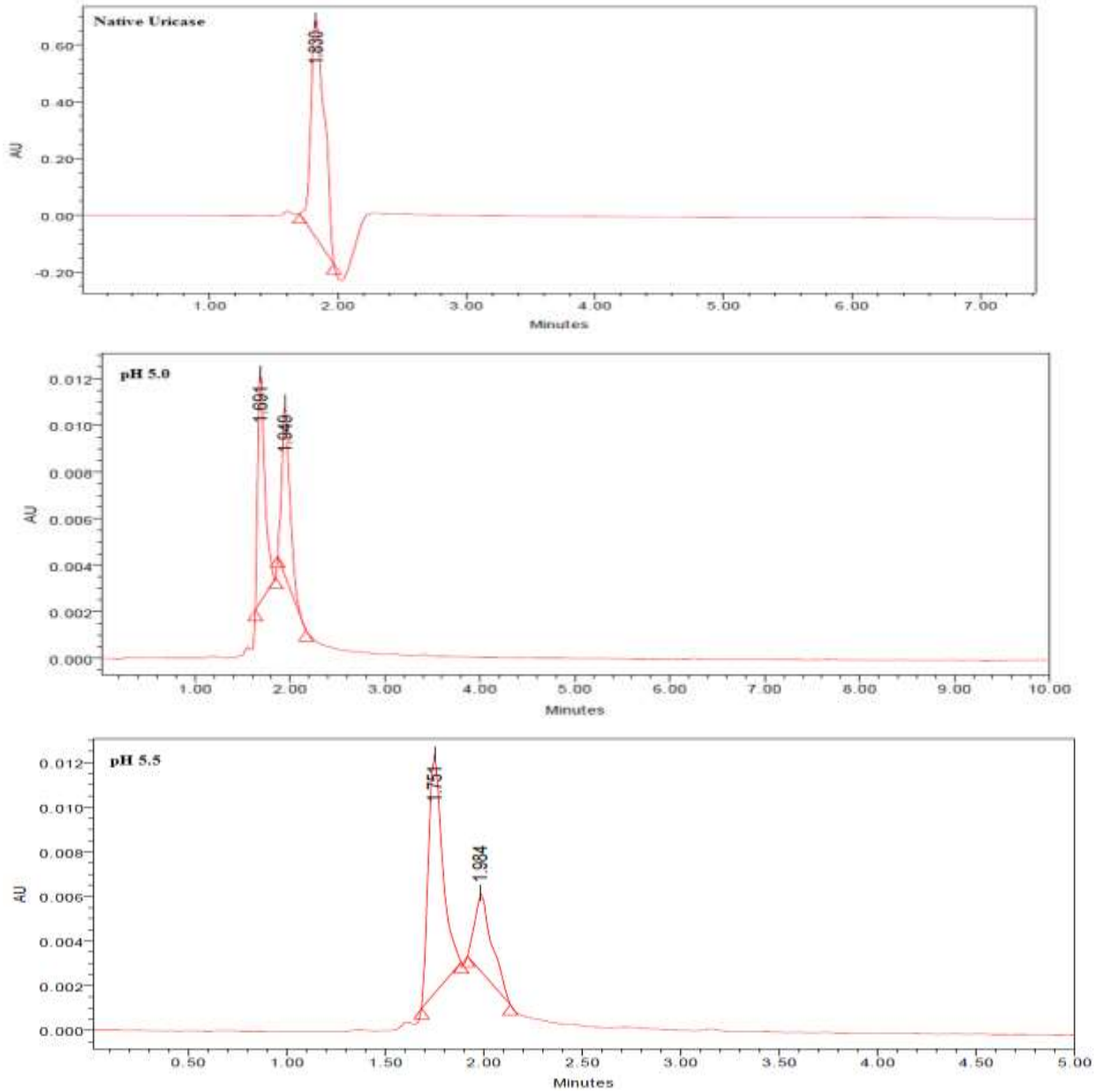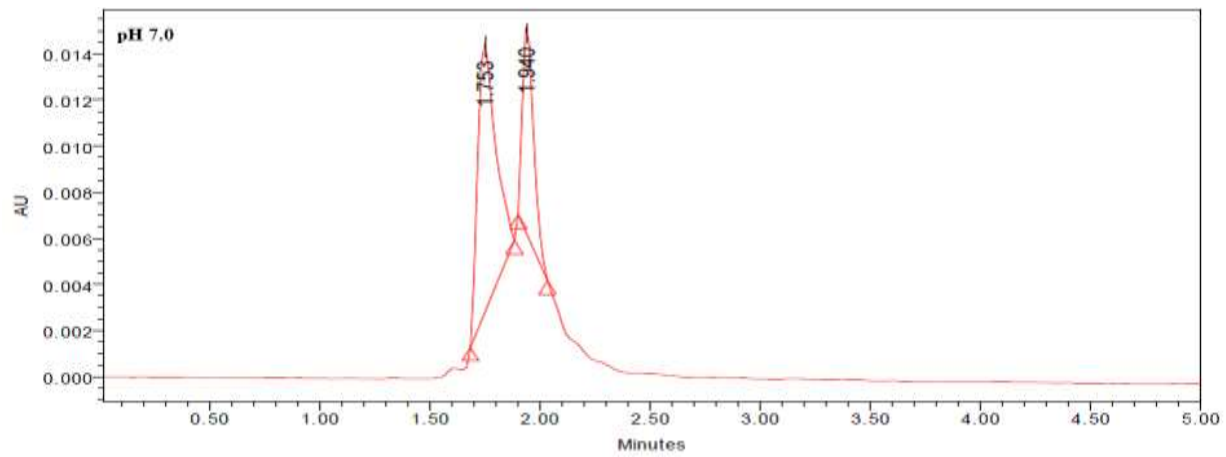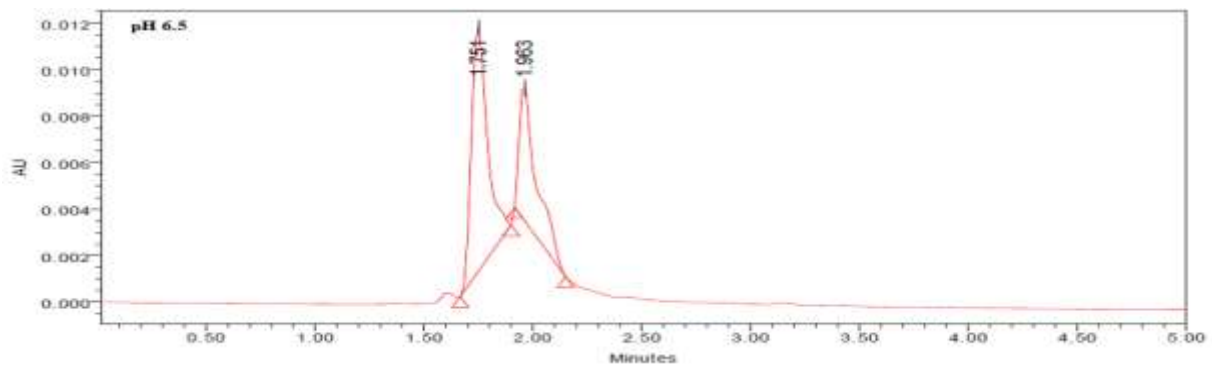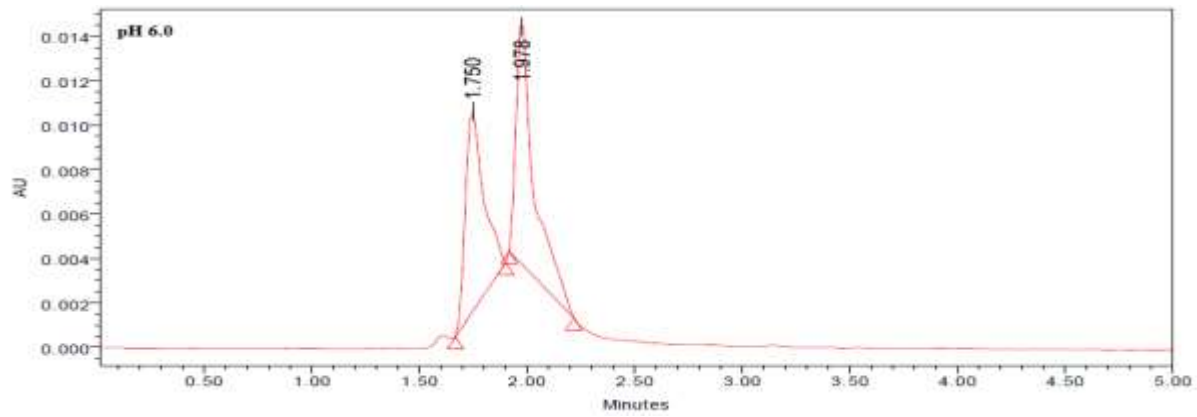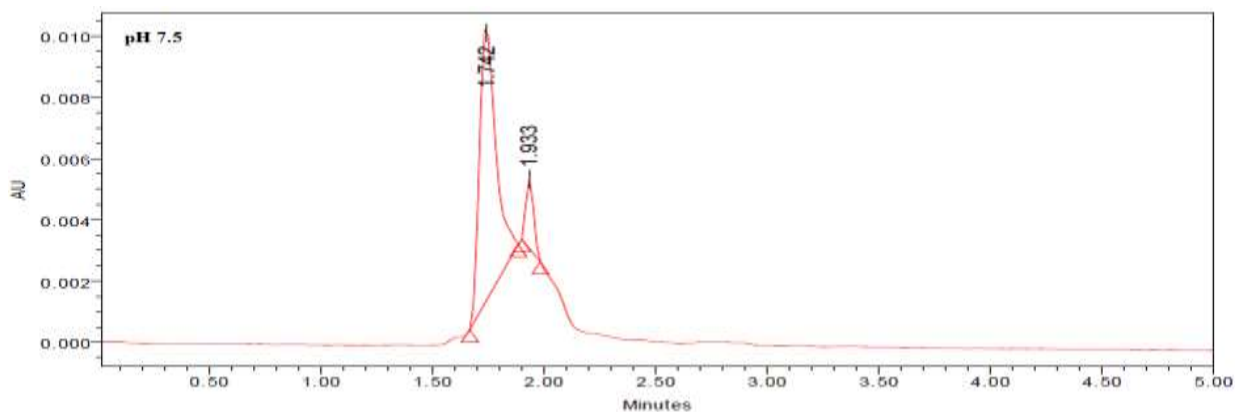
Figure 4.29: RP-HPLC chromatograms of native uricase and Uc-mPEG-prop conjugates synthesized in buffer solutions of different pH values

Table 4.14: Yields of Uc-mPEG-prop conjugates obtained by conducting the PEGylation reaction at different pH conditions

Sl no	Reaction buffer pH	Yield of Uc-mPEG-prop conjugates (%)
1	5.0	76.88
2	5.5	42.35
3	6.0	72.53
4	6.5	59.65
5	7.0	56.68
6	7.5	7.78

The yields of Uc-mPEG-prop conjugates could be correlated to the percentage substitution obtained from TNBS colorimetric analysis. The values of percentage substitutions of the N-terminal amine in a given fixed concentrated uricase solution as represented in **Figure 4.28.** Considering the above results, the subsequent PEGylation reactions were conducted at pH 5.0 and later diluted using SP buffer of pH 8.5 to restore its activity.

4.5.2. Purification of Uc-mPEG-prop conjugates:

The separation and purification of the Uc-mPEG-prop conjugate reaction mixture was carried out based on the differences in molecular weights of the reaction components using centrifugal ultrafiltration, SE-HPLC and SE-FPLC chromatographic procedures. **Figure 4.30** and **Figure 4.31** indicate the SE-HPLC chromatogram for native uricase and Uc-mPEG-prop PEGylation reaction mixture with elution time and apparent molecular weight data. **Table 4.15** and **Table 4.16** represent the corresponding peak number, retention times and the molecular weights of the components of the sample. Native uricase (tetramer) possessed a molecular weight of 161.98 kDa. The presence of very minute quantities of broken peptide fragments was also observed.
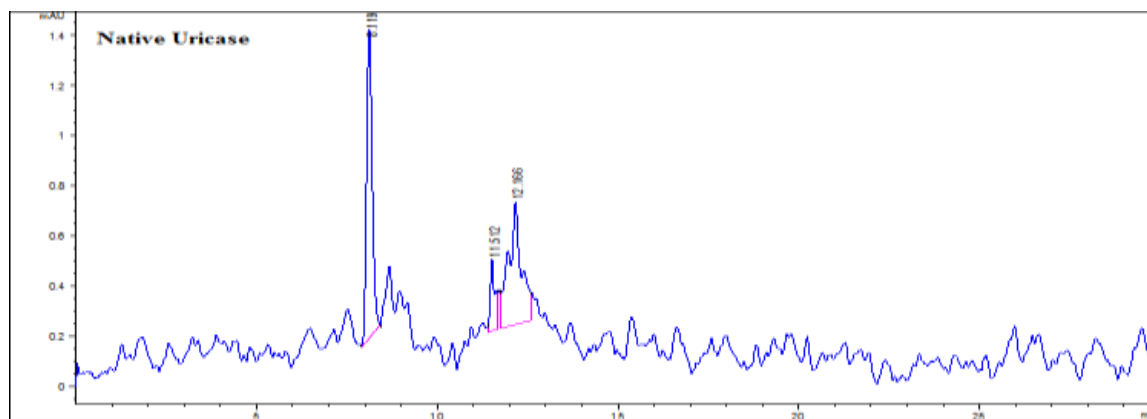
Figure 4.30: SE-HPLC chromatogram for native uricase

Table 4.15: Elution time and molecular weight data obtained after SE-HPLC for native uricase

Peak number	Time	Height	Width	Symmetry	Mol wt
1	8.119	1.2	0.1698	0.808	161.98
2	11.512	2.80E-01	0.1313	0.47	4.14
3	12.166	4.90E-01	0.3277	1.131	2.04
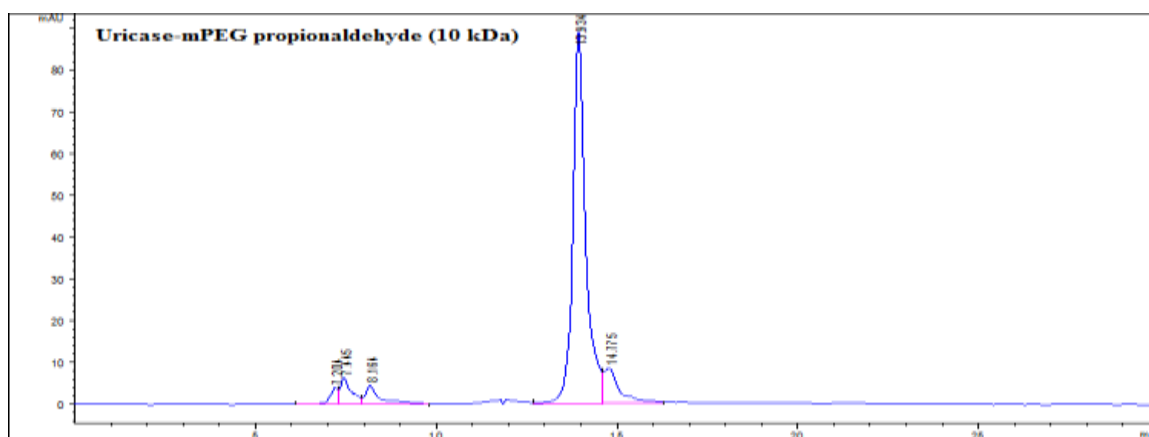
Figure 4.31: SE-HPLC chromatogram for Uc-mPEG-prop conjugate reaction mixture

The SE-HPLC chromatogram of PEGylated uricase reaction mixture indicated the formation of Uc-mPEG-prop conjugates as well as their aggregates. Partial purification of the Uc-mPEG-prop conjugates was achieved with trace amounts of smaller peptide molecules. The fractions of native uricase and Uc-mPEG-prop conjugates were collected and subjected to further purification by SE-FPLC.

Table 4.16: Elution time and molecular weight data obtained after SE-HPLC for native uricase

Peak number	Time	Height	Width	Symmetry	Mol wt
1	7.204	4	0.2163	2.462	432.2945
2	7.445	6.3	0.2962	0.458	335.5104
3	8.164	4.5	0.3833	0.395	154.2948
4	13.934	89	0.3712	0.761	0.302738

Figure 4.32 represents the SE-FPLC chromatogram of native uricase detected at 280 nm. **Figure 4.33 (a)** and **(b)** represent the chromatograms of PEGylation reaction mixture (using mPEG-prop-10 and 20 kDa) for the separation of the conjugates by SE-FPLC as detected at 280 (for protein detection) and 214 nm (for mPEG detection). Components of the PEGylation reactions such as native uricase and its conjugates and other reaction components were separated and pooled efficiently. In the chromatograms detected at 214 nm, the presence of mPEG molecules was also detected **[Figure 4.33 (b)** and **Figure 4.34 (b)]. Figure 4.35** represents the gel documentation image for SDS-PAGE gel of Uc-mPEG-prop conjugates before and after purification by SE-FPLC chromatography. From the SDS-PAGE gel images, it can be observed that the unmodified uricase was completely eliminated after purification using SE-FPLC.
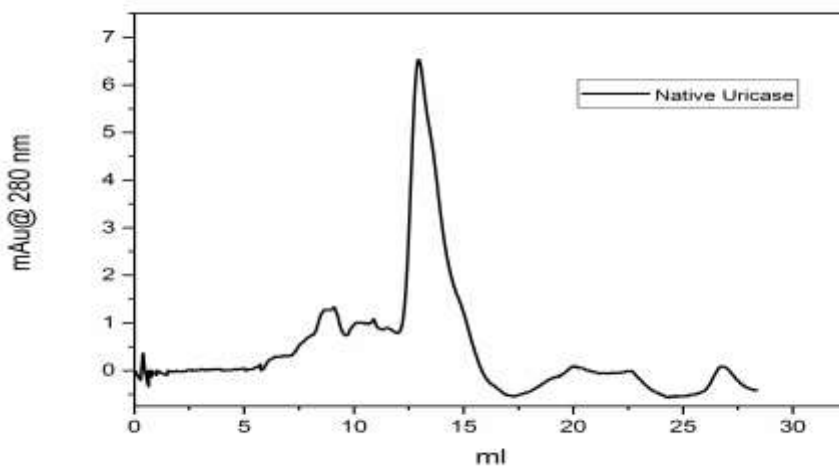
Figure 4.32: SE-FPLC chromatogram of native uricase
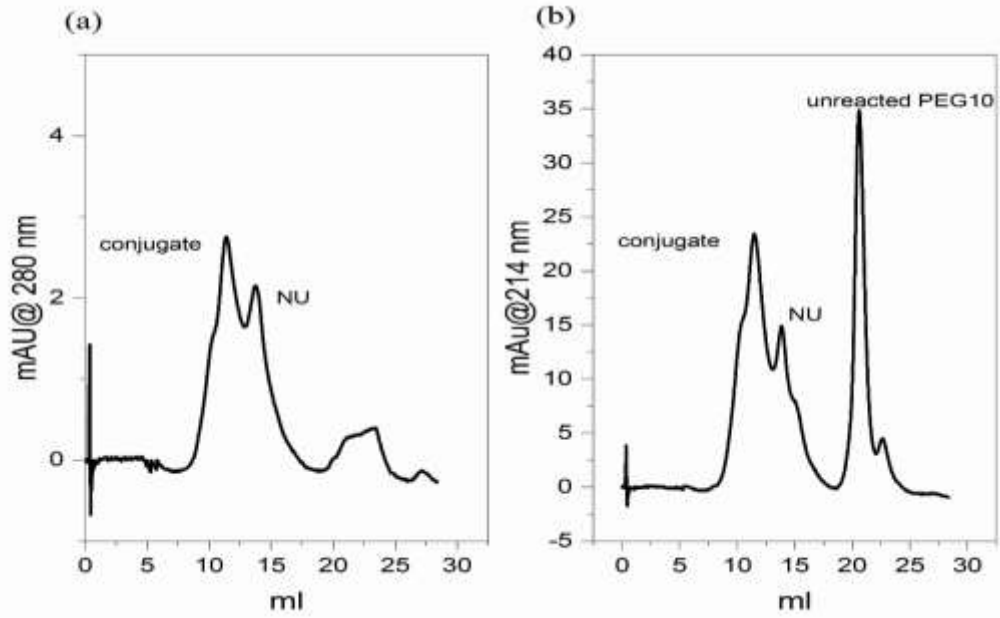
Figure 4.33: SE-FPLC chromatogram of PEGylated mixture with mPEG-prop 10kDa at (a) 280nm and (b) 214nm
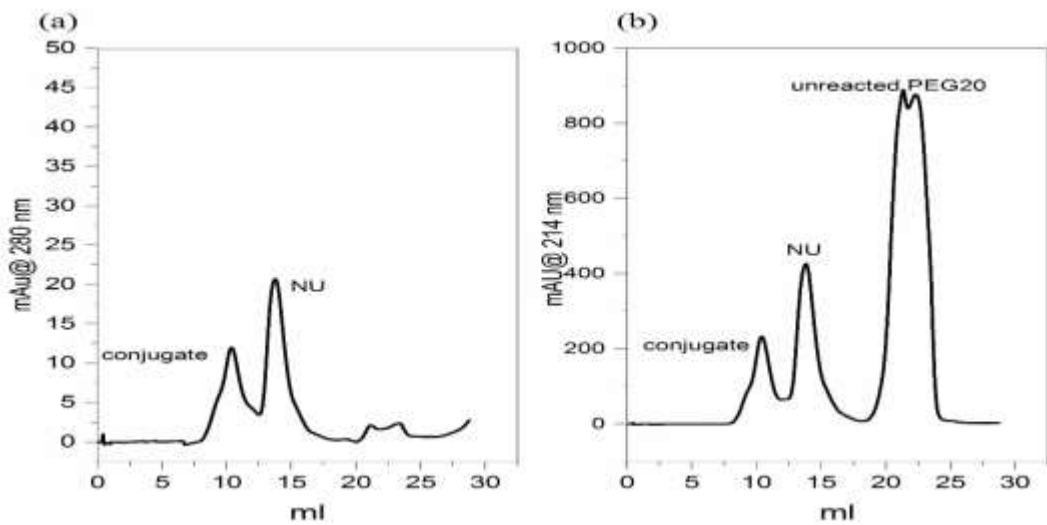
Figure 4.34: SE-FPLC chromatogram of PEGylated mixture with mPEG-prop 20kDa at (a) 280nm and (b) 214nm

Figure 4.34 (a) and **(b)** represent the chromatograms of PEGylation reaction mixture (using mPEG-prop-20 kDa) for the separation of the conjugates by SE-FPLC as detected at 280 and 214 nm. **Figure 4.35** represents the gel documentation image of native uricase and Uc-mPEG-prop (10 and 20 kDa) conjugates obtained after purification by SE-FPLC. Since native uricase and Uc-mPEG-prop conjugate were co-eluted, there were small traces of native uricase detected in the samples containing conjugates (**Figure 4.34**).
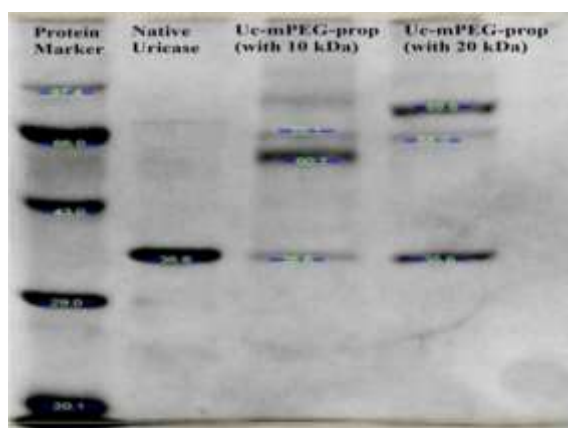
Figure 4.35: Gel documentation image for SDS-PAGE gel of native uricase and Uc-mPEG-prop conjugates before and after purification by SE-FPLC chromatography

From the SDS-PAGE analysis, the molecular weight of the conjugates obtained by conjugation with mPEG-prop (10 kDa) was determined to be in the range of 60-70 kDa and 63-89 kDa per subunit upon PEGylation with mPEG-prop (20 kDa). The conjugate molecular weight has a direct influence on its hydrodynamic radius and consequently its kidney ultrafiltration rate.

In the present study, a wide variety of mPEG-derivatives mPEG was successfully conjugated to uricase which reflects the scope of conjugation to any other therapeutic protein. Also, the size analysis results indicated that the Uc-mPEG-prop (10 and 20 kDa) conjugates had a size of 63.98 and 72 nm respectively (**Fig 4.36**). The surge in the molecular weight of the single subunit of the conjugate by double (Uc-mPEG-prop-10 kDa) and triple folds (Uc-mPEG-prop-20 kDa) indicated a desirable conjugate size which can prevent early kidneys ultrafiltration.

4.5.3. Characterization of Uc-mPEG-prop conjugates:

4.5.3.1. Determination of residual activity:

The residual activities of Uc-mPEG-prop conjugate indicated that even after PEGylation, the uricase molecule possessed significant uricolytic activity (**Table 4.17**). The residual uricolytic activity displayed by Uc-mPEG-prop (10 kDa) conjugates was 84%, which is similar to that obtained from the residual activities displayed by Uc-mPEG-mal conjugates. The result indicates that the post-PEGylation uricolytic activity displayed by the uricase molecule was desirably high, which is a very important criterion for a PEGylated drug to be directed as a therapeutic agent. In contrast to the previously reported post PEGylation residual uricolytic activities profiles of randomly PEGylated uricase conjugates (less than 70 %) (Zhang et al. 2010) (Freitas et al. 2010) Uc-mPEG-prop conjugates (10 and 20 kDa) obtained in the present study displayed desirable residual uricolytic activities.

Table 4.17: Residual activities of native uricase and Uc-mPEG-prop conjugates

Sample	Residual Activity (%)
Native Uricase	100
Uc-mPEG-prop (10 kDa)	83.46
Uc-mPEG-prop (20 kDa)	80.87

4.5.3.2. Size analysis of native uricase and Uc-mPEG-prop conjugates:

The size of native uricase and Uc-mPEG-prop conjugates are represented in **Table 4.18.** The results indicated that the hydrodynamic radius of native uricase increased upon PEGylation with mPEG-prop of 10 and 20 kDa molecular weights. It was observed that there was a 4-5 fold increase in the size of native uricase after PEGylation. The molecular weight of the PEGylating reagents used was directly proportional to the increase in the

hydrodynamic radius of Uc-mPEG-prop conjugates and inversely proportional to the residual activity of the conjugate. **Figure 4.36** represents the size distribution of **(a)** native uricase **(b)** Uc-mPEG-prop-10 kDa and **(c)** Uc-mPEG-prop-20 kDa conjugates.

Table 4.18: Size of native uricase and Uc-mPEG-prop conjugates

Sample	Size (nm)
Native Uricase	12.8
Uc-mPEG-prop (10 kDa)	63.98
Uc-mPEG-prop (20 kDa)	72.0
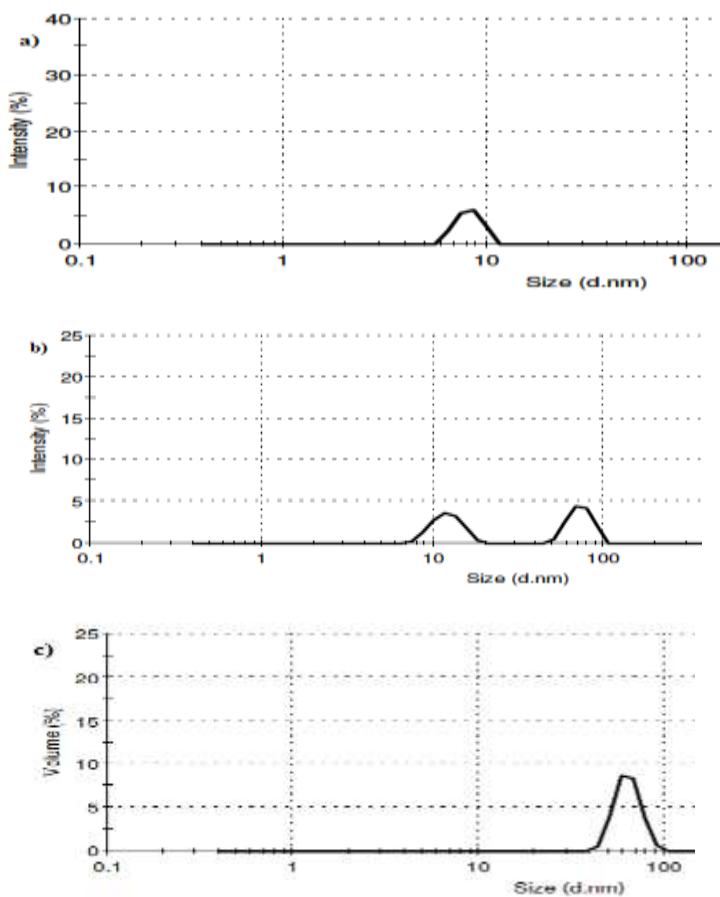
Figure 4.36: Size distribution of (a) native uricase, (b) Uc-mPEG-prop-10 kDa and (C) Uc-mPEG-prop-20 kDa

However, doubling the mPEG-prop molecular weight from 10 kDa to 20 kDa resulted in a very slight increase in the radius (by 8 nm). In a report by Chiu et al. (2010), computational analysis of functionalized hemoglobin indicated that covalently attached PEG chains (with a molecular weight ranging from 5-20 kDa) condensed onto the surface of the hemoglobin molecule. In the present study, as a result of the folding of mPEG chains, there was no significant increase in the hydrodynamic radius of the conjugate with higher molecular weight PEG.

4.5.3.3. Circular dichroism for the determination of PEGylation induced conformational changes:

Circular dichroism spectroscopic studies were completed to determine the PEGylation induced conformational changes in the secondary structure of uricase molecule. It was observed that PEGylation had caused variations in the secondary structure of the uricase molecule which is apparent from the CD spectra obtained for native uricase and Uc-mPEG-prop conjugates. Native uricase and Uc-mPEG-prop conjugates showed changes in their secondary structure with increase in operational temperature during the CD analysis. The α-helix content of the native uricase was found to change due to PEGylation and was highly dependent on the molecular weight of mPEG-prop used. The CD spectra of the native uricase and Uc-mPEG-prop conjugates (10 and 20 kDa) at different temperatures are shown in **Figure 4.37**. **Table 4.19** represents valuation of the percentage of α-helix content in native uricase, Uc-mPEG-prop-10, and Uc-mPEG-prop-20 at different temperatures.
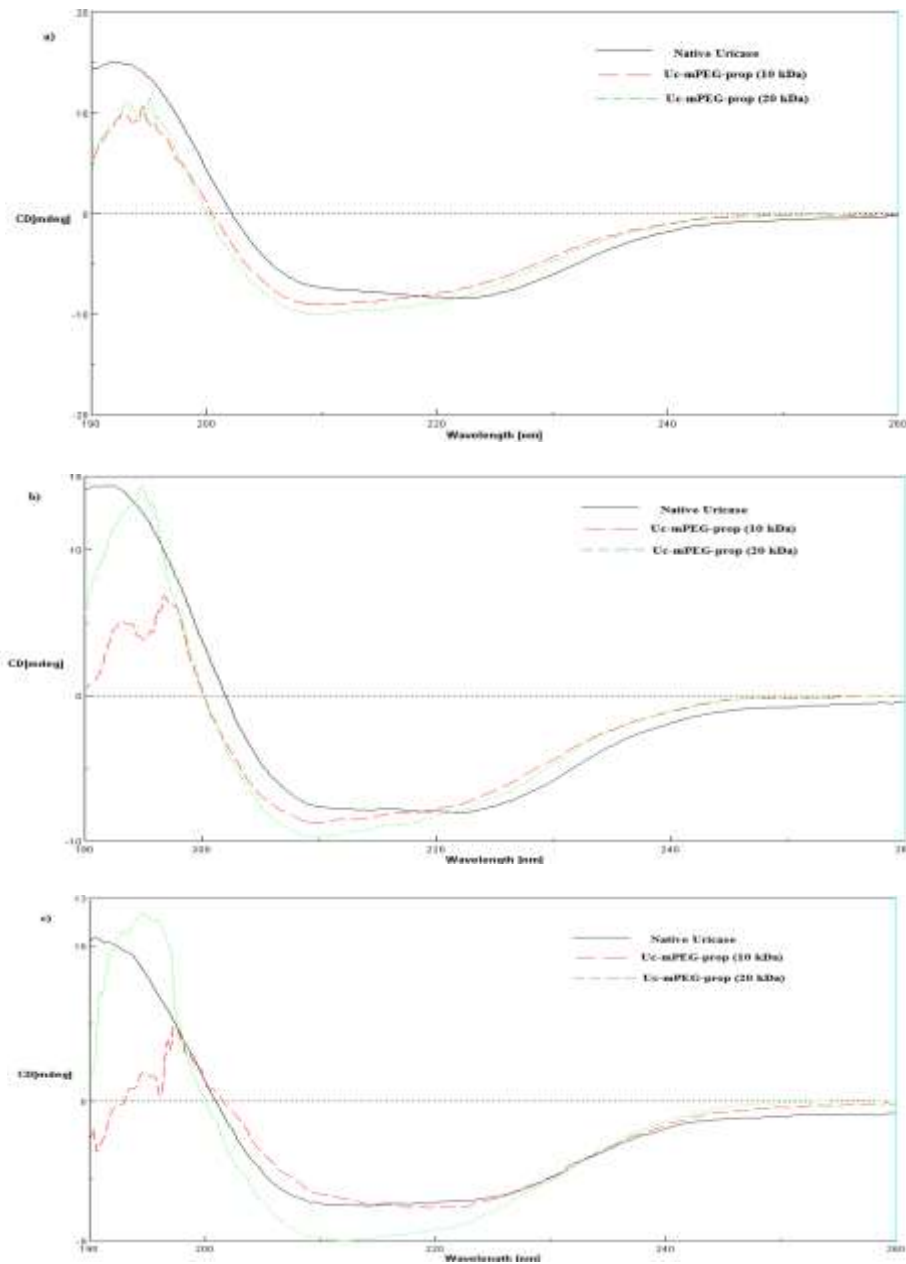
Figure 4.37: Circular dichroism (CD) of native uricase and Uc-mPEG-prop (10 kDa and 20 kDa) at different temperatures (a) 20°C (b) 50°C and (c) 70°C

Table 4.19: Assessment of the percentage of α-helix content in native uricase, Uc-mPEG-prop-10, and Uc-mPEG-prop-20 at different temperatures

Sample	α-helix content		
	20°C	**50°C**	**70°C**
Native Uricase	50%	47%	28%
Uc-mPEG-prop-10	48%	46%	33%
Uc-mPEG-prop-20	44%	43%	41%

In the present study, Uc-mPEG-prop conjugates had a higher thermal stability than native enzyme (upon treatment at 50°C and 70°C) indicating that PEGylation could induce higher stability to native uricase by slight changes in secondary structure. Similar results were also obtained in the case of Uc-mPEG-mal conjugates, wherein insignificant alterations (up to 2 %) were observed in the secondary structures of the native uricase and PEGylated uricase. Also, there was a slight decrease in the α-helix content after PEGylation, which was in turn highly dependent on the temperature at which CD spectrometry was conducted.

4.5.4. Determination of kinetic parameters:

The kinetic constant K_m was determined from the classic enzyme kinetic analysis based on initial velocity measurements using LB plot. Different uric acid concentrations (5-30 μM) were applied to study the effect of substrate concentration on the activity of the native uricase and PEGylated enzyme at standard assay conditions. **Figure 4.38** represents the Lineweaver-Burk plot for estimation of kinetic constants for Uc-mPEG-mal conjugates. The K_m value of the conjugate has decreased slightly from 4.899×10^{-5} M of the native uricase to 4.208×10^{-5} M for Uc-mPEG-prop-10 and 3.902×10^{-5} M for Uc-mPEG-prop-20. This suggested an augmentation in the affinity of the modified uricase towards uric acid.
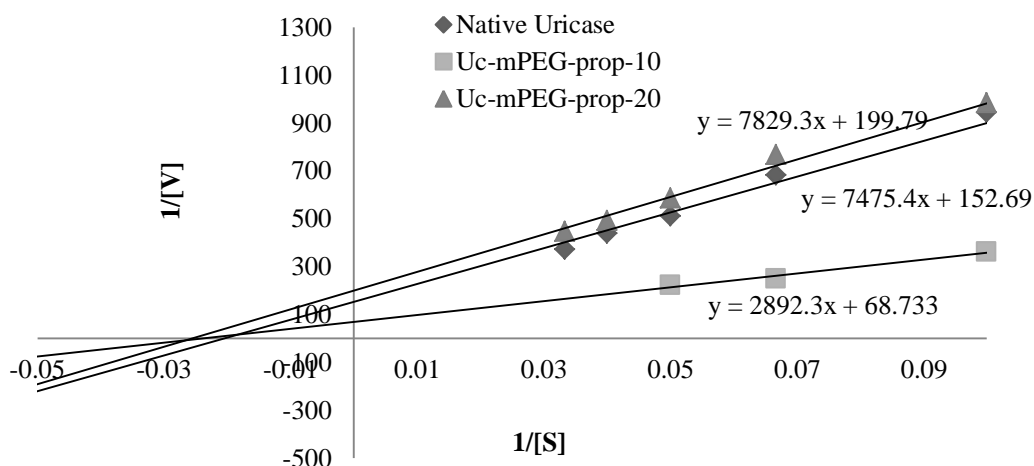
Figure 4.38: Lineweaver-Burk plot for estimation of kinetic constants for native uricase and Uc-mPEG-prop conjugates

Table 4.20 represents the K_m values of native uricase and mPEG-prop conjugates indicating that the PEGylation reaction improved the affinity of uricase towards uric acid (**Figure 4.35**).

Table 4.20: K_m values of native uricase and Uc-mPEG-prop conjugates

Sl no	Sample	K_m
1	Native Uricase (NU)	4.899×10^{-5} M
2	mPEG-prop-10	4.208×10^{-5} M
3	mPEG-prop-20	3.902×10^{-5} M

The increased affinity may be attributed to the flexible shell formed by PEG chains around the enzyme. The elastic shell constrains the conformational changes in the uricase molecule and provides a favorable microenvironment for uricase binding to uric acid. In the previously reported literature, a decrease in the K_m value of uricase after its immobilization provided a similar microenvironment for the enzyme. Previously reported

literature also reported a lowering of the K_M of uricase after its immobilization (Arora et al. 2007) (Freitas et al. 2010).These results about the K_m of Uc-mPEG-prop conjugates are of physiological and clinical applicability since they display higher affinity towards uric acid.

The Uc-mPEG-mal conjugates synthesized in our study displayed a K_m value of 4.347 $\times 10^{-5}$ M through which an enhancement in the affinity of uricase towards uric acid was confirmed (**Figure 4.23).** In conclusion, it can be stated that the PEGylation provided a favorable environment for uricase and uric acid to react and form allantoin.

4.5.5. Evaluation of storage stability of native uricase and Uc-mPEG-prop conjugates:

In the current work, we explored the effects of incubating native uricase and Uc-mPEG-prop (10 kDa) conjugates at different temperatures conditions (4°C, 25°C and 37°C) for about 18 days which is as represented in **Figure 4.39 (a), (b)** and **(c)**. The Uc-mPEG-prop (10 kDa) conjugates retained around approximately 40% uricolytic activity even after incubation at 4°C for 18 days and were more stable in comparison to native uricase.

The Uc-mPEG-prop-10 kDa conjugates retained around 10.83 % residual uricolytic activity on the 12'th day of incubation at physiological conditions of pH 7.5 and 37°C, whereas the Uc-mPEG-mal conjugates retained approximately 21.26 % residual uricolytic activity on the 12'th day of incubation at the same physiological conditions. But all the conjugates displayed a complete loss of activity by the end of 18 days of incubation. It can be concluded that, Uc-mPEG-mal conjugates have a better stability, due to a higher number of mPEG-mal strands (2-4) attached per subunit.

However, the stability was studied in sodium phosphate buffer of pH 7.4 only, but more efforts on the formulation development part can be worked upon to enhance the overall stability.
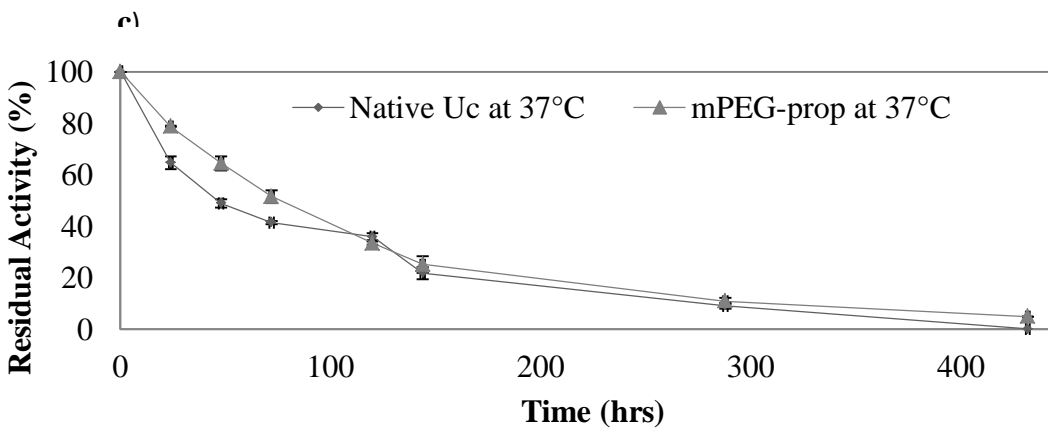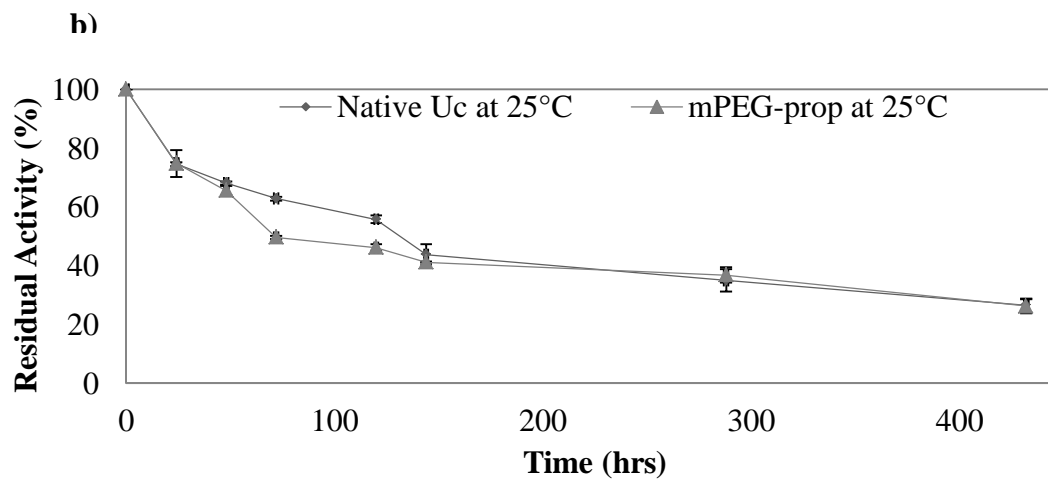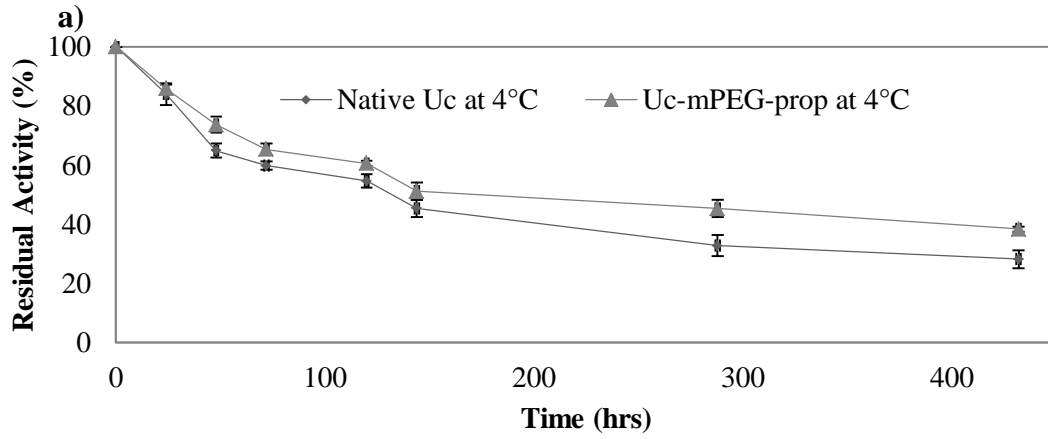
Figure 4.39: Storage stability of native uricase and Uc-mPEG-prop (10 kDa) at a) 4°C, b) 25°C and c) 37°C

4.5.6. Immunological evaluation of native uricase and Uc-mPEG-prop conjugates:

A calibration curve was constructed using serially diluted serum solution (as shown in **Figure 4.24**) and used as a standard for determination of antibody titer. **Figure 4.40** indicates the antibody titer of serum samples containing anti-uricase antibodies produced against native uricase and Uc-mPEG-prop conjugates. It can be observed that the antibody count of native uricase was high since it has a microbial origin and hence was identified as an antigen by the immune system of the animal. In contrast to native uricase, the serum sample with Uc-mPEG-prop conjugates displayed a lesser antibody count and the optical density displayed by the conjugates were 3-5 folds lesser than that of native uricase samples.

There was no correlation between the immunogenicity caused and the molecular weight of the PEGylating reagent used for conjugation. The Uc-mPEG-prop conjugates displayed approximately 60 % lesser immunogenicity in comparison to that displayed by its native counterpart.

The optical density displayed by the Uc-mPEG-mal conjugates were 3-5 folds lesser than that of native uricase samples. Uc-mPEG-mal conjugates displayed a 73 % reduction in the antibody titer in comparison to native uricase.
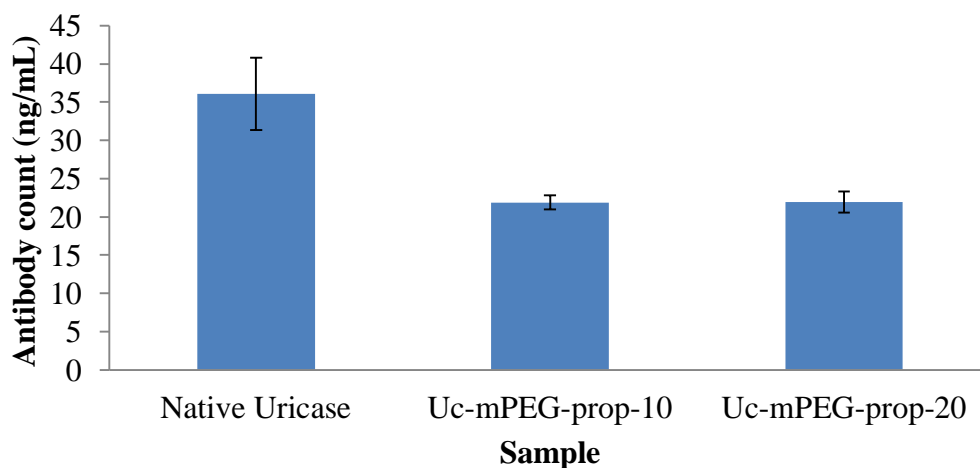
Figure 4.40: Antibody titer of native uricase and Uc-mPEG-prop conjugates

In the present study, the N-terminal PEGylation strategy used for PEGylation targeted N-terminal amino group of lysine residue of uricase wherein PEG wrapped around the protein molecule. The attachment and wrapping around of PEG molecules generally increases the hydrodynamic radius of the conjugate due to hydrophilic property of the PEG molecule, which result in steric hindrance and masking of the protein molecule (Sapan et al. 1999). Thus, the immunogenicity caused by native uricase is reduced due to its entrapment within the PEG strands and thus rendering the developed conjugates more favorable to be used for therapeutic purposes. Also, the larger amount of mPEG strands attached to the uricase molecule increased the risk of immunogenicity caused (Zhang et al. 2012).

According to a report by Ganson et al. (2006) on the antigenicity of PEG-uricase, antibodies were directed against PEG itself rather than the uricase protein. Uc-mPEG-conjugates synthesized in the present study had a very minute amount of mPEG-prop attached; hence the above-stated demerit of randomly PEGylated uricase conjugate can be deciphered. The conjugates can be further verified for their pharmaceutical properties and advanced for clinical trials.

N-terminal PEGylation is one such strategy which can aid in the maintenance of residual uricolytic activity of uricase, as the mPEG-prop chain mainly wrapped around it without causing any steric hindrance. The results indicate that Uc-mPEG-prop conjugates appear to be highly beneficial long-acting uricolytic effects for curing hyperuricemia and gout in comparison to the commercially available random PEGylated uricase 'Krystexxa®'.

Table 4:21 represents a comparative analysis of all the conjugates synthesized in the present study.

Table 4.21: Comparative analysis of the conjugates synthesized in the present study

Sl no	Property	Native uricase	Random Uricase conjugates	Uc-mPEG-mal-	Uc-mPEG-prop-10	Uc-mPEG-prop-20
1	Residual activity (%)	100	10-63%	84	83.46	80.87
2	Molecular weight per subunit (kDa)	35	96	39.2 (750 Da) 46.7 (5 kDa) 52.3 (10 kDa)	60.65	72.34
3	Structural Changes at 20 °C	0%	50%	3 %	2 %	6%
4	Km	4.899×10^{-5} M		4.347×10^{-5} M	4.208×10^{-5} M	3.902×10^{-5} M
5	Size (nm)	8-12		11.01	63.98	72.0
6	Storage stability at 4°C	2 % for 18 days		39 % for 18 days	38 % for 18 days	-
7	Immunogenicity	Highly immunogenic		73 % lesser compared to native uricase	60 % lesser compared to native uricase	60 % lesser compared to native uricase
8	PEG-induced immunogenicity	Nil	May be very high	Lesser due to lesser amount of PEG attached	Lesser due to lesser amount of PEG attached	Lesser due to lesser amount of PEG attached

5.1 Summary:

The present work encompassed the development of site-specific monoPEGylated uricase conjugates for the purposes of delivery of the therapeutic enzyme uricase. The uricase conjugates were developed using the second generation of PEGylation with the implementation of two different synthesis strategies namely Thiol and N-terminal PEGylation. In the Thiol PEGylation reaction, mPEG-maleimide was used as a PEGylating reagent which targeted the free thiols group of the cysteine residues of uricase from *Bacillus fastidious*. The synthesis of monoPEGylated uricase conjugates was confirmed using SDS-PAGE and SE-HPLC. The reaction conditions of the PEGylation reaction for the synthesis of the above conjugates namely molecular weight of the PEGylation reagents, EDTA concentration and the molar ratios of uricase to mPEG-maleimide were optimized using response surface methodology. The optimum values of reaction conditions were determined as 1:12 concentration ratio of Uc to mPEG-mal, 2.76 kDa as mPEG-mal molecular weight and 3.55 mM EDTA concentration which resulted in a very high conjugate yield of 95.16 %. The conjugates synthesized using the optimized method retained a residual uricolytic activity of 84 % and a thiol group modification extent of 68.3 %. The conjugates obtained after optimization were purified and recovered from the PEGylation reaction mixture via purification techniques like centrifugal ultrafiltration, SE-HPLC, and SEC. The monoPEGylated conjugates were extensively characterized for their residual activity, molecular weight, the degree of modification, size/hydrodynamic radius, PEGylation induced alterations in the secondary structure. The conjugates were found to retain 84 % of their residual activity in comparison to native uricase, a thiol modification extent of 68.3 %, a molecular weight 42.5 kDa and a hydrodynamic radius of 11.01 nm. It was also found that slight changes in the secondary structure of uricase molecule were induced due to PEGylation. The storage stability of the conjugate was assessed at various conditions like temperature for a span of 18 days, wherein the conjugates were more stable in comparison to native uricase at all the temperature conditions. The conjugates retained around 21.26 % residual activity on the 12'th day of incubation at physiological conditions. The studies related to the kinetic

properties of PEGylated conjugates indicated that the conjugates possessed more affinity towards the substrate uric acid in comparison to their native counterpart. The conjugates displayed a 73 % reduction in the antibody titer produced in comparison to native uricase. N-terminal PEGylation strategy was also implemented with a scope of improving the residual activity of uricase conjugates. In the N-terminal PEGylation reaction, mPEG-propionaldehyde was used as a PEGylating reagent which targeted the ε-amine group of the N-terminal lysine residue of uricase from *Bacillus fastidious*. The synthesis of PEGylated uricase conjugates was confirmed using SDS-PAGE and SE-HPLC. PEGylation reaction parameters like reaction mixture pH and molar ratio of uricase and mPEG-prop concentration ratio were optimized. The maximum yield of monoPEGylated uricase (76.8 %) was obtained at a pH value of 5.0 as determined by RP-HPLC experiments. The conjugates obtained after optimization were purified and recovered from the PEGylation reaction mixture via purification techniques like centrifugal ultrafiltration, SE-HPLC, and SEC. The monoPEGylated conjugates were extensively characterized further for their residual activity, molecular weight, the degree of modification, size/hydrodynamic radius, PEGylation induced changes in the secondary structure etc. The conjugates were found to retain 76.88 % of their residual activity in comparison to native uricase, a modification extent of 80.7 %, a molecular weight 60.1 kDa and a hydrodynamic radius of 66.0 nm. It was also found that slight alterations in the secondary structure of uricase molecule were induced due to PEGylation. The storage stability of the conjugates was assessed at various conditions of temperature for a span of 18 days, wherein the conjugates were more stable in comparison to native uricase at all the temperature conditions. The conjugates retained around 10.83 % residual activity on the 12'th day of incubation at physiological conditions. The Uc-mPEG-prop conjugates displayed approximately 60 % lesser immunogenicity in comparison to its native counterpart.

5.2 Conclusion:

- This is the first study on optimization of PEGylation reaction conditions for the production of site-specific PEGylated uricase.

- It was found that the reaction parameters and the operating conditions had a direct influence on the yield of PEGylated uricase which helped in maximizing the yield of PEGylated species (which may eventually bring down the production costs). The developed RP–HPLC method could further be applied to bioanalytical method development for large scale production of monoPEGylated uricase.

- The PEGylated uricase conjugates obtained by both thiol and N-terminal PEGylation strategies possessed higher residual activities in comparison to all the residual activities of the PEGylated uricase conjugates reported till date.

- The properties displayed by the monoPEGylated uricase conjugates were much superior to the ones displayed by native uricase in terms of secondary structure maintenance and storage stability.

- In comparison to native uricase, Uc-mPEG-mal and Uc-mPEG-prop conjugates displayed a 73 % and 60 % reduction in the antibody titer respectively. This immunogenicity analysis proves that the conjugates synthesized in the present study appeared to have highly beneficial long-acting uricolytic effects for curing hyperuricemia and gout in comparison to the random PEGylated uricase.

5.3 Future scope:

- Implementation of more efficient purification techniques for conjugate purification.
- Implemented of optimization of the final formulation development for prolonged stability of synthesized conjugates.
- Further *invivo* pharmacokinetic trials to access the efficacy of the PEGylated uricase molecule.
- Exploitation of new sites on the uricase molecule for PEGylation to achieve substantial uricolytic residual activity and prolonged storage stability.

Abuchowski, J.R., McCoy, N.C., Palczuk, T.V. and F.F. Davis. (1977). "Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase." *J. Biol. Chem.*, 252, 3582–3586.

Abzalimov, R. R., Frimpong, A., & Kaltashov, I. A. (2012). "Structural characterization of protein–polymer conjugates. I. Assessing heterogeneity of a small PEGylated protein and mapping conjugation sites using ion exchange chromatography and top-down tandem mass spectrometry." *International Journal of Mass Spectrometry*, *312*, 135-143.

Alcalá, J. M. L., Puerta-Vizcaíno, M. C., González-Vílchez, F., Duesler, E. N. and Tapscott. R. E. (1984). "A redetermination of sodium aqua [ethylenediaminetetraacetato (4-)] ferrate(III) dihydrate, Na[Fe($C_{10}H_{12}N_2O_8$)(H_2O)].2H_2O." *Acta Cry.,* C40, 939-941.

Alexander, N. M. (1958). "Spectrophotometric assay for sulfhydryl groups using N-ethylmaleimide." *Analytical Chemistry*, *30*(7), 1292-1294.

Al-Hanbali, O., Onwuzo, N.M., Rutt, K.J. and Dadswell, C.M. (2007). "Modification of Stewards Biphasic Colorimetric Assay for Stable and Accurate Quantitative Determination of Pluronic and Tetronic Block Copolymers for Application in Biological Systems." *Anal. Biochem.,* 361, 287–293.

Alliegro, M.C. (2000). "Effects of dithiothreitol on protein activity unrelated to thiol-disulfide exchange: for consideration in the analysis of protein function with Cleland's reagent." *Anal Biochem.* 15, 282(1), 102-6.

Alamillo JM, Cárdenas J, Pineda M. (1991). "Purification and molecular properties of urate oxidase from *Chlamydomonas reinhardtii*." *Biochim Biophys Acta.* 1076(2):203-8.

Aly, M., Tork, S., Al-Garni, S., & Allam, R. (2013). "Production and characterization of uricase from Streptomyces exfoliatus UR10 isolated from farm wastes." *Turkish Journal of Biology*, *37*(5), 520-529.

An, Q., Lei, Y., Jia, N., Zhang, X., Bai, Y., Yi, J., Chen, R., Xia, A., Yang, J., Wei, S., Cheng, X., Fan, A., Mu, S. and Xu, Z. (2007). "Effect of site-directed PEGylation of trichosanthin on its biological activity, immunogenicity, and pharmacokinetics." *Biomol Eng.,* 24(6), 643-9.

Arakawa, T., Prestrelski, S.J., Narhi, L.O., Boone, T.C., Kenny, W.C. (1993). "Cysteine 17 of recombinant human granulocyte –colony stimulating factor is partially solvent exposed." *J. Protein Chemistry.*, 12,525-531.

Arora, K., Saxena, S.V., Gupta, R.K., Gupta, S.K., Yakhmi, J.V., Pandey, M.K., Chand, S.,Malhotra, B.D. (2007). "Improved performance of polyaniline-uricase biosensor." *Anal. Chim. Acta.*, 594, 17–23.

Azarkan, M., Nijs, M., Smolders, N., Guermant, J., Vlncentelli, J., & Looze, Y. (1996). "Chromatographic behavior of mPEG-papaya proteinases conjugates examined on ion-exchange and hydrophobic gel media." *Applied biochemistry and biotechnology*, *60*(2), 167-183.

Balan, S., Choi, J.W., Godwin, A., Teo, I., Laborde, C.M., Heidelberger, S., Zloh, M., Shaunak, S. and Brocchini, S. (2007). "Site-specific PEGylation of protein disulfide bonds using a three-carbon bridge." *Bioconjug Chem*. 18(1):61-76.

Banci, L., Bertini, I., Caliceti, P., Monsu, S.L., Schiavon, O. and Veronese F.M. (1990). "Spectroscopic characterization of polyethylene glycol modified superoxide dismutase: ^{1}HNMR studies on its Cu_2Co_2 derivative." *J Inorg Biochem*. 39, 149–59.

Bangham, A. D. and Horne, R. W. (1964). *J. Mol. Biol*. 1964, 8, 660–668.

Banik, S.P., Pal, S., Ghorai, S., Chowdhury, S. and Khowala, S. (2009). "Interference of sugars in the Coomassie Blue G dye binding assay of proteins." *Anal Biochem.*, 386(1), 113-5.

Barana, E. T., Ozer, N. and Hasircia, V. (2003). "Solid phase enzyme modification via affinity chromatography." *J. Chromatograph B*, 794, 311-322.

Baraf, H. S., Yood, R. A., Ottery, F. D., Sundy, J. S., & Becker, M. A. (2014). "Infusion-related reactions with pegloticase, a recombinant uricase for the treatment of chronic gout refractory to conventional therapy." *JCR: Journal of Clinical Rheumatology*, 20(8), 427-432.

Barbosa, H, Slater, N.K.H. and Marcos, J.C. (2009). "Protein quantification in the presence of poly(ethylene glycol) and dextran using the Bradford method." *Anal. Biochem.* 395, 108–110.

Barz, M., Luxenhofer, R.,Zentel, R.,Vicent, M. J. (2011**). "**Overcoming the PEG Addiction: well defined alternatives to PEG, from structure-property relationships to better defined therapeutics." *Polym. Chem.*, 2*,* 1900-1918.

Beauchamp, C.O., Gonias, S.L., Menapace, D.P., Pizzo, S.V. (1983). "A new procedure for the synthesis of polyethylene glycol-protein adducts, elects on function, receptor recognition and clearance of superoxide dismutase, lactoferrin and a2-macroglubulin." *Anal Biochem.,*131, 25-33.

Bell, S.J., Fam, C.M., Chlipala, E.A., Carlson, S.J., Lee, J.I., Rosendahl, M.S., Doherty, D.H., and Cox, G.N. (2008). "Enhanced circulating half-life and antitumor activity of a site-specific PEGylated interferon-alpha protein therapeutic." *Bioconjug. Chem*. 19, 299–305.

Bendele, A., Seely, J., Richey, C., Sennello, G., & Shopp, G. (1998). "Short communication: renal tubular vacuolation in animals treated with polyethylene-glycol-conjugated proteins. *Toxicological sciences*," 42(2), 152-157.

Bentley, M.D. , Harris, J.M. and Kozlowski, A. (1999). "Heterobifunctional poly(ethylene glycol) derivatives and methods for their preparation." P.C.T. US99/23536.

Bessmertny, O., Robitaille, L.M. and Cairo, M.S. (2005). "Rasburicase: a new approach for preventing and/or treating tumor lysis syndrome." *Curr Pharm Des*., 11(32), 4177–85.

Bian, X., Shen, F., Chen, Y., Wang, B., Deng, M., & Meng, Y. (2010). "PEGylation of alpha-momorcharin: synthesis and characterization of novel anti-tumor conjugates with therapeutic potential." *Biotechnology letters*, *32*(7), 883-890.

Bomalaski, J.S., Holtsberg, F.W., Ensor, C.M. and Clarck, M.A. (2002). "Uricase formulated with polyethylene glycol (Uricase-PEG 20): biochemical rationale and preclinical studies." *J. Rheumatol.*, 29, 1942–1948.

Blick, S. K., & Curran, M. P. (2007). "Certolizumab pegol." *BioDrugs*, *21*(3), 195-201.

Braxton, M., Scott Michael; Murry, Lynn E. (*1998)*. "*Cysteine*-PEGylated proteins." U.S. Patent No. 5,766,897.

Brière, C., Richette, P. and Clavert,V. H. (2006). "Efficacité et tolérance de la rasburicase (Fasturtec) chez des patients goutteux intolérants ou réfractaires à l'allopurinol." *Rev Rhum.,*73,1033.

Caliceti, F.M. Veronese and Jonak, Z. (1999). "Imunogenic and tolerogenic properties of monomethoxypoly (ethylene glycol) conjugate proteins." Farmaco., 54, 430–437.

Caliceti, P., Schiavon, O., and Veronese, F.M. (2001). "Immunological properties of uricase conjugated to neutral soluble polymers." *Bioconjug. Chem.*, 12, 515–522.

Caliceti, P., & Veronese, F. M. (2003). "Pharmacokinetic and biodistribution properties of poly (ethylene glycol)–protein conjugates." *Advanced drug delivery reviews*, *55*(10), 1261-1277.

Cannella, A. C., & Mikuls, T. R. (2005). "Understanding treatments for gout." *Am J Manag Care*, *11*(15 Suppl), S451-8.

Cammalleri, L. and Malaguarnera, M. (2007). "Rasburicase represents a new tool for hyperuricemia in tumor lysis syndrome and in gout." *Int. J. Med. Sci.*, 4(2), 83-93.

Carlsson, N A. Borde, S. Wolfel, B. Kerman and A. Larsson. (2011). "Quantification of protein concentration by the Bradford method in the presence of pharmaceutical polymers." *Anal. Biochem.*, 411(1), 116–121.

Carpenter, C. P., Woodside, M. D., Kinkead, E. R., King, J. M. and Sullivan, L. J. (1971). "Response of dogs to repeated intravenous injection of polyethylene glycol 4000 with notes on excretion and sensitization." *Toxicol. Appl. Pharmacol.*, 18, 35.

Caserman. S., Kusterle, M., Kunstelj, M., Milunović, T., Schiefermeier, M., Jevsevar, S. and Porekar, V.G. (2009). "Correlations between in vitro potency of polyethylene glycol-protein conjugates and their chromatographic behavior." *Anal Biochem.*, 389(1), 27-31.

Caspi, D., Lubart, E., Graff, E., Habot, B., Yaron, M., & Segal, R. (2000). "The effect of mini-dose aspirin on renal function and uric acid handling in elderly patients." *Arthritis & Rheumatism*, *43*(1), 103-108.

Caves, M. S., Derham, B. K., Jezek, J., & Freedman, R. B. (2013). "Thermal inactivation of uricase (urate oxidase): mechanism and effects of additives." *Biochemistry*, *52*(3), 497-507.

Chae, S. Y., Kim, T. H., Park, K., Jin, C. H., Son, S., Lee, S., Chen, X. (2010). "Improved Antitumor Activity and Tumor Targeting of NH2-Terminal–Specific PEGylated Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand." *Molecular cancer therapeutics*. *9*(6),1719-1729.

Chen, R.H.L., Abuchowski, A., Van Es, T., Palczuk, N.C. and Davis, F.F. (1981). "Two urate oxidase modified by the covalent attachment of poly(ethylene glycol)." *Biochim.Biophys. Acta.*, 660, 293–298.

Chen, L. X., & Schumacher, H. R. (2008). "Gout: an evidence-based review.*JCR: Journal of Clinical Rheumatology*" *14*(5S), S55-S62.

Cheng, T.L., Chen, B.M., Chern, J.W., Wu, M.F. and Roffler, S.R. (2000). "Efficient clearance of poly(ethylene glycol)- modified immunoenzyme with anti-PEG monoclonal antibody for prodrug cancer therapy." *Bioconjug Chem.*, 11(2), 258–66.

Chial, H. J. and Splittgerber, A. G. (1993) *Anal.Biochem.,* 213**,** 362–369.

Childs, C.E. (1975). "The determination of polyethylene glycol in gamma globulin solutions." *Microchem. J.*, 20, 190-192.

Chohan, S. and Becker, M.A. (2009). "Update on emerging urate-lowering therapies." *Curr Opin Rheumatol.,* 21(2), 143–149.

Chua, C. C., Greenberg, M. L., Viau, A. T., Nucci, M., Brenckman Jr, W. D. and Hershfield, M. S. (1988). "Use of polyethylene glycol-modified uricase (PEG-uricase) to treat hyperuricemia in a patient with non-Hodgkin lymphoma." *Ann. Intern. Med*., 109, 114–117.

Chun, Z., Xiao, L.Y, Juan, F., Yonghua, Y., Xiang, L., Youquan, B., Yanling, X., Huidong, Y. and Fei, L. (2010). "Effects of modification of amino groups with Polyethylene Glycol on a recombinant Uricase from *Bacillus fastidiosus." Biosci.Biotechnol.Biochem*., 74(6), 1298-1301.

Clark, R., Olson, K., Fuh, G., Marian, M., Mortensen, D., Teshima, G., Chang, S., Chu, H., Mukku, V., Canova-Davis, E., Somers, T., Cronin, M., Winkler, M. and Wells, J.A. (1996). "Long-acting growth hormones produced by conjugation with poly(ethylene glycol)." *Journal of Biological Chemistry*, 271(21), 969-77.

Cleland, W. W. (1964). "Dithiothreitol, a new protective reagent for SH groups*." *Biochemistry*, *3*(4), 480-482.

Cohan, R.A., Madadkar, A.S., Khanahmad, H., Roohvand, F., Aghasadeghi, M.R., Hedayati, M.H., Barghi, Z., Ardestani, M.S., Inanlou, D.N. and Norouzian, D. (2011). "Design, modeling, expression, and chemoselective PEGylation of a new nanosize cysteine analog of erythropoietin." *Int J Nanomedicine.,* 6, 1217-1227.

Collen, D., Sinnaeve, P., Demarsin, Moreau, De Maeyer, M., Jespers, L., Laroche, Y. and Van de Werf, F. (2000). "Polyethylene glycol-derivatized cysteine-substitution variants of

recombinant staphylokinase for single-bolus treatment of acute myocardial infarction." *Circulation*. 102(15), 1766-1772.

Colloc'h, N., El Hajji, M., Bachet, B., L'Hermite, G., Schlitz, M., Prangé, T., Mornon, J. P. (1997). "Crystal structure of the protein drug urate oxidase-inhibitor complex at 2.05 A resolution." *Nature structural biology*, *4*, 947-952.

Colonna, C., Conti, B., Perugini, P., Pavanetto, F., Modena, T., Dorati, R., Ladarola, P., Genta, I., (2008). "Site-directed PEGylation as successful approach to improve the enzyme replacement in the case of prolidase." *Int. J. Pharm.*, 358, 230–237.

Compton, S.J. and Jones, C.G. (1985). "Mechanism of dye response and interface in the Bradford protein assay." *Anal. Biochem.*, 51, 369–374.

Cong Y, Pawlisz E, Bryant P, Balan S, Laurine E, Tommasi R, Singh R, Dubey S, Peciak K, Bird M, Sivasankar A, Swierkosz J, Muroni M, Heidelberger S, Farys M, Khayrzad F, Edwards J, Badescu G, Hodgson I, Heise C, Somavarapu S, Liddell J, Powell K, Zloh M, Choi JW, Godwin A, Brocchini S. (2012). "Site-specific PEGylation at histidine tags." *Bioconjug Chem*, 23(2):248-63.

Coppola, D., Bruno, S., Ronda, L., Viappiani, C., Abbruzzetti, S., di Prisco, G., Verde, C. and Mozzarelli, A. (2011). "Low affinity PEGylated hemoglobin from Trematomusbernacchii, a model for hemoglobin-based blood substitutes." *BMC Biochem.*, 12, 66.

Davidson, M.B., Thakkar, S., Hix, J.K., Bhandarkar, N.D., Wong, A. and Schreiber, M.J. (2004). "Pathophysiology, clinical consequences, and treatment of tumor lysis syndrome." *Am J Med*., 116(8), 546–54.

Davis, S., Park, Y.K., Abuchowski, A. and Davis, F.F. (1981). "Hypouricaemic effect of polyethylene glycol modified urate oxidase." *Lancet.,* 8241, 281–283.

Delgado, C., Francis, G.E., Derek FF (1992). "The uses and properties of PEG-linked proteins." *Critical Rev Ther Drug Carrier Syst*, 9, 249, 304.

Deiters, A., Cropp, T. A., Summerer, D., Mukherji, M., & Schultz, P. G. (2004). "Site-specific PEGylation of proteins containing unnatural amino acids." *Bioorganic & medicinal chemistry letters*, *14*(23), 5743-5745.

Deng, X., He, D., Xiong, H. R., Zhou, Y. L., & Zhang, J. Q. (2015). "The Pharmacokinetics and Pharmacodynamics of Intravenous Uricase Multivesicular Liposomes". *Sichuan da xue xue bao. Yi xue ban Journal of Sichuan University. Medical science edition*, *46*(5), 688-691.

Dhal, P.K., Polomoscanik, S.C., Avila LZ, Holmes-Farley SR, Miller RJ. (2009). "Functional polymers as therapeutic agents: concept to market place." *Adv Drug Deliv Rev.,* 12, 61(13), 1121-30.

Doherty, D. H., Rosendahl, M. S., Smith, D. J., Hughes, J. M., Chlipala, E. A., & Cox, G. N. (2005). "Site-specific PEGylation of engineered cysteine analogues of recombinant human granulocyte-macrophage colony-stimulating factor." *Bioconjugate chemistry*, *16*(5), 1291-1298.

Doherty, M. (2009). "New insights into the epidemiology of gout." *Rheumatol*, 48, 2–8.

Dolence, E.K., Hu, C., Tsang, R., Sanders, C.G. and Osaki, S. (1997). "Electrophilic polyethylene oxides for the modification of polysaccharides, polypeptides (proteins) and surfaces." US Patent 5.650.234.

Duncan, R. (2003). "The dawning era of polymer therapeutics." *Nature Reviews Drug Discovery*, 2, 347-360.

Edwards, N.L. (2008). "Treatment-failure gout: a moving target." *Arthritis Rheum.*, 58, 2587–2590.

Ellman, G.L. (1959). "Tissue sulfhydryl groups." *Arch Biochem Biophys*. 82, 70-77.

Fan, K., Zhang, C., Ma, X., Mei, X., & Hu, C. (2013). *U.S. Patent No. 8,586,535*. Washington, DC: U.S. Patent and Trademark Office.

Fee, C. J.; Damodaran, V. B. (2012). "Production of PEGylated Proteins, in Biopharmaceutical Production Technology" G. Subramanian, Ed. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany.

Fee, C.J. J.M. Van Alstine (2004). "Prediction of the viscosity radius and the size exclusion chromatography behavior of PEGylated proteins." *Bioconjugate Chem*. 15, 1304–1313.

Fee, C.J. and VanAlstine, J.M. (2006). "PEG-proteins: Reaction engineering and separation issues." *Chem. Engg. Sc.* 61(3), 924-939.

Feig, D.I. (2009). "Uric acid: a novel mediator and marker of risk in chronic kidney disease?" *Curr Opin Nephrol Hypertens*., 18(6), 526–530.

Fels, E. and Sundy, J.S. (2008). "Refractory gout: what is it and what to do about it?" *Curr Opin Rheumatol*., 20(2), 198–202.

Feng, J., Wang, L., Liu, H., Yang, X., Liu, L., Xie, Y., Liu, M., Zhao, Y., Li, X., Wang, D., Zhan, C.G. and Liao, F (2015). "Crystal structure of *Bacillus fastidious* uricase reveals an unexpected folding of the C-terminus residues crucial for thermostability under physiological conditions." *Appl Microbiol Biotechnol*, 99(19):7973-86.

Feng, J., Li, X., Yang, X., Zhang, C., Yuan, Y., Pu, J., Liao, F. (2010). "A new practical system for evaluating the pharmacological properties of uricase as a potential drug for hyperuricemia." *Archives of pharmacal research*, *33*(11), 1761-1769.

Fernandes, A.I. and Gregoriadis, G (2001). "The effect of polysialylation on the immunogenicity and antigenicity of asparaginase: implication in its pharmacokinetics." *Int J Pharm.,* 17; 217(1-2):215-24.

Filpula, D., and Zhao, H. (2008). "Releasable PEGylation of proteins with customized linkers." *Advanced Drug Delivery Reviews*, *60*(1), 29-49.

Fishbane, S., Schiller, B., Locatelli, F., Covic, A. C., Provenzano, R., Wiecek, A., Mayo, M. R. (2013). "Peginesatide in patients with anemia undergoing hemodialysis." *New England Journal of Medicine*, *368*(4), 307-319

Fodje, M.N. and Al-Karadaghi, S. (2002). "Occurrence, conformational features and amino acid propensities for the pi-helix." *Protein Eng.,* 15(5); 353-358.

Fontana, A., Spolaore, B., Mero, A., & Veronese, F. M. (2008). "Site-specific modification and PEGylation of pharmaceutical proteins mediated by transglutaminase." *Advanced drug delivery reviews*, *60*(1), 13-28.

Foster, G.R. (2010). "PEGylated interferons for the treatment of chronic hepatitis C: pharmacological and clinical differences between peginterferon-alpha-2a and peginterferon-alpha-2b." *Drugs*, 70(2), 147–165.

Foster, L.J., de Hoog, C.L. and Mann, M. (2003). "Unbiased quantitative proteomics of lipid rafts reveals high specificity for signaling factors." *Proc. Natl. Acad. Sci. USA*. 100, 5813–5818.

Freitas, D.S., Spencer, P.J., Vassao, R.C. and Abrahao, N. J. (2010). "Biochemical and biopharmaceutical properties of PEGylated uricase." *Int J Pharm.,* 387(1-2), 215-222.

Fridovich I. (1965). "The Competitive Inhibition of Uricase by Oxonate and by Related Derivatives of Striazines." *J Biol Chem*., 240, 2491–2494.

Friedman, T.B., Polanco, G.E., Appold, J.C. and Mayle, J.E. (1985). "On the loss of uricolytic activity during primate evolution-I. Silencing of urate oxidase in a hominoid ancestor." *Comp. Biochem. Phys. B*., 81, 653-659.

Fujita, H., Okamoto, K., & Takao, A. (1991). "Pharmacokinetics of adriamycin-oxidized dextran conjugate (ADM-OXD) in experimental animals." *Drug Delivery System*, *6*, 133-138.

Ganson, N.J., Kelly, S.J., Scarlett, E., Sundy, J.S. and Hershfield, M.S. (2006). "Control of hyperuricemia in subjects with refractory gout, and induction of antibody against poly(ethylene glycol) (PEG), in a phase I trial of subcutaneous PEGylated urate oxidase." *Arthritis Res Ther.,*8(1), 12.

Gao, M., Tian, H., Ma, C., Gao, X., Guo, W., & Yao, W. (2010)." Expression, purification, and C-terminal site-specific PEGylation of cysteine-mutated glucagon-like peptide-1." *Applied biochemistry and biotechnology*, *162*(1), 155-165.

Garay, R.P. and Labaune, J.P. (2011). "Immunogenicity of Polyethylene Glycol (PEG)." *The Open Conference Proceedings Journal*, 2, 104–107.'

Gaspar, H.B., Aiuti, A., Porta, F., Candotti, F., Hershfield, M.S. and Notarangelo, L.D. (2009). "How I treat ADA deficiency." *Blood*., 114(17), 3524–32.

Garratty, G. (2008). "Modulating the red cell membrane to produce universal/stealth donor red cells suitable for transfusion." *Vox sanguinis*,*94*(2), 87-95.

Ghosh, S. S., Gepstein, J.J. Heikkila and E.B. Dumbroff. (1988). "Use of a scanning densitometer or an ELISA plate reader for measurement of nanogram amounts of protein in crude extracts from biological tissues." *Anal Biochem.,* 169(2):227-33.

Gong, N., Ma, A.N. and Zhang, L.J. (2011). "Site-specific PEGylation of exenatide analogues markedly improved their glucoregulatory activity." *Br J Pharmacol*. 163(2): 399–412.

Grace, M.J., Lee, S., Bradshaw, S., Chapman, J., Spond, J., Cox, S., DeLorenzo, Marc., Wylie, D., Carlson, S.C., Cullen, C., Indelicato, S., Voloch, M. and Bordens, R.(2001). "Site of PEGylation and Polyethylene Glycol Molecule Size Attenuate Interferon-α Antiviral and

Antiproliferative Activities through the JAK/STAT Signaling Pathway." *The Journal of Biological Chemistry,* 280, 6327-6336.

Graham, M. L. (2003). "Pegaspargase: a review of clinical studies." *Advanced drug delivery reviews*, *55*(10), 1293-1302.

Habeeb, A.F.S.A. (1966). "Determination of free amino groups in protein by trinitrobenzene sulfonic acid." *Anal. Biochem.* 14, 328-336.

He, X. H., Shaw, P. C., & Tam, S. C. (1999). "Reducing the immunogenicity and improving the in vivo activity of trichosanthin by site-directed pegylation.' *Life sciences*, *65*(4), 355-368.

Hemmerich, S., Verdugo, D., and Rath, V.L. (2004). "Strategy for drug discovery by targeting sulfation pathways." *Drug Discov. Today.,* 9, 967–975.

Hermanson, G. T. (1996). "Antibody modification and conjugation." *Bioconjugate techniques*, 456-493.

Hershfield, M. S., Ganson, N. J., Kelly, S. J., Scarlett, E. L., Jaggers, D. A., & Sundy, J. S. (2014). "Induced and pre-existing anti-polyethylene glycol antibody in a trial of every 3-week dosing of pegloticase for refractory gout, including in organ transplant recipients." *Arthritis research & therapy*, *16*(2), 1

Hill, C. L., Lu, T. Y., Cervelli, M., & Mathew, T. (2008). "Failure of rasuricase therapy in recurrent acute gout with tophi." *International Journal of Rheumatic Diseases*, *11*(3), 315-317.

Holm, L.S., Thulstrup, P.W., Kasimova, M.R. and van de Weert M. (2015). "Preferential Interactions and the Effect of Protein PEGylation." PLoS One, 10(7).

Huang, Z., Zhu, C., Sun, C., Zhang, J., Zhang, Y., Zhang, Y., Ye, C., Wang, X., Ilghari, D. and Xiaokun Li. (2012). "A Novel Solid-Phase Site-Specific PEGylation Enhances the *In Vitro* and *In Vivo* Biostabilty of Recombinant Human Keratinocyte Growth Factor 1." PLoS ONE, 7(5).

Huang, L., Gough, P. C., & DeFelippis, M. R. (2009). "Characterization of poly (ethylene glycol) and PEGylated products by LC/MS with postcolumn addition of amines." *Analytical chemistry*, *81*(2), 567-577.

Humphreys, D.P., Heywood, S.P., Henry, A., Ait-Lhadj, L., Antoniw, P., Palframan, R., Greenslade, K.J., Carrington, B., Reeks, D.G., Bowering, L.C., West, S. and H.A. Brand. (2007). "Alternative antibody Fab' fragment PEGylation strategies: combination of strong reducing agents, disruption of the interchain disulphide bond and disulphide engineering." *Protein Eng Des Sel.*, 20(5), 227-34.

Hundekar, Y. R., Nanjwade, B. K., Mohamied, A. S., Idris, N. F., Srichana, T., & Shafioul, A. S. M. (2015). "Nanomedicine to Tumor by Enzymosomes." *J Nanotechnol Nanomed Nanobiotechnol*, *2* (004).

Hunter, T. (1995). "Protein kinases and phosphatases; the yin and yang of protein phosphorylation and signalling." *Cell.,* 80, 225–236.

Iafelice, R., Cristoni, S., Caccia, D., Russo, R., Rossi-Bernardi, L., Lowe, K.C. and Perrella, M. (2007). "Identification of the sites of deoxyhaemoglobin PEGylation." *Biochem J.,* 403(1):189-96.

Harris, J. M. (1992). "Poly(ethylene glycol) Chemistry, Introduction to Biotechnical and Biomedical Applications of Poly(Ethylene Glycol)". Plenum Press, New York.

Harris, J. M., Martin, N. E., & Modi, M. (2001). "Pegylation." *Clinical pharmacokinetics*, *40*(7), 539-551.

Jevsevar, S., Kunstelj, M. and Porekar, V.G. (2010). "PEGylation of therapeutic proteins." *Biotechnology Journal.*, 5; 113–128.

Kale, A. A. and Torchilin, V.P. (2007). "Design, synthesis, and characterization of pH sensitive PEG-PE conjugates for stimuli-sensitive pharmaceutical nanocarriers: the effect of substitutes at the hydrazone linkage on the pH stability of PEG-PE conjugates." *Bioconjug Chem.*, 18(2):363-70.

Kalia, J., & Raines, R. T. (2007). Catalysis of imido group hydrolysis in a maleimide conjugate. *Bioorganic & medicinal chemistry letters*, *17*(22), 6286-6289.

Khan, M. N. (1984). "Kinetics and mechanism of the alkaline hydrolysis of maleimide." *Journal of pharmaceutical sciences*, *73*(12), 1767-1771.

Kaji, E.H. and Lodish, H.F. (1993). "Unfolding of newly made retinol-binding protein by dithiothreitol, sensitivity to retinoids." *J. Biol. Chem.*, 268 (29) 22188–22194.

Karas, M., Gluckmann, M., and Schafer, J. (2000). "Ionization in matrix-assisted laser desorption/ionization: Singly charged molecular ions are the lucky survivors." *J. Mass Spectrom.* 35, 1–12.

Kelly, S. J., Delnomdedieu, M., Oliverio, M. I., Williams, L. D., Saifer, M. G., Sherman, M. R., Hershfield, M. S. (2001). "Diabetes insipidus in uricase-deficient mice: a model for evaluating therapy with poly (ethylene glycol)-modified uricase." *Journal of the American Society of Nephrology*, *12*(5), 1001-1009.

Kerwin, B. A., Chang, B. S., Gegg, C. V., Gonnelli, M., Li, T., & Strambini, G. B. (2002). "Interactions between PEG and type I soluble tumor necrosis factor receptor: modulation by pH and by PEGylation at the N terminus.*Protein science*". *11*(7), 1825-1833

Kim, Y., Sam O. Ho, Natalie R. Gassman, You Korlann, Elizabeth V. Landorf, Frank R. Collart, Shimon Weiss. (2008). "Efficient Site-Specific Labeling of Proteins via Cysteines." *Bioconjug Chem.*, 19(3): 786–791.

Kim, S. W., Ramasamy, K., Bouamar, H., Lin, A. P., Jiang, D., & Aguiar, R. C. (2012). "MicroRNAs miR-125a and miR-125b constitutively activate the NF-κB pathway by targeting the tumor necrosis factor alpha-induced protein 3 (TNFAIP3, A20)." *Proceedings of the National Academy of Sciences*,*109*(20), 7865-7870.

Kinstler, O. B. (1998). "N-terminally chemically modified protein compositions and methods." U.S. Patent Serial No. 5824784.

Knop, K., Hoogenboom, R., Fischer, D. and Schubert, U.S. (2010). "Poly(ethylene glycol) in Drug Delivery: Pros and Cons as Well as Potential Alternatives." *Angew. Chem. Int. Ed.*, 49, 6288 – 6308.

Kochendoerfer, G.G. (2005). "Site specific polymer modification of therapeutic proteins." *Curr Opin Chem Biol*, 9, 555-560.

Kodera, Y., Matsushima, A., Hiroto, M., Nishimura, H., Ishii, A., Ueno, T., & Inada, Y. (1998). "PEGylation of proteins and bioactive substances for medical and technical applications." *Progress in Polymer Science*, *23*(7), 1233-1271.

Kontermann, R. (2012). "Therapeutic Proteins: Strategies to Modulate Their Plasma Half-lives." *John Wiley & Sons*., Science, 354.

Korzhavin, D. V., Chernovskaya, T. V., Efanov, Y. G., Rudenko, E. G., Ivanov, R. A., Pshenichnikova, A. B., & Shvets, V. I. (2015). "Preparation of monoPEGylated human interferon beta-1a: Optimization of the conditions for N-terminal PEGylation." *Applied Biochemistry and Microbiology*, *51*(7), 774-785

Kouchakzadeh, H., Shojaosadati, S.A., Maghsoudi, A. and Farahani, V. E. (2010). "Optimization of PEGylation conditions for BSA nanoparticles using response surface methodology." *AAPS PharmSciTech.*, 11(3), 1206-11.

Kozlowski, A., & Harris, J. M. (2001). "Improvements in protein PEGylation: pegylated interferons for treatment of hepatitis C." *Journal of Controlled Release*, *72*(1), 217-224.

Kratzer, J. T. (2013). "Reengineering a human-like uricase for the treatment of gout."

Kurfurst, M.M. (1992). "Detection and molecular weight determination of polyethyleneglycol-modified hirudin by staining after sodium dodecyl sulfate polyacrylamide gel electrophoresis." *Anal Biochem.,* 200, 244–8.

Kusterle, M., Jevsevar, S., Porekar, V. G. (2008). "Size of PEGylated Protein Conjugates Studied by Various Methods." *Acta Chim Sloven.*, 55, 594-601.

Kwon, B., Molek, J. and Zydney, A.L. (2008). "Ultrafiltration of PEGylated proteins: Fouling and concentration polarization effects." *Journal of Membrane Science*, 319 (1–2), 206–213.

Laemmli, U.K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." *Nature.,* 227, 680–685.

Lee, Y. H., Perry, B. A., Labruno, S., Lee, H. S., Stern, W., Falzone, L. M., & Sinko, P. J. (1999). Impact of regional intestinal pH modulation on absorption of peptide drugs: oral absorption studies of salmon calcitonin in beagle dogs. *Pharmaceutical research*, *16*(8), 1233-1239.

Lee, H. and Park, T.G. (2002). "Preparation and characterization of mono-PEGylated epidermal growth factor: evaluation of in vitro biologic activity." *Pharm Res*., 19(6):845-51.

Lee, B. K., Kwon, J. S., Kim, H. J., Yamamoto, S. and Lee, E. K. (2007). "Solid-phase PEGylation of recombinant interferon alpha-2a for site-specific modification: Process performance, characterization, and in-vitro bioactivity." *Bioconjugate Chemistry,* 18, 1728-1734.

Levy, Y., Hershfield, M. S., Fernandez-Mejia, C., Polmar, S. H., Scudiery, D., Berger, M., & Sorensen, R. U. (1988). "Adenosine deaminase deficiency with late onset of recurrent infections: response to treatment with polyethylene glycol-modified adenosine deaminase." *The Journal of pediatrics*, *113*(2), 312-317.

Lewis, A., Tang, Y., Brocchini, S., Choi, J. W., & Godwin, A. (2008). "Poly (2 methacryloyloxyethyl phosphorylcholine) for protein conjugation." *Bioconjugate chemistry*, *19*(11), 2144-2155.

Li, S., Yang, Z., Sun, X., Tan, Y., Yagi, S. and Hoffman, R.M. (2003). "A simultaneous colorimetric assay of free and protein-coupled polyethylene glycol." *Anal. Biochem*. 313(2), 335-337.

Li, N., Ziegemeier, D., Bass, L. and Wang W. (2008). "Quantitation of free polyethylene glycol in PEGylated protein conjugate by size exclusion HPLC with refractive index (RI) detection." *J Pharm Biomed Anal.,* 48(5), 1332-1338.

Li, Y. P., Zhang, Y.H., Jiang, L. Y., Rong, L.F., Du, X.Y and Song, L.H. (2012). "Effects of PEGylation on the Lowry method for the content determination of trichosanthin." *African Journal of Pharmacy and Pharmacology*., 6(38), 2724-2728.

Li, L., Shang, B., Hu, L., Shao, R., & Zhen, Y. (2015). "Site-specific PEGylation of lidamycin and its antitumor activity." *Acta Pharmaceutica Sinica B*, *5*(3), 264-269.

Liao, F., Zhu, X.Y., Wang, Y.M. and Zuo, Y.P. (2005). "The comparison of the estimation of enzyme kinetic parameters by fitting reaction curve to the integrated Michaelis-Menten rate equations of different predictor variables." *J Biochem Biophys Methods*., 62(1), 13–24.

Lin, Z., Pan, X., Wu, F., Ye, D., Zhang, Y., Wang, Y., Triggle, C. (2015). "Fibroblast growth factor 21 prevents atherosclerosis by suppression of hepatic sterol regulatory element-binding protein-2 and induction of adiponectin in mice." *Circulation*, CIRCULATIONAHA-115.

Lin, W., Zhang, H., Wu, J., Wang, Z., Sun, H., Yuan, J., & Chen, S. (2013). "A novel zwitterionic copolymer with a short poly (methyl acrylic acid) block for improving both conjugation and separation efficiency of a protein without losing its bioactivity." *Journal of Materials Chemistry B*, *1*(19), 2482-2488.

Liu, J., Hamza, A. and Zhan, C.G. (2009). "Fundamental reaction mechanism and free energy profile for (-)-cocaine hydrolysis catalyzed by cocaine esterase." *J Am Chem Soc.*, 131(33), 11964–75.

Liu, R., Li, D., Wang, J., Qiu, R., Lin, Q., Zhang, G., Hu, T. (2012). "Preparation, characterization and in vitro bioactivity of N-terminally PEGylated staphylokinase dimers." *Process Biochemistry*, *47*(1), 41-46.

Lobo, D. N. (2004). "Fluid, electrolytes and nutrition: physiological and clinical aspects." *Proceedings of the Nutrition Society*, *63*(03), 453-466.

London, M. and Hudson, P.B. (1957). "Uricolytic activity of purified uricase in two human beings." *Science.,* 125, 937–938.

Long, D.L., Doherty, D.H., Eisenberg, S.P., Smith, D.J., Rosendahl, M.S., Christensen, K.R., Edwards, D.P., Chlipala, E.A. and Cox, G.N. (2006). "Design of homogeneous, mono-pegylated erythropoietin analogs with preserved *invitro* bioactivity." *Exp. Hematol.*, 34(6), 697-704.

Lonsdale, R., Harvey, J. N., & Mulholland, A. J. (2010). "Inclusion of dispersion effects significantly improves accuracy of calculated reaction barriers for cytochrome P450 catalyzed reactions." *The Journal of Physical Chemistry Letters*, *1*(21), 3232-3237.

Lotfy, W.A. (2008). "Production of a thermostable uricase by a novel *Bacillus thermocatenulatus* strain." *Bioresource. Technol* ., 99, 699-702.

Lu, Y. A. and Felix, A. M. (1994). "Solid-phase synthesis with PEGylating reagents of varying molecular weight: synthesis of multiply PEGylated peptides." *Reactive Polymers,* 22(3), 221-9.

Lu, J., & Deutsch, C. (2001). "Pegylation: a method for assessing topological accessibilities in Kv1." 3. *Biochemistry*, *40*(44), 13288-13301.

Lu, M., Li, X.-F., Le, X. C., Weinfeld, M. and Wang, H. (2010). "Identification and characterization of cysteinyl exposure in proteins by selective mercury labeling and nano-

electrospray ionization quadrupole time-of-flight mass spectrometry." *Rapid Commun. Mass Spectrom.,* 24: 1523–1532.

Lundblad, R.L. and N.C. Price (2004). "Protein concentration determination." *Bioprocess Int*. 2, 38–46.

Luxenhofer, R., Han, Y., Schulz, A., Tong, J., He, Z., Kabanov, A.V. and Jordan, R. (2012). "Poly(oxazoline)s as polymer therapeutics." *Macromol Rapid Commun*. 15, 33(19), 1613-31.

Luxenhofer, R., Fetsch, C., Grossmann, A. (2013). "Polypeptoids: A Perfect Match for Molecular Definition and Macromolecular Engineering?" *J. Polym. Sci.: Part A: Polym. Chem.,* 51, 2731-2752.

M.R. (2010). "PEG-urate oxidase conjugates and use thereof." US 6576235 B1

Ma, C., Gao, M., Liu, W., Zhu, J., Tian, H., Gao, X. and Yao, W. (2010). "Intein-mediated expression and purification of an analog of glucagon-like peptide-1 in *Escherichia coli*." *Protein Pept. Lett*., 17(10), 1245-50.

Macdougall, I. C., Robson, R., Opatrna, S., Liogier, X., Pannier, A., Jordan, P., Reigner, B. (2006). "Pharmacokinetics and pharmacodynamics of intravenous and subcutaneous continuous erythropoietin receptor activator (CERA) in patients with chronic kidney disease." *Clinical journal of the American Society of Nephrology*, *1*(6), 1211-1215.

Magda, A.L.Y., Sanaa, T.O.R.K., Saleh, AL-GARNI and Reda, ALLAM. (2013). "Production and characterization of uricase from *Streptomyces exfoliatus* UR10 isolated from farm wastes." *Turk J Biol., 37*, 520- 529.

Magnani, M., Mancini, U., Bianchi, M., & Fazi, A. (1992). "Comparison of uricase-bound and uricase-loaded erythrocytes as bioreactors for uric acid degradation." In *The Use of Resealed Erythrocytes as Carriers and Bioreactors* (pp. 189-194). Springer US.

Mahdi Al, S.A., Atraqchi, and Mosawi, E.A.A. (2012). "Purification and characterization of uricase enzyme produced from *Pseudomonas aeruginosa.*" *Kerbala Journal of Pharmaceutical Sciences*, 3, 64-76.

Mahler, H., Hubscher, G., and Baum, H. (1955). "Studies on Uricase. I. Preparation, Purification and Properties of a Cuproprotein." *J. Biol. Chem.*, 216, 625.

Malzert, A., Boury, F., Renard, D., Robert, P., Lavenant, L., Benoıt, J. P., & Proust, J. E. (2003). "Spectroscopic studies on poly (ethylene glycol)–lysozyme interactions." *International journal of pharmaceutics*, *260*(2), 175-186.

Maiser, B., Dismer, F. and Hubbuch, J. (2014). "Optimization of random PEGylation reactions by means of high throughput screening." *Biotechnol. Bioeng.*, 111: 104–114.

Manjula, B.N., Tsai, A.G., Intaglietta, M., Tsai, C.H., Ho, C., Smith, P.K., Perumalsamy, K., Kanika, N.D., Friedman, J.M. and Acharya, S.A. (2005). "Conjugation of multiple copies of polyethylene glycol to hemoglobin facilitated through thiolation: influence on hemoglobin structure and function." *Protein J.*, 24(3):133-46.

Marshall, T. and Williams, K.M. (2004). "Interference in the coomassie brilliant blue and pyrogallol red protein dye-binding assays is increased by the addition of sodium dodecyl sulfate to the dye reagents." *Anal. Biochem.*, 331, 255– 259.

McGoff, P., Baziotis, A.C. and Maskiewicz, R. (1988). "Analysis of polyethylene glycol modified superoxide dismutase by chromatographic, electrophoretic, light scattering, chemical and enzymatic methods." *Chem Pharm Bull.*, 36, 3079–91.

McDonnell, T., Ioannou, Y., & Rahman, A. (2014). "PEGylated drugs in rheumatology—why develop them and do they work?." *Rheumatology*, *53*(3), 391-396.

McGrath, M.B. and Walsh, G. (2005). "Directory of Therapeutic Enzymes." *Taylor and Francis Group.*, 165-176.

Mehvar, R. (2000). "Modulation of the pharmacokinetics and pharmacodynamics of proteins by polyethylene glycol conjugation." *J Pharm Pharmaceut Sci.*, 3,125–136.

Mero, A., Clementi, C., Veronese, F.M. and Pasut, G. (2011). "Covalent conjugation of Poly(ethylene glycol) to proteins and peptides: Strategies and methods." *Bioconjugation Protocols,* Humana Press, second edition.

Michael S. Caves; Barry K. Derham; Jan Jezek; Robert B. Freedman. (2013). "Thermal Inactivation of Uricase (Urate Oxidase): Mechanism and Effects of Additives." *Biochemistry*, 52 (3), 497–507.

Milunovic, T., Kunstelj, M., Fidler, K.,Anderluh, G. and Porekar, V. G. (2012). *Anal. Biochem.* 430, 105-107.

Mikuls, T. R., & Saag, K. G. (2006). "New insights into gout epidemiology." *Current opinion in rheumatology*, *18*(2), 199-203.

Molek, J.R. and Zydney A.L. (2006). "Ultrafiltration characteristics of pegylated proteins." *Biotechnol. Bioeng.*, 95, 474–482.

Moolenburgh, J., Reinders, M. and Jansen, T. (2006). "Rasburicase treatment in severe tophaceous gout: a novel therapeutic option." *Clin Rheumatol.*, 25, 749–752.

Monkarsh, S. P., Spence, C., Porter, J. E., Palleroni, A., Nalin, C., Rosen, P. and Bailon, P. (1997). "Isolation of positional isomers of monopoly(ethylene glycol)ylated interferon alpha-2a and the determination of their biochemical and biological characteristics." *ACS Symposium Series 680 (Poly(ethylene glycol) Chemistry and biological applications)*, 207-216.

Morris, C. J., Beck, K., Fox, M. A., Ulaeto, D., Clark, G. C., & Gumbleton, M. (2012). "PEGylation of Antimicrobial Peptides Maintains the Active Peptide Conformation, Model Membrane Interactions, and Antimicrobial Activity while Improving Lung Tissue Biocompatibility following Airway Delivery." *Antimicrobial Agents and Chemotherapy*, *56*(6), 3298–3308.

Mu Q, Hu T, Yu J. (2013). "Molecular insight into the steric shielding effect of PEG on the conjugated staphylokinase: biochemical characterization and molecular dynamics simulation." *PLoS One*, 8(7).

Mughal, T.I., Ejaz, A.A, Foringer, J.R. and Coiffier, B. (2010). "An integrated clinical approach for the identification, prevention, and treatment of tumor lysis syndrome." *Cancer Treat Rev.*, 36(2), 164–76.

Na, D. H., & DeLuca, P. P. (2005). "PEGylation of octreotide: I. Separation of positional isomers and stability against acylation by poly (D, L-lactide-co-glycolide)." *Pharmaceutical research*, *22*(5), 736-742.

Nag, A., Mitra, G. and Ghosh, P.C. (1996). "A colorimetric assay for estimation of polyethylene glycol and polyethylene glycolated protein using ammonium ferrothiocyanate." *Anal Biochem.*, 237, 224-231.

Nanda, P., Babu, P.E.J., Tekalkote, S., Kunnummal, B.M. and Jacob, N. (2012). "Bioconjugation of *Bacillus fastidiosus* uricase with methoxy polyethylene glycol derivative and study of physiochemical properties." *Int J Pharm PharmSci*, 4(4), 290-295.

Nanda, P., JagadeeshBabu, P. E., & Raju, J. R. (2016). "Production and Optimization of Site-Specific monoPEGylated Uricase Conjugates Using mPEG-Maleimide Through RP–HPLC Methodology." *Journal of Pharmaceutical Innovation*, 1-10.

Nanda, P., & JagadeeshBabu, P. E. (2016). "Studies on the Site-specific PEGylation Induced Interferences Instigated in Uricase Quantification Using the Bradford Method." *International Journal of Peptide Research and Therapeutics*, 1-8.

Natalello, A., Ami, D., Collini, M., D'Alfonso, L., Chirico, G., Tonon, G., Doglia, S. M. (2012). "Biophysical characterization of Met-G-CSF: effects of different site-specific mono-pegylations on protein stability and aggregation." *PloS one*, *7*(8), e42511.

Niculescu-Duvaz, D., Getaz, J. and Springer, C. J., (2008). "Long functionalized Poly(ethylene glycol)s of defined molecular weight: Synthesis and application in solid-phase synthesis of conjugates." *Bioconjugate Chemistry*, 19(4), 973-981.

Nishikawa, M. and Hashida, (2002). *M. Biol. Pharm. Bull.*, 25, 275–283.

Nishimura, H., Ashihara, Y., Matsushima, A., Inada, Y., (1979). "Modification of yeast uricase with polyethylene glycol: disappearance of binding ability towards antiuricase serum." *Enzyme.*, 24, 261–264.

Nishimura, H., Matsushima, A., & Inada, Y. (1980). Improved modification of yeast uricase with polyethylene glycol, accompanied with nonimmunoreactivity towards anti-uricase serum and high enzymic activity.*Enzyme*, *26*(1), 49-53.

Oh, E. J., Park, K., Kim, K. S., Kim, J., Yang, J. A., Kong, J. H., ... & Hahn, S. K. (2010). "Target specific and long-acting delivery of protein, peptide, and nucleotide therapeutics using hyaluronic acid derivatives." *Journal of Controlled Release*, *141*(1), 2-12.

O'LOUGHLIN, J. A., Bruder, J. M., & Lysaght, M. J. (2004). "Degradation of low molecular weight uremic solutes by oral delivery of encapsulated enzymes." *ASAIO journal*, *50*(3), 253-260.

Orsattie, *L.* and Veronese, F.M. (199). "An unusual coupling of poly(ethylene glycol) to tyrosine residues in epidermal growth factor." *J Bioact C*, 14(5), 429-436.

Pacher, P., Nivorozhkin, A., & Szabó, C. (2006). "Therapeutic effects of xanthine oxidase inhibitors: renaissance half a century after the discovery of allopurinol." *Pharmacological reviews*, *58*(1), 87-114.

Pai, S. S., Hammouda, B., Hong, K., Pozzo, D. C., Przybycien, T. M., & Tilton, R. D. (2011). "The conformation of the poly (ethylene glycol) chain in mono-PEGylated lysozyme and mono-PEGylated human growth hormone." *Bioconjugate chemistry*, *22*(11), 2317-2323.

Pan, L.Q., Wang, H.B., Xie, Z.M., Li, Z.H., Tang, X.J., Xu, Y.C., Zhang, C., Naranmandura, H. and Chen, S.Q. (2003). "Novel conjugation of tumor-necrosis-factor-related apoptosis-inducing ligand (TRAIL) with monomethyl auristatin E for efficient antitumor drug delivery." *Adv Mater*., 25(34):4718-22.

Park, E.J. and Na, D.H. (2008). "Optimization of octreotide PEGylation by monitoring with fast reversed-phase high-performance liquid chromatography." *Anal Biochem*., 380(1):140-142.

Pasut, G. and Veronese, F.M. (2007). "Polymer–drug conjugation, recent achievements and general strategies." *Prog Polym Sci*., 32, 933–961.

Pasut, G. and Veronese, F.M. (2012). "State of the art in PEGylation: the great versatility achieved after forty years of research." *J Control Release.,* 161(2):461-72.

Pepinsky, R. B., LePage, D. J., Gill, A., Chakraborty, A., Vaidyanathan, S., Green, M., Martin, P. (2001). "Improved pharmacokinetic properties of a polyethylene glycol-modified form of interferon-β-1a with preserved in vitro bioactivity." *Journal of Pharmacology and Experimental Therapeutics*, *297*(3), 1059-1066.

Pfister, D., & Morbidelli, M. (2014). "Process for protein PEGylation." *Journal of Controlled Release*, *180*, 134-149.

Pitts, O. M., & Priest, D. G. (1974). "A steady-state kinetic investigation of the uricase reaction mechanism." *Archives of biochemistry and biophysics*,*163*(1), 359-366.

Plesner, B., Fee, C.J., Westh, P. and Nielsen, A.D. (2011). "Effects of PEG size on structure, function and stability of PEGylated BSA." *Eur J Pharm Biopharm*., (2):399-405.

Puchkova, I.A, Kononovaa, N. V., Bobruskina, A. I., Bairamashvilib, D. I., Martyanova, V. A. and Shustera, A. M. (2012). "Recombinant Granulocyte Colony Stimulating Factor (Filgrastim):

Optimization of Conjugation Conditions with Polyethylene Glycol." *Bioorg Khim*. 38(5), 479–487.

Pui, C.H., Jeha, S., Irwin, D. and Camitta, B. (2001). "Recombinant urate oxidase (rasburicase) in the prevention and treatment of malignancy-associated hyperuricemia in pediatric and adults patients: results of a compassionate-use trial." *Leukemia*, 15, 1505–1509.

Punnappuzha, A., PonnanEttiyappan, J.B., Nishith, R.S., Hadigal, S and Pai, P.G. (2014). "Synthesis and Characterization of Polysialic Acid–Uricase Conjugates for the Treatment of Hyperuricemia." *International Journal of Peptide Research and Therapeutics*, 1-8.

Qian, X H. Dong, X. Hu, H. Tian, L. Guo, Q. Shen, X. Gao and W. Yao. (2014). "Analysis of the interferences in quantitation of a site-specifically PEGylated exendin-4 analog by the Bradford method." *Anal. Biochem*., 465; 50–52.

Rajan, R. S., Li, T., Aras, M., Sloey, C., Sutherland, W., Arai, H., Yeghnazar, H. (2006). "Modulation of protein aggregation by polyethylene glycol conjugation: GCSF as a case study." *Protein science*, *15*(5), 1063-1075.

Reinders, M. K., & Jansen, T. L. (2010). "Management of hyperuricemia in gout: focus on febuxostat." *Clin Interv Aging*, *5*(5), 7-18.

Resch, G., Moreillon, P., & Fischetti, V. A. (2011). "PEGylating a bacteriophage endolysin inhibits its bactericidal activity." *AMB Express*, *1*, 29.

Richette, P. and Bardin, T. (2006). "Successful treatment with rasburicase of a tophaceous gout in a patient allergic to allopurinol." *Nat Clin Pract Rheumatol*., 2(6), 338–342.

Richette, P., Brière, C. and Clavert, H .V. (2007). "Rasburicase for tophaceous gout not treatable with allopurinol: an exploratory study." *J Rheumatol*., 34, 2093–2098.

Riddles, P.W., Blakeley, R.L. and Zerner, B. (1979). "Ellman's reagent: 5,5′-dithiobis(2-nitrobenzoic acid) - A re-examination." *Anal Biochem*., 94,75–81.

Roberts, M.J., Bently, M.D. and Harris, J.M. (2002). "Chemistry for peptide and protein PEGylation." *Advanced drug delivery reviews.*, 54, 459-476.

Robinson, D. H., & Mauger, J. W. (1991). "Drug delivery systems." *American Journal of Health-System Pharmacy*, *48*(10 Suppl), S14-S23.

Rodriguez, T.M., Jeffers, L.J., Sheikh, M.Y., Rossaro, L., Ankoma, S.V., Hamzeh, F.M. and Martin, P. (2009). "PEGinterferon alfa-2a and ribavirin in Latino and non-Latino whites with hepatitis C." *N Engl J Med.*, 360(3), 257–67.

Roelfsema, F., Biermasz, N. R., Pereira, A. M., & Romijn, J. M. (2006). "Nanomedicines in the treatment of acromegaly: focus on pegvisomant.*International journal of nanomedicine.*" *1*(4), 385.

Rosendahl, M.S., Doherty, D.H., Smith, D.J., Carlson, S.J., Chlipala, E.A. and Cox, G.N. (2005). "A long-acting, highly potent interferon alpha-2 conjugate created using site-specific PEGylation." *Bioconjug Chem.,* 16(1), 200-207.

Rutkoski, T.J., Kink, J.A., Strong, L.E. and R.T. Raines. (2011). "Site-specific PEGylation endows a mammalian ribonuclease with antitumor activity." *Cancer Biol Ther*. 1, 12(3), 208-214.

Salmaso, S., Bersani, S., Scomparin, A., Mastrotto, F., Scherpfer, R., Tonon, G. and Caliceti, P. (2009). "Tailored PEG for rh-G-CSF Analogue Site-Specific Conjugation." *Bioconjug. Chem., 20,* 1179–1185.

Sapan, C.V., Lundblad R. L. and N.C. Price. (1999). "Colorimetric protein assay techniques." *Biotechnol Appl Biochem.*, 99-108.

Sapra, B., Jain, S., & Tiwary, A. K. (2008). "Transdermal delivery of carvedilol containing glycyrrhizin and chitosan as permeation enhancers: biochemical, biophysical, microscopic and pharmacodynamic evaluation." *Drug delivery*, *15*(7), 443-454.

Sapra, P., Kraft, P., Mehlig, M.. (2009). "Marked therapeutic efficacy of a novel polyethylene glycol-SN38 conjugate, EZN-2208, in xenograft models of B-cell non-Hodgkin's lymphoma." *Haematologica*., 94(10):1456–1459.

Savoca, K. V., Davis, F. F., & Palczuk, N. C. (1984). "Induction of tolerance in mice by uricase and monomethoxypolyethylene glycol-modified uricase." *International Archives of Allergy and Immunology*, *75*(1), 58-67

Schiavon, O., Caliceti, P., Ferruti, P. and Veronese, F.M. (2000). "Therapeutic proteins: a comparison of chemical and biological properties of uricase conjugate to linear or branched poly(ethyleneglycol)andpoly(N-acryloylmorpholine)." *Farmaco.,* 55, 264–269.

Schlesinger, N., Yasothan, U and Kirkpatrick, P. (2011). "PEGloticase." *Nature Reviews Drug Discovery.,* 10, 17–18.

Seely, J.E. and Richey, C.W. (2001). "Use of ion-exchange chromatography and hydrophobic interaction chromatography in the preparation and recovery of polyethylene glycol-linked proteins." *J. Chromatogr. A*, 908, 235–241.

Shaunak, S., Godwin, A., Choi, J.W., Balan, S., Pedone, E., Vijayarangam, D., Heidelberger, S., Teo, I., Zloh, M. and Brocchini, S. (2006). "Site-specific PEGylation of native disulfide bonds in therapeutic proteins." *Nat Chem Biol.,* 2(6), 312-3.

Sherman, M. R., Saifer, M. G., & Williams, L. D. (2004). *U.S. Patent No. 6,783,965*. Washington, DC: U.S. Patent and Trademark Office.

Sherman, M.R., Saifer, M.G.P. and Perez-Ruiz, F. (2008). "PEG-uricase in the management of treatment-resistant gout and hyperuricemia." *Adv. Drug Deliv. Rev*., 60, 59–68.

Sherman, M. R., Saifer, M. G., Williams, L. D., Hershfield, M. S., & Kelly, S. J. (2011). *U.S. Patent No. 7,927,852*. Washington, DC: U.S. Patent and Trademark Office.

Sims, G.E.C. and Snape, T.J. (1980). "A method for the estimation of polyethylene glycol in plasma protein fractions." *Anal.Biochem.*, 107, 60–63.

Sinclair, D. S., & Fox, I. H. (1975). "The pharmacology of hypouricemic effect of benzbromarone." *The Journal of rheumatology*, 2(4), 437-445

Sivakolundu, S. G., and Mabrouk, P.A. (2003). "Proton NMR study of chemically modified horse heart ferricytochrome C confirms the presence of histidine and lysine ligated conformers in 30% acetonitrile solution." *J. Inorg. Biochem*. 94, 381-385.

Smith, P.K. (1985). "Measurement of protein using bicinchoninic acid." *Anal. Biochem.*, 150,76-85.

Soares, A. L., Guimarães, G. M., Polakiewicz, B., de Moraes Pitombo, R. N., & Abrahão-Neto, J. (2002). "Effects of polyethylene glycol attachment on physicochemical and biological stability of E. colil-asparaginase." *International journal of pharmaceutics*, 237(1), 163-170.

Sun, F., Chai, S., Li, L., Yu, K., Yang, Z., Wu, S., Zhan, S. (2015). "Effects of glucagon-like peptide-1 receptor agonists on weight loss in patients with type 2 diabetes: a systematic review and network meta-analysis." *Journal of diabetes research*, *2015*.

Sundy, J.S., Ganson, N.J., Kelly, S.J., Scarlett, E.L., Rehrig, C.D., Huang, W. and Hershfield, M.S. (2007). "Pharmacokinetics and pharmacodynamics of intravenous PEGylated recombinant mammalian urate oxidase in patients with refractory gout." *Arthritis Rheum.,* 56(3), 1021–1028.

Suo, X., Zheng, C., Yu, P., Lu, X., Ma, G. and Su, Z. (2009). "Solid-phase PEGylation of hemoglobin." *Artificial Cells, Blood Substitutes, and Biotechnology*., 37(4), 147-155.

Tan, Q.-Y., Zhang, J.-Q., Wang, N., Yang, H., Li, X., Xiong, H.-R. and Yin, H.-F. (2012). "Improved biological properties and hypouricemic effects of uricase from *Candida utilis* loaded in novel alkaline enzymosomes." *International Journal of Nanomedicine*., 7, 3929–3938.

Tao, L., Liu, J., Xu, J., & Davis, T. P. (2009). "Synthesis and bioactivity of poly (HPMA)–lysozyme conjugates: the use of novel thiazolidine-2-thione coupling chemistry." *Organic & biomolecular chemistry*, *7*(17), 3481-3485.

Tang, L., Jariwala, F., Bolgar, M. and Lloyd, D.K. (2012). "Separation and detection of bis-maleimide-polyethylene glycol and mono-maleimide-polyethylene glycol by reversed-phase high pressure liquid chromatography*." J Chromatogr A.,* 1246, 117-122.

Terkeltaub, R. (2010). "Update on gout: new therapeutic strategies and options." *Nat Rev Rheumatol*., 6(1), 30–38.

Tiwari, K., Tiwari, D., Shebannavar, S., Pokalwar, S. and Mishra, M. (2011). "Interference of components of PEGylation reaction mixture in colorimetric protein assays." *Int. J. Curr. Res.* 4(03), 24-26

Tiwari, S., Dwivedi, H., Kymonil, K. M., & Saraf, S. A. (2015). "Urate crystal degradation for treatment of gout: a nanoparticulate combination therapy approach." *Drug delivery and translational research*, *5*(3), 219-230.

Treetharnmathurot, B., Dieudonné, L., Ferguson, E.L., Schmaljohann, D., Duncan, R. and Wiwattanapatapee, R. (2009). "Dextrin-trypsin and ST-HPMA-trypsin conjugates: enzyme activity, autolysis and thermal stability." *Int J Pharm*., 373(1-2), 68-76.

Trivedi, R.K. and Goel, P.K. (1986). "Chemical and Biological Methods for Water Pollution Studies." *Maharashtra Environmental Publication Karad.*

Trivedi, M. S., Shah, J. S., Al-Mughairy, S., Hodgson, N. W., Simms, B., Trooskens, G. A., Deth, R. C. (2014). "Food-derived opioid peptides inhibit cysteine uptake with redox and epigenetic consequences." *The Journal of nutritional biochemistry*, *25*(10), 1011-1018.

Tsuji, J. I., Hirose, K., Kasahara, E., Naitoh, M., & Yamamoto, I. (1985). "Studies on antigenicity of the polyethylene glycol (PEG)-modified uricase." *International journal of immunopharmacology*, *7*(5), 725-730.

Tsui, S. M., Lam, W.M., Lam, T.L., Chong, H.C., Kwok, S.U., Arnold, S., Cheng, P.M.N., Lo, W.H. and Leung. Y.C. (2009). "PEGylated derivatives of recombinant human arginase (rhArg1) for sustained *invivo* activity in cancer therapy: preparation, characterization and analysis of their pharmacodynamics *invivo* and *invitro* and action upon hepatocellular carcinoma cell (HCC)." *Cancer cell International.* 2009, 9:9.

Tsutsumi, Y., Onda, M., Nagata, S., Lee, B., Kreitman, R.J. and Pastan, I. (2000). "Site-specific chemical modification with polyethylene glycol of recombinant immunotoxin anti-Tac(Fv)-PE38 (LMB-2) improves antitumor activity and reduces animal toxicity and immunogenicity." *Proc Natl Acad Sci U S A.*, 97(15):8548-53.

Ulbricht, J., Jordan, R., & Luxenhofer, R. (2014). "On the biodegradability of polyethylene glycol, polypeptoids and poly (2-oxazoline)s." *Biomaterials*,*35*(17), 4848-4861.

Usuda, N., Usman, M.I., Reddy, M.K., Hashimoto, T., Reddy, J.K. and Rao, M.S. (1988). "Immunocytochemical localization of urate oxidase, fatty acyl-CoA oxidase and catalase in bovine kidney peroxisomes." *J. Histoch. Cytoch.* 36,253–258.

Van Arnum, P. (2011). Quality by design in APIs.

Van der Wal, S. and Snyder, L. R. (1983). "Photometric detection at 185 nm for high-performance liquid chromatography with either isocratic or gradient elution : Assay of mixtures of polyethylene glycol oligomers." *J.Chromatogr.,* 255, 463–474.

Varki, A., Cummings, R.D., Esko, J.D., Freeze, H.H, Stanley, P., Bertozzi, C.R., Hart, G.W. and Etzler, M.E. (2009). "Essential of Glycobiology 2*." Cold spring Harbor Laboratory Press.*

Vellard, M. (2003). "The enzyme as drug: application of enzymes as pharmaceuticals." *Current Opinion in Biotechnology.*, 14, 444–450.

Veronese, F. M., Largajolli, R., Boccu, E., Benassi, C. A., & Schiavon, O. (1985). "Surface modification of proteins activation of monomethoxy-polyethylene glycols by phenylchloroformates and modification of ribonuclease and superoxide dismutase." *Applied biochemistry and biotechnology*, *11*(2), 141-152.

Veronese, F. M., & Morpurgo, M. (1999). "Bioconjugation in pharmaceutical chemistry." *Il Farmaco*, *54*(8), 497-516.

Veronese, F.M. (2001). "Peptide and Protein PEGylation: A review of problems and solutions." *Biomaterials.*, 22, 405-417.

Veronese, F.M., Saccà, B., Polverino, L. P., Sergi, M., Caliceti, P., Schiavon, O. and Orsolini P. (2001). "New PEGs for peptide and protein modification, suitable for identification of the PEGylation site." *Bioconjug Chem.*, 12(1):62-70.

Veronese, M.F. and Pasut, G. (2005). "PEGylation, successful approach to drug delivery." *Drug Delivery Today.*, 10(21), 1451-1458.

Viegas, T. X., Bentley, M. D., Harris, J. M., Fang, Z., Yoon, K., Dizman, B,. Weimer, R., Mero, A., Pasut, G. and Veronese, F.M. (2011). Polyoxazoline: chemistry, properties, and applications in drug delivery. *Bioconjugate chemistry*, *22*(5), 976-986.

Vine, A. K., Maguire, P. T., Martonyi, C., & Kincaid, M. C. (1988). "Recombinant tissue plasminogen activator to lyse experimentally induced retinal arterial thrombi." *American journal of ophthalmology*, *105*(3), 266-270.

Vogt, B. (2005). "Urate oxidase (rasburicase) for treatment of severe tophaceous gout." *Nephrol Dial Transplant.,*20, 431–433.

Volkl, A., Baumgart, E. and Fahimi, D. (1988). "Localization of urate oxidase in the crystalline cores of rat liver peroxisomes by immunocytochemistry and immunoblotting." *J. Histoch. Cytoch.* 36, 329–336.

Wang, Y.S., Youngster, S., Grace, M., Bausch, J., Bordens, R. and Wyss, D.F. (2002). "Structural and biological characterization of PEGylated recombinant interferon alpha-2b and its therapeutic implications." *Advanced Drug Delivery Reviews.*, 54, (4), 547-570.

Wang, J., Wang, Y., Hu, T., Li, X., Huang, Y., Liu, Y., Ma, G. and Su, Z. (2012). "An oriented adsorption strategy for efficient solid-phase PEGylation of recombinant staphylokinase by immobilized metal-ion affinity chromatography." *Process Biochemistry*, 47, 106-112.

Watson, E., Shah, B., DePrince, R., Hendren, R.W. and Nelson, R. (1994). "Matrix-assisted laser desorption mass spectrometric analysis of a PEGylated recombinant protein." *Biotechniques.,* 16, 278–281.

Williams, L. D., Hershfield, M. S., Kelly, S. J., Saifer, M. G., & Sherman, M. R. (2003). *U.S. Patent No. 6,576,235*. Washington, DC: U.S. Patent and Trademark Office.

Williams, L. D., Hershfield, M. S., Kelly, S. J., Saifer, M. G., & Sherman, M. R. (2010). *U.S. Patent No. 7,723,089*. Washington, DC: U.S. Patent and Trademark Office.

Wong, S.S. (1991). "Chemistry of protein conjugation and cross-linking." CRC press.

Wood, A. and Shilatifard, A. (2004). "Post-traslational modifications of histones by methylation." *Adv. Protein Chem.,* 67, 201–222.

Woodburn, K. W., Fong, K. L., Wilson, S. D., Sloneker, S., Strzemienski, P., Solon, E. and Tagawa, Y. (2013). "Peginesatide clearance, distribution, metabolism, and excretion in monkeys following intravenous administration." *Drug Metabolism and Disposition*, *41*(4), 774-784.

Working, P.K., Newman, M.S., Johnson, J. (1997). "Safety of poly(ethylene glycol) and poly(ethylene glycol) derivatives, in: J.M. Harris, S. Zalipsky (Eds.), Poly(ethylene glycol)." *Chemistry and Biological Applications*, ACS Books, Washington, DC, 45–57.

Wu, X., Wakamiya, M., Vaishnav, S., Geske, R. and Montgomery, C. (2004). "Hyperuricemia and urate nephropathy in urate oxidase deficient mice." *Proc. Nat. Acad. Sci. USA*, 91, 742-746.

Xian, H. H., Pang C. S., Li, H, X. and Siu, C. T. (1999). "Site Directed polyethylene glycol modification of trichosanthine: Effects on its biological activities, pharmacokinetics and antigenicity." *Life. Science*., 64, 1163-1175.

Yamaoka, T., Tabata, Y., & Ikada, Y. (1994). "Distribution and tissue uptake of poly (ethylene glycol) with different molecular weights after intravenous administration to mice." *Journal of pharmaceutical sciences*, *83*(4), 601-606.

Yang, K., Basu, A., Wang, M., Chintala, R., Hsieh, M.C., Liu, S., Hua, J., Zhang, Z., Zhou, J., Li, M., Phyu, H., Petti, G., Mendez, M., Janjua, H., Peng, P., Longley, C., Borowski, V., Mehlig, M. and Filpula, D. (2003). "Tailoring structure-function and pharmacokinetic properties of single-chain Fv proteins by site-specific PEGylation." *Protein Eng*., 16(10):761-70.

Yang, X., Yuan, Y., Zhan, C. G. and Liao, F. (2013). "Uricases as therapeutic agents to treat refractory gout: Current states and future directions." *Drug Dev Res*., 73(2): 66–72.

Yoshida, K., Nishimura, H., Takahashi, K., Matsushima, A. and Inada, Y. (1981). "Modification of amino groups in Candida utilis uricase with naphthoquinone disulfonic acid in relation to the enzymic activity." *FEBS Lett.* 134(1)., 50-52.

Yoshioka, Y., Tsunoda, S. and Tsutsumi, Y. (2011). "Development of a novel DDS for site specific PEGylated proteins." *Chemistry Central Journal*, 5:25, 1-6.

Yu, P., Zheng, C., Chen, J., Zhang, G., Liu, Y., Suo, X., ... & Su, Z. (2007). "Investigation on PEGylation strategy of recombinant human interleukin-1 receptor antagonist." *Bioorganic & medicinal chemistry*, *15*(16), 5396-5405.

Yu, P., Qin, D., Qin, G., Fan, B., Ma, G., & Su, Z. (2009). "PEGylation of rhIL-1RA increased its solution stability at room temperature." *Process Biochemistry*, *44*(12), 1340-1345.

Yu, X., Shang, X. and Ghosh, R. (2010). "Fractionation of different PEGylated forms of a chromatography using environment- responsive membranes." *J. Chromatograph A*, 1217, 5595-5601.

Yue, C.S., Huang, W., Alton, M., Maroli, A.N., Waltrip, R.W., Wright, D., Marco, M.D. (2008). "Population pharmacokinetic and pharmacodynamic analysis of PEGloticase in subjects with hyperuricemia and treatment-failure gout." *J Clin Pharmacol,* 48(6), 708–718.

Zalipsky, S. (1995). "Chemistry of Poly(etheneglycol) conjugates with biologically active molecules." *Advanced drug delivery reviews*, 16 (2-3), 157-182.

Zhang, Y. Q., Zhou, W. L., Shen, W. D., Chen, Y. H., Zha, X. M., Shirai, K., & Kiguchi, K. (2005). "Synthesis, characterization and immunogenicity of silk fibroin-L-asparaginase bioconjugates." *Journal of biotechnology*, *120* (3), 315-326.

Zhang, W., Doherty, M., Bardin, T., Pascual, E., Barskova, V., Conaghan, P., McCarthy, G. (2006). "EULAR evidence based recommendations for gout. Part II: Management. Report of a task force of the EULAR Standing Committee for International Clinical Studies Including Therapeutics (ESCISIT)." *Annals of the rheumatic diseases*, *65*(10), 1312-1324.

Zhang, C., Yang, X., Feng, J., Yuan, Y., Li, X., Bu, Y., Xie, Y., Yuan, H. and Liao, F. (2010). "Effects of modification of amino groups with Polyethylene Glycol on a recombinant Uricase from *Bacillus fastidiosus." Biosci. Biotechnol. Biochem.*, 74(6), 1298-1301.

Zhang, P., Sun, F., Tsao, C., Liu, S., Jain, P., Sinclair, A., ... & Jiang, S. (2015). "Zwitterionic gel encapsulation promotes protein stability, enhances pharmacokinetics, and reduces immunogenicity." *Proceedings of the National Academy of Sciences*, *112*(39), 12046-12051.

Zhai, Y., Zhao, Y., Lei, J., Su, Z. and Ma, G. (2009). "Enhanced circulation half-life of site-specific PEGylated rhG-CSF: optimization of PEG molecular weight." *J Biotechnol.,* 15:142(3-4):259-66.

Zhao, Y., Zhao, L., Yang, G., Tao, J., Bu, Y. and Liao, F. (2006). "Characterization of a uricase from Bacillus fastidious A.T.C.C. 26904 and its application to serum uric acid assay by a patented kinetic uricase method." *Biotechnol Appl Biochem.*, 45(2), 75–80.

Zhou, J. G., & Chen, Y. M. (2011). "Research on PEGylation of porcine prothrombin for improving biostability and reducing animal immunogenicity." *Bioorganic & medicinal chemistry letters*, *21*(11), 3268-3272

Zhou, Y., Zhang, M., He, D., Hu, X., Xiong, H., Wu, J., Zhang, J. (2016). "Uricase alkaline enzymosomes with enhanced stabilities and anti-hyperuricemia effects induced by favorable microenvironmental changes." *Scientific reports*, *7*.

Zor, T. and Selinger, Z. (1996). "Linearization of the Bradford protein assay increases its sensitivity: theoretical and experimental studies." *Anal. Biochem.,* 236, 302–308.

APPENDIX I

Enzymatic Assay of URICASE (EC 1.7.3.3)

Principle: Urate + H_2O + O_2 Uricase -------> Allantoin + H_2O_2 + CO_2

Conditions: $T = 25^oC$, pH = 9.0, A293nm, Light path = 1 cm

Method: Continuous Spectrophotometric Rate Determination

A. 20 mM Boric Acid Buffer, pH 9.0 at 25^oC

B. 3.57 mM Uric Acid Solution (Urate) (The solution may require heat and vortexing in order to effect complete dissolution.)

C. Uricase Enzyme Solution

PROCEDURE:

Pipette (in milliliters) the following reagents into suitable cuvettes:

	Test	Blank
Reagent A (Buffer)	3.00	3.00
Reagent B (Urate)	0.075	0.075

Mix by swirling and equilibrate to 25 ^oC. Monitor the A293nm until constant using a suitably thermo-statted spectrophotometer. Then add:

	Test	**Blank**
Reagent C (Enzyme)	0.02	------
Reagent A (Buffer)	------	0.02

Immediately mix by inversion and record the decrease in A293nm for approximately 5 minutes. Obtain the ΔA293nm/minute using the maximum linear rate for both the Test and Blank.

CALCULATIONS

$$\text{Units/mL enzyme} = \frac{(\Delta A_{293nm}/\text{min Test} - \Delta A_{293nm}/\text{min Blank})*(3.095)*(df)}{(12.6)*(0.02)}$$

3.095 = Volume of reaction mixture

df = Dilution factor

12.6 = Millimolar extinction coefficient of UA at

293nm

0.02 = Volume of enzyme used

$$\text{Units/mg solid} = \frac{\text{Units/mL enzyme}}{\text{mg solid/mL enzyme}}$$

UNIT DEFINITION

One unit will convert 1.0 μmole of uric acid to allantoin per minute at pH 9.0 at 25 ºC.

Total protein Estimation by Bradford Analysis

The Bradford Reagent can be used to determine the concentration of proteins in solution. The procedure is based on the formation of a complex between the dye, Brilliant Blue G, and proteins in solution. The protein dye complex causes a shift in the absorption maximum of the dye from 465 to 595 nm. BSA (bovine serum albumin) and Uricase from *Bacillus fastidiosus* were used as the standard proteins for developing the standard curve.

Procedure

The standard 3.1 mL Bradford assay consisted of mixing 1 part of the protein sample with 30 parts of the Bradford reagent. The blank sample consisted of buffer with no protein. The protein standard consisted of a known concentration of protein with the unknown

sample in the solution to be assayed. Bradford assays were routinely performed at room temperature. Color development began immediately and recorded at 595 nm with the protein concentration determined by comparison to a standard curve.

Tube no	BSA/ Uricase (µL)	Distilled Water (µL)	Bradford Reagent (mL)	OD @ 595 nm	Difference in Abs
Blank	0	100	3	0.430	-
1	20	80	3	0.625	0.195
2	40	60	3	0.823	0.393
3	60	40	3	0.979	0.549
4	80	20	3	1.120	0.69
5	100	0	3	1.297	0.867

1. Bradford reagent bottle was gently mixed in the bottle and brought to room temperature.

2. Protein standards of appropriate concentrations in the same buffer as the unknown samples were prepared. The standards were created by serially diluting 1 mg/mL BSA as a protein standard. Deionized water was substituted for the buffer.

3. After adding 3 mL of Bradford Reagent to each tube, they were vortexed gently for thorough mixing. The total liquid volume in each tube was 3.1 mL.

4. The samples were incubated at room temperature for 5–45 minutes and transferred into cuvettes.

5. The absorbance was measured at 595 nm within 10 minutes of incubation.

6. The protein concentration was determined by comparison of the unknown samples to the standard curve prepared using the protein standards.

Bradford calibration curve for BSA

$y = 8.9605x$
$R^2 = 0.9962$

(Abs-Blank) @ 595 nm

BSA Concentration (mg/mL)
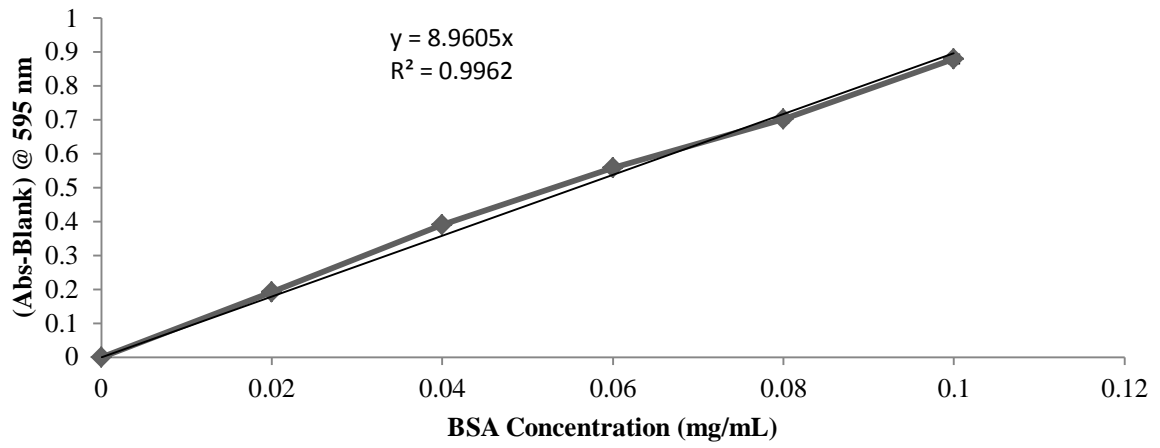
Bradford standard curve for Uricase from *Bacillus fastidiosus*

$y = 0.1821x$
$R^2 = 0.9951$

(Abs-Blank) @ 696 nm

Uricase Concentration (mg/mL)
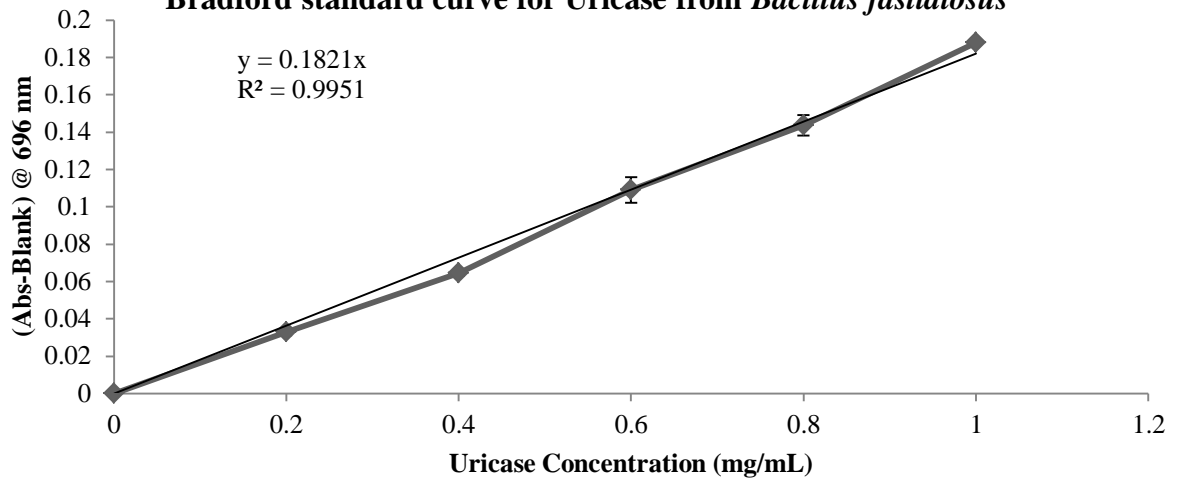
Determination of % degree of amine substitution by TNBS assay
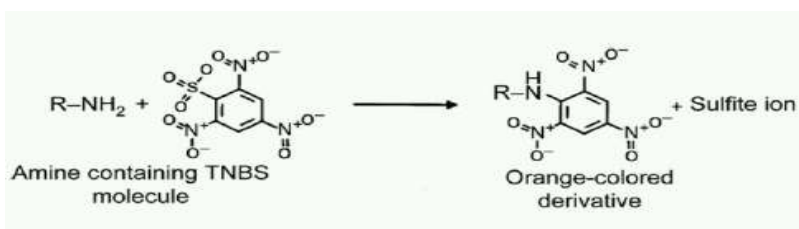
2,4,6-Trinitrobenzene Sulfonic Acid (TNBSA or TNBS) is a rapid and sensitive assay reagent for the determination of free amino groups (Habeeb et al. 1966). Primary amines, upon reaction with TNBSA, form a highly chromogenic derivative, which can be measured at 335 nm. The reaction is as represented in Figure.

Materials Required:

Reaction Buffer: 0.1 M sodium bicarbonate, pH 8.5

TNBSA: 0.01% (w/v) solution of TNBSA. Prepare using reaction buffer as a diluent. Prepare fresh for each reaction.

10% solution of SDS (Sodium dodecyl sulphate) in distilled water

1 N HCl (Hydrochloric acid)

Method

1. The proteins were dissolved directly in reaction buffer at a concentration of 20-200 μg/ml. Alternatively, for proteins already in solution, the buffer was changed by dialysis.

2. 0.25 ml of the 0.01% solution of TNBSA was added to 0.5 ml of each sample solution and mixed well.

3. Test tubes were incubated at 37°C for two hours.

4. 0.25 ml of 10% SDS and 0.125 ml of 1 N HCl was added to each sample and the absorbance of the solution was measured at 335 nm.

Quantitative determination of the number of amines contained within a sample can be accomplished through comparison to a standard curve generated by the use of an amine

containing compound (*e.g*., amino acid) dissolved in a series of known concentrations. The standards should be dissolved or dialyzed into the reaction buffer and must be assayed under reaction conditions identical to those utilized for the samples.

The calibration curve for TNBS Assay was plotted using BSA (Bovine Serum Albumin) (1 mg/mL) as a protein standard. The procedure for plotting the calibration curve is as follows.

Sl no	Vol of BSA solution (mL)	Vol of Reaction Buffer (mL)	Vol of Bicarbonate Buffer (mL)	Vol of TNBSA solution (mL)
1	0	1.0	1.0	0.5
2	0.1	0.9	0.9	0.5
3	0.2	0.8	0.8	0.5
4	0.3	0.7	0.7	0.5
5	0.4	0.6	0.6	0.5
6	0.5	0.5	0.5	0.5
7	0.6	0.4	0.4	0.5
8	0.7	0.3	0.3	0.5
9	0.8	0.2	0.2	0.5
10	0.9	0.1	0.1	0.5
11	1.0	0	0	0.5

After the addition of all the above stated reagents, the reaction mixtures were incubated for 2 hours at 37ºC. 0.5 mL of 10% SDS was added to all the test tubes, followed by the addition of 0.25 mL of 1 N HCl. The absorbance of all the samples was measured at 335 nm. A calibration curve of OD of samples at 335 nm versus the protein concentration was plotted.

Calibration Curve for TNBS Assay

y = 0.8567x
R² = 0.9889

Y-axis: **(Abs-Blank) @ 335**

X-axis: **BSA Concentration (mg/mL)**
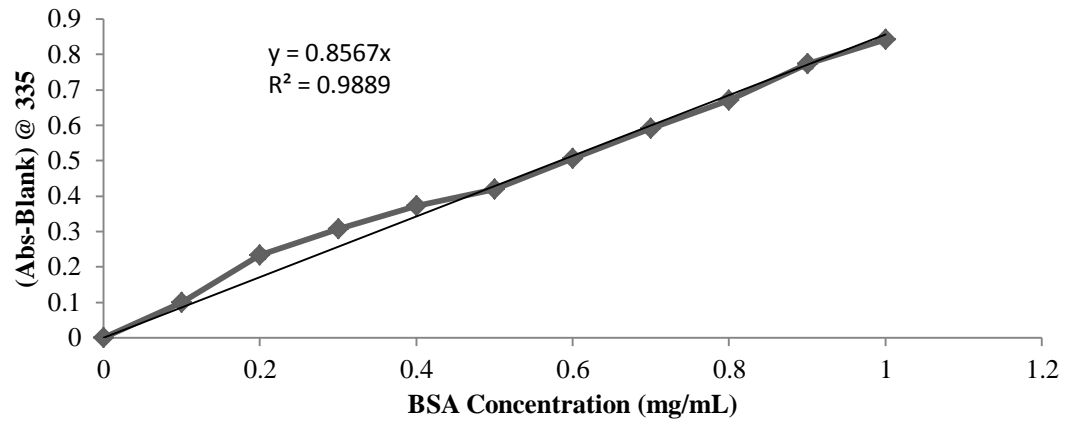
Appendix III

Ellman's Assay for the determination of sulfdryl concentration

In 1959, Ellman introduced 5,5'-dithio-*bis*-(2-nitrobenzoic acid), also known as DTNB, as a versatile water-soluble compound for quantitating free sulfhydryl groups in solution. A solution of this compound produces a measurable yellow-colored product when it reacts with sulfhydryls. Consequently, Ellman's Reagent is very useful as a sulfhydryl assay reagent because of its specificity for -SH groups at neutral pH, high molar extinction coefficient and short reaction time. Sulfhydryl groups may be estimated in a sample by comparison to a standard curve composed of known concentrations of a sulfhydryl-containing compound such as cysteine.
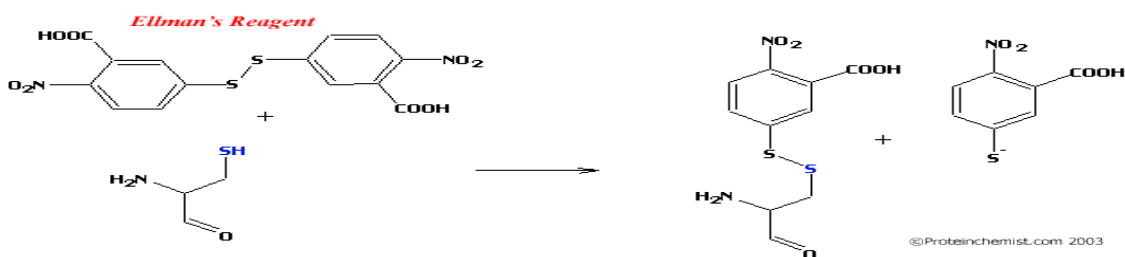
Procedure for Quantitating Sulfhydryl Groups Using a Cysteine Standard

A. Material Preparation

• Reaction Buffer: 0.1M sodium phosphate, pH 8.0, containing 1mM EDTA

• L-Cysteine (for biochemistry): M.W. = 121.16, 10 µg/mL stock

• Ellman's Reagent Solution: Dissolve 4mg Ellman's Reagent in 1mL of Reaction Buffer

B. Procedure

1. A set of cysteine standards by dissolving L-Cysteine at the following concentrations in was prepared in a reaction buffer:

Standard	Volume of Reaction Buffer	Amount of Cysteine (M.W. = 121.16)	Cysteine Concentration(μg/mL)
A	50mL	0.5 mg	10
B	2.5mL	12.5mL of Standard A	8.33
C	5mL	10mL of Standard A	6.67
D	7.5mL	7.5mL of Standard A	5
E	10mL	5mL of Standard A	3.33
F	12.5mL	2.5mL of Standard A	1.67
G	15mL	0ml	0

2. A set of test tubes, each containing 50μL of Ellman's reagent solution and 2.5mL of reaction buffer was prepared.

3. 250μL of each standard or unknown to the separate test tubes prepared in step 2 and to blank add 250 μL reaction buffer were added.

4. The reaction mixtures were incubated at room temperature for 15 minutes and absorbance measured at 412nm.

5. The values obtained were plotted for the standards to generate a standard curve. The experimental sample concentrations were determined from this curve.

Concentration (mg/mL)	Abs @ 412 nm-Blank
0	0
1.67	0.027
3.33	0.0515
5	0.063
6.67	0.0785
8.33	0.1025
10	0.123

Calibration Curve for Ellmans Assay

$y = 0.0124x$
$R^2 = 0.9848$

Y-axis: (Abs-Blank) @ 412 nm

X-axis: Cysteine Concentration (μg/mL)

Procedure for Quantitating Sulfhydryl Groups Based on Molar Absorptivity

A. Material Preparation

• Reaction Buffer: 0.1M sodium phosphate, pH 8.0, containing 1mM EDTA

• Ellman's Reagent Solution: Dissolve 4mg Ellman's Reagent in 1mL of Reaction Buffer.
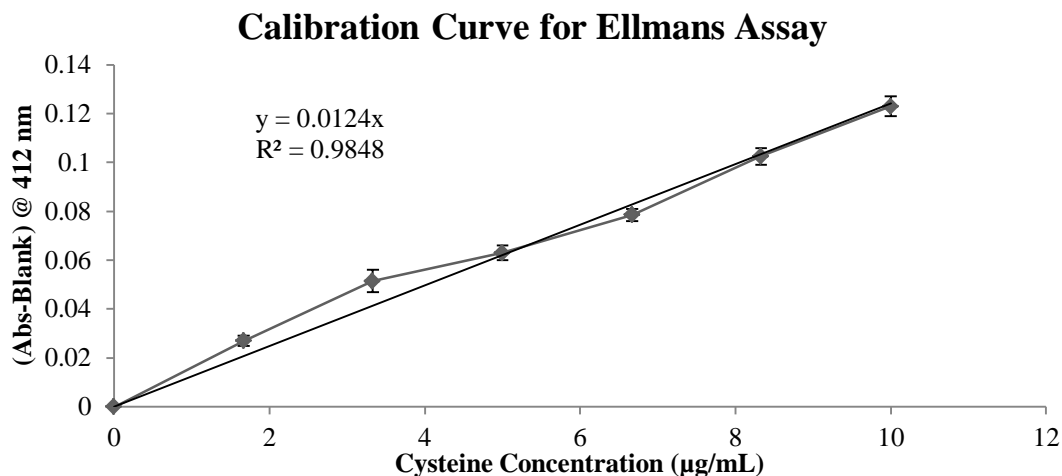
B. Measure Absorbance

1. For each unknown sample to be tested, a tube containing 50μL of Ellman's reagent solution and 2.5mL of reaction buffer was prepared.

2. 250μL of each unknown to the separate test tubes prepared in step 1 was added. As a blank, 250μL of reaction buffer were added to a separate test tube prepared in Step 1.

3. The reaction mixtures were mixed and incubate at room temperature for 15 minutes.

4. With a spectrophotometer set to 412nm, the instrument was zeroed on the blank and then absorbance of each sample was measured.

5. The amount and concentration of sulfhydryls in the sample was calculated from the molar extinction coefficient of TNB ($14,150 \ M^{-1} \ cm^{-1}$) as exemplified below:

a) A 30 μL aliquot of the unknown mixed with 970 μL of reaction buffer and 50μL of Ellman's reagent solution gave an absorbance of A (after subtracting the blank) using a 1cm Spectrophotometric cuvette.

b) The sulfhydryl concentration in μmoles per mL of unknown were calculated. The reported molar absorptivity (molar extinction coefficient, which is expressed in units of M^{-1} cm^{-1}) of TNB in this buffer system at 412nm is 14,150.

Molar absorptivity, E, is defined as follows: $E = \frac{A}{bc}$

where A = absorbance, b = path length in centimeters, c = concentration in moles/liter (=M)

Solving for concentration gave the following formula: $c = A/bE$

This value represented the concentration of the solution in the spectrophotometric cuvette.

c) To calculate the concentration of the unknown sample, it was necessary to account for dilution factors as follows:

The total volume of the solution being measured was: 1.05 mL

970 μL of Reaction Buffer + 30μL of Unknown Sample + 50μL of Ellman's Reagent Solution

If the concentration of the assay solution was B Moles/L, then 1.05 mL of that solution contained

$$1.05 \text{ mL} \times \frac{1\ L}{1000\ mL} \times B \text{ Moles/L} = C \text{ moles}$$

These C moles of sulfhydryl in the assay solution were contributed by the original 30μL sample. The concentration of free sulfhydryl in the original unknown sample was

$$\frac{C\ Moles}{0.03\ mL} \times \frac{1000\ mL}{1\ L} = D \text{ Moles}$$

Appendix IV

IODINE ASSAY

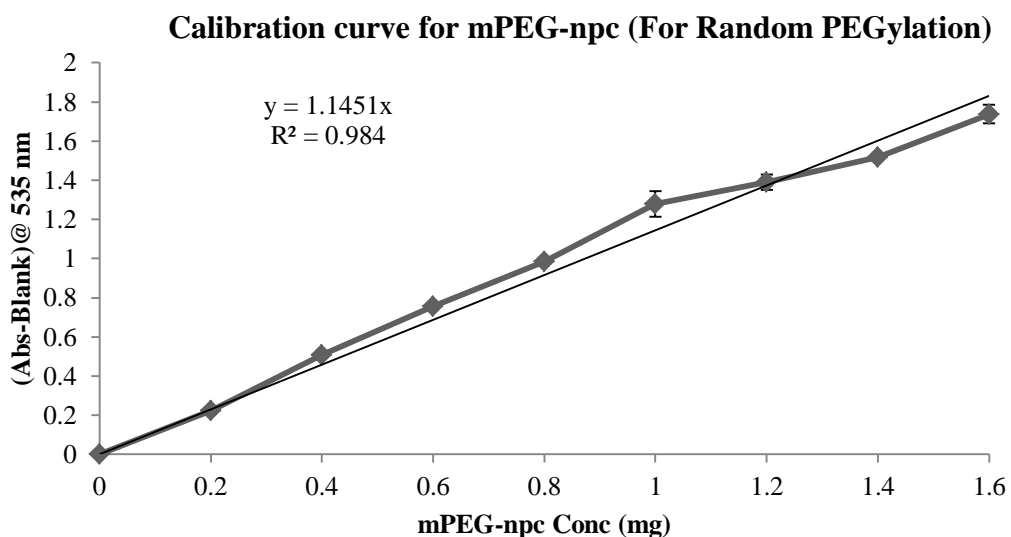A rapid, qualitative analysis of the total PEG content in a sample was performed as follows:

1) To a clean tube, 975 μL of distilled water, 250 μL of barium chloride solution and 250 μL of iodine solution were added.

2) To the above mixture, 25 µL of PEG-protein conjugate solution was added.

3) The test was positive if the final mixture formed a dark precipitate or showed increased absorbance at 535 nm.

Blank	Sample
925 µL of milli-Q water	900 µL of milli-Q water
250 µL of BaCl$_2$ solution	250 µL of BaCl$_2$ solution
250 µL of Iodine solution	250 µL of Iodine solution
	25 µL of PEG solution

Calibration curve for Iodine assay:

1) A blank, PEG standard solutions (0.2-0.5 mg/mL PEG) and unknown sample solutions were prepared as described above.

2) The solutions were incubated for 15 minutes and then the absorbance values were read at 535 nm versus the known concentration values of PEG standards.

3) The amount of PEG present in the unknown sample solution was determined from comparison of the measured absorbance values against the standard curve generated.

Calibration curve for mPEG-npc (For Random PEGylation)

y = 1.1451x
R^2 = 0.984

(Abs-Blank) @ 535 nm

mPEG-npc Conc (mg)

Appendix V

SDS-PAGE PROTOCOL

Acrylamide gels are composed of a mixture of two chemicals: acrylamide and bis-acrylamide. These components will form a cross linked network in the presence of Ammonium persulfate (APS), which is a polymerizing agent. A catalyst called TEMED increases the rate at which the acrylamide solution will polymerize to form a polyacrylamide gel matrix. SDS is an anionic detergent which denatures proteins by "wrapping around" the polypeptide backbone - and SDS binds to proteins fairly specifically in a mass ratio of **1.4**:**1**. In so doing, SDS confers a negative charge with equal charge or charge densities per unit amino acid length. In most proteins, the binding of SDS to the polypeptide chain imparts an even distribution of charge per unit mass, thereby resulting in a fractionation by approximate size during electrophoresis.

A molecular weight size marker (a set of different proteins of different molecular weights) was used to identify the approximate size of the PEGylated complex run on a gel, using the principle that molecular weight is inversely proportional to migration rate through a gel matrix. Commercially obtained protein markers were in the molecular weight range of 14 kDa-110 kDa. The marker consisted of β-galactosidase (110 kDa), Phosphorylase b (97 kDa), Bovine Serum Albumin (66 kDa), Amylase (51 kDa), Carbonic anhydrase (30 kDa), Recombinant Protein (25 kDa), Lactoglobulin (18 kDa), Lysozyme (14 kDa).

Procedure in detail

1. All the vials of marker protein provided by the manufacturer were spun before use (short spin 10,000 rpm for 1 min).

2. The gel casting assembly was prepared as per the requirements. It was ensured that the assembly is leakproof by filling water between the plates. Approximately, 6mL of separating and 4 mL of stacking gel were used. The composition of the gels of 12%, 7% and 5% is as given in the table below.

Components (For 8 mL of Gel)	5 %	7 %	10 %	12 %
Water (mL)	4.5	4.0	3.2	2.6
30 % Acrylamide-Bisacrylamide Gel mix	1.33	1.87	2.67	3.2
1.5 M Tris Buffer (pH 8.8) (mL)			2.0	2.0
0.5 M Tris Buffer (pH 6.8) (mL)	2.0	2.0		
10 % Sodium Dodecyl Sulphate (μL)	80	80	80	80
10 % Ammonium Per Sulphate (μL)	80	80	80	80
TEMED (μL)	8	8	8	8

4. The plate assembly was carefully fixed to the PAGE apparatus.

5. After the gel was set (approximately 45 minutes), the top of separating gel was washed with distilled water and drained off completely.

6. Stacking gel mix was poured on top of the polymerized separating gel. The gel assembly was allowed to solidify.

7. The teflon comb was inserted immediately in the gel solution carefully without trapping the air bubbles and the stacking gel was allowed to polymerize for about 45 minutes.

8. Meanwhile, the samples of unmodified uricase and the PEGylated uricase were prepared. For the samples to be made ready to be loaded onto the gel, the samples were mixed in the sample loading buffer [1 M Tris-HCl pH 6.8 (2.5 mL); Glycerol, 100% (4 mL); β-mercaptoethanol (2 mL); Bromophenol Blue, 0.1% (0.8 mL); Distilled water (0.5 mL) and SDS (1 gm)]. The samples were boiled for 5 minutes and suddenly cooled on an ice bath for 5 minutes. The samples incubated for 10 minutes at room temperature and were ready to be loaded onto the gel.

9. After the stacking gel was solidified and set, the comb was carefully removed. The wells were washed immediately with distilled water to remove the non- polymerized acrylamide.

10. The gel running apparatus was filled with 1X gel running buffer (755 mg Tris base, 3.6 g glycine and 250 mg SDS added in 250 mL of distilled water and stored at 4^0C).

11. 20 μL of protein marker, 20 μL of unmodified uricase sample, and 20 μl of bioconjugated uricase complexes were pipette into into individual vials and labelled

appropriately. These samples were loaded into different wells formed in the gel matrix accordingly.

12. The cords were connected to the power supply according to the convention red for anode and   black for cathode.

13. The current was set to 20mA and voltage to 70 V. The electrophoresis procedure was carried out for 4-5 hours, depending on the time taken by the dye front to reach the end of the separating gel. After the electrophoresis process was over, the cords were disconnected and the plates were removed gently from the PAGE apparatus.

15. The two plates were held under running water and a spatula was used to open up the glass plates and the gel was recovered and immediately transferred to a trough of distilled water, where the gel was washed 2-3 times in distilled water.

16. The water was discarded and replaced with 25 mL of staining solution (0.25 gm coomassie brilliant blue R-250 dissolved in a solution consisting of 400 mL methanol, 70 mL acetic acid and 530 mL of distilled water) and stained overnight. The gel was destained using a destaining solution (consisting of 400 mL methanol, 70 mL acetic acid and 530 mL of distilled water) and later washed with distilled water. The gel was washed repeatedly in distilled water, till the bands were clearly visible and the excess staining dye disappeared.

17. Relative mobility (Rf) is the ratio of distance migrated by the protein molecule to the distance migrated by the tracking dye.

Standard Curve for Estimation of Mol Wt by SDS-PAGE
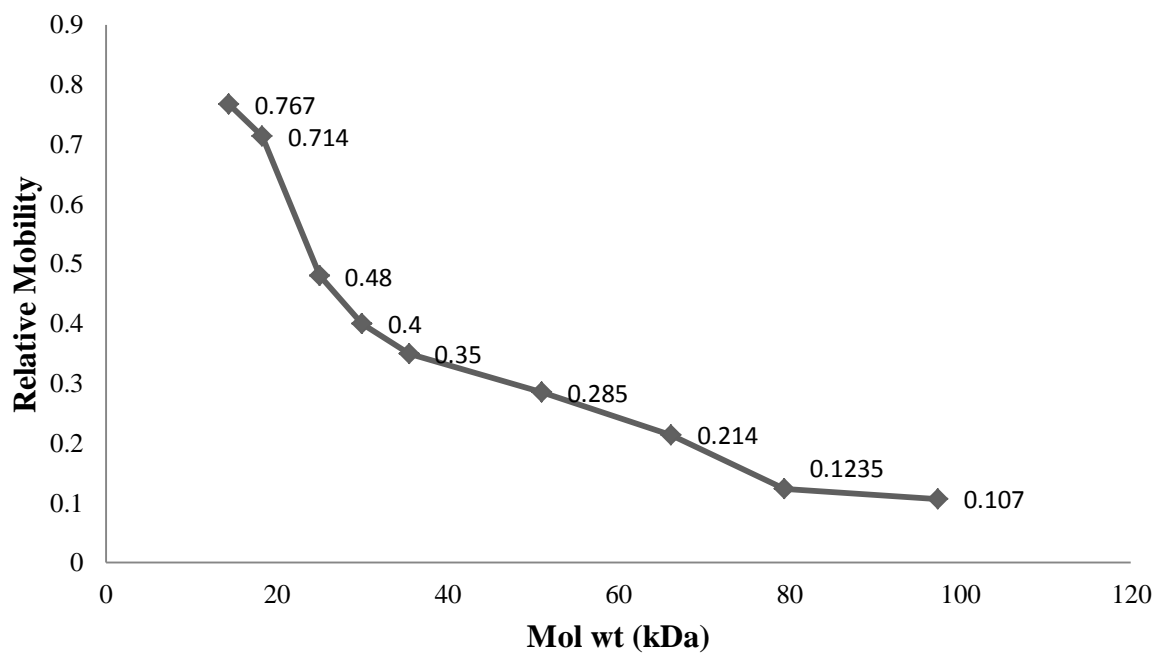
Sl no	Sample	Retention time at 214	AUC Native Uricase at 214 nm	Retention time at 214	AUC mono PEGylated Uricase at 214 nm	Retention time at 214 nm	AUC di PEGylated Uricase at 214 nm
1	Native Uricase	1.332	2455186				
2	Rxn 1	a) 1.336	a) 2470629	a) 1.479	a) 1287271	a) 1.615	a) 4379950
3	Rxn 2	a) 1.324 b) 1.265	a) 3496202 b) 265458	a) 1.478 b) 1.479	a) 2348292 b) 469924	a) 1.607 b) 1.598	a) 7013964 b) 176890
4	Rxn 3	a) 1.330 b) 1.318	a)1319124 b) 2448998				
5	Rxn 4	a) 1.322 b) 1.321	a) 2854380 b) 2951146				
6	Rxn 5	a) 1.307 b) 1.305	a) 1642311 b) 1675787	a) 1.424 b) 1.415	a) 999248 b) 738895		
7	Rxn 6	a) 1.304 b) 1.320	a) 1438559	a) 1.425 b) 1.434	a) 2150271 b) 2547488		

			b) 1472043				
8	Rxn 7	a) 1.317 b) 1.316	a) 4220902 b) 4180811				
9	Rxn 8	a) 1.316 b) 1.314	a) 3667406 b) 3597742	a) 1.400 b) 1.400	a) 162625 b) 75378		
10	Rxn 9	a) 1.305 b) 1.313	a) 1739805 b) 1686364	a) 1.459 b) 1.459	a) 1722890 b) 1865867	a) 1.593 b) 1.600	a) 6807822 b) 8448083
11	Rxn 10	a) 1.317 b) 1.317	a) 3228851 b) 3199700				
12	Rxn 11	a) 1.299 b) 1.294	a) 5089406 b) 5021945	a) 1.604 b) 1.609	a) 1006615 b) 1001588		
13	Rxn 12	a) 1.302 b) 1.299	a) 6608540 b)				

			5906879				
14	Rxn 13	a) 1.304 b) 1.303	a) 4321076 b) 4414103	a) 1.453 b) 1.453	a) 1724039 b) 1857966		
15	Rxn 14	a) 1.293 b) 1.291	a) 4656574 b) 4314049	a) 1.451 b) 1.452	a) 1493899 b) 2235739		
16	Rxn 15	a) 1.289 b) 1.288	a) 4042843 b) 4165588	a) 1.453 b) 1.453	a) 2459573 b) 2226403		
17	Rxn 16	a) 1.285 b) 1.285	a) 4333947 b) 4331992	a) 1.459 b) 1.458	a) 3338772 b) 3628948		
18	Rxn 17	a) 1.278 b) 1.286	a) 3846996 b) 4109405	a) 1.452 b) 1.456	a) 2009729 b) 1748863		

Retention time data at 214 nm for all the trial of statistical optimization

Sl no	Sample	Retention time at	AUC Native	Retention time at	AUC mono PEGylated	Retention time at	AUC di PEGylated

		280 nm	Uricase at 280 nm	280 nm	Uricase at 280nm	280 nm	Uricase at 280 nm
1	Native Uricase	1.267	295540				
2	Rxn 1	a) 1.273 b) 1.270	a) 251940 b) 254089	a) 1.487 b) 1.484	a) 138720 b) 174045	a) 1.605 b) 1.602	a) 64621 b) 61853
3	Rxn 2	a) 1.269 b)	a) 261272	a) 1.485	a) 423362	a) 1.604	a) 177288
4	Rxn 3	a) 1.268 b) 1.256	a) 219228 b) 198769	a) 1.470 b) 1.454	a) 24493 b) 27421		
5	Rxn 4	a) 1.264 b) 1.260	a) 170694 b) 203514	a) 1.459 b) 1.455	a) 80496 b) 80593		
6	Rxn 5	a) 1.263 b) 1.262	a) 58393 b) 62514	a) 1.438 b) 1.432	a) 167355 b) 161515		
7	Rxn 6	a) 1.260 b) 1.275	a) 56586 b) 54976	a) 1.436 b) 1.447	a) 327144 b) 304657		
8	Rxn 7	a) 1.268 b) 1.267	a) 305230 b) 269026	a) 1.417 b) 1.450	a) 53363 b) 56842		
9	Rxn 8	a) 1.261	a) 280792	a) 1.421	a) 163540		

		b) 1.261	b) 272305	b) 1.431	b) 155037		
10	Rxn 9	a) 1.259	a) 46134	a) 1.458	a) 582874	a) 1.589	a) 85668
		b) 1.268	b) 36822	b) 1.458	b) 523338	b) 1.596	b) 166004
11	Rxn 10	a) 1.200	a) 1984	a) 1.288	a) 11989	a) 1.398	a) 35165
		b) 1.274	b) 25807	b) 1.385	b) 52768		
12	Rxn 11	a) 1.258	a) 295247	a) 1.396	a) 78962	a) 1.603	a) 123928
		b) 1.258	b) 254786	b) 1.387	b) 74076	b) 1.607	b) 210398
13	Rxn 12	a) 1.268	a) 229430				
		b) 1.267	b) 220168				
14	Rxn 13	a) 1.263	a) 175319	a) 1.440	a) 167994		
		b) 1.263	b) 144978	b) 1.441	b) 179799		
15	Rxn 14	a) 1.261	a) 224110	a) 1.443	a) 173395		
		b) 1.260	b) 234841	b) 1.444	b) 156142		
16	Rxn 15	a) 1.260	a) 204504	a) 1.446	a) 206662		
		b) 1.261	b) 201471	b) 1.447	b) 178456		
17	Rxn 16	a) 1.258	a) 274211	a) 1.455	a) 256500		
		b) 1.259	b)	b) 1.453	b) 238252		

			271361				
18	Rxn 17	a) 1.260 b) 1.267	a) 224409 b) 72903	a) 1.444 b) 1.446	a) 113305 b) 111480		

Retention time data at 280 nm for all the trial of statistical optimization

Appendix VI

## HPLC chromatograms for optimization experiments

Native uricase

SAMPLE INFORMATION

Sample Name:	native_uricase
Sample Type:	Unknown
Vial:	1
Injection #:	2
Injection Volume:	10.00 ul
Run Time:	10.0 Minutes

Acquired By:	System
Sample Set Name:	pooja_nitk
Acq. Method Set:	NITK_pooja_method
Processing Method	native_uricase_ch2
Channel Name:	2487Channel 2
Proc. Chnl. Descr.:	

Date Acquired: 7/17/2015 12:10:08 PM IST
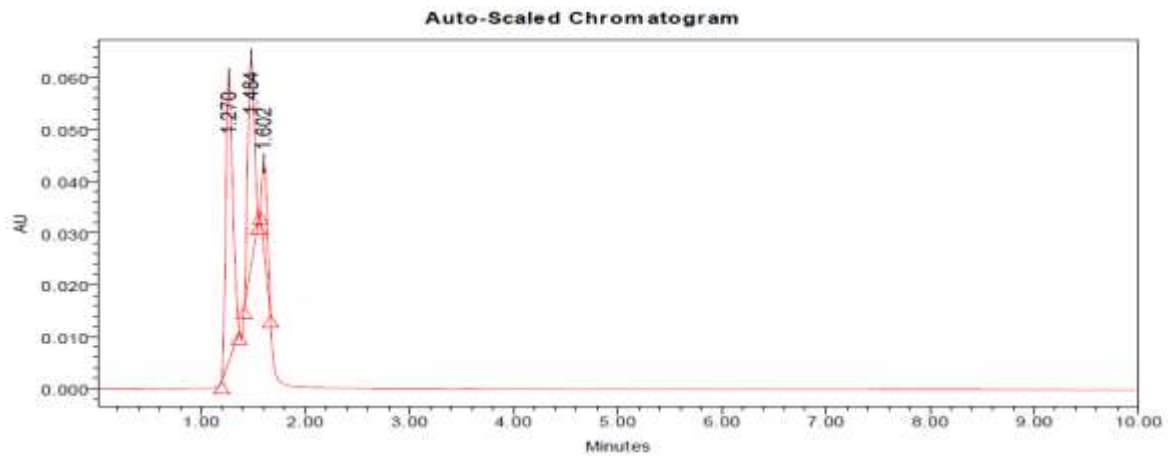Date Processed: 7/17/2015 3:42:03 PM IST

Auto-Scaled Chromatogram
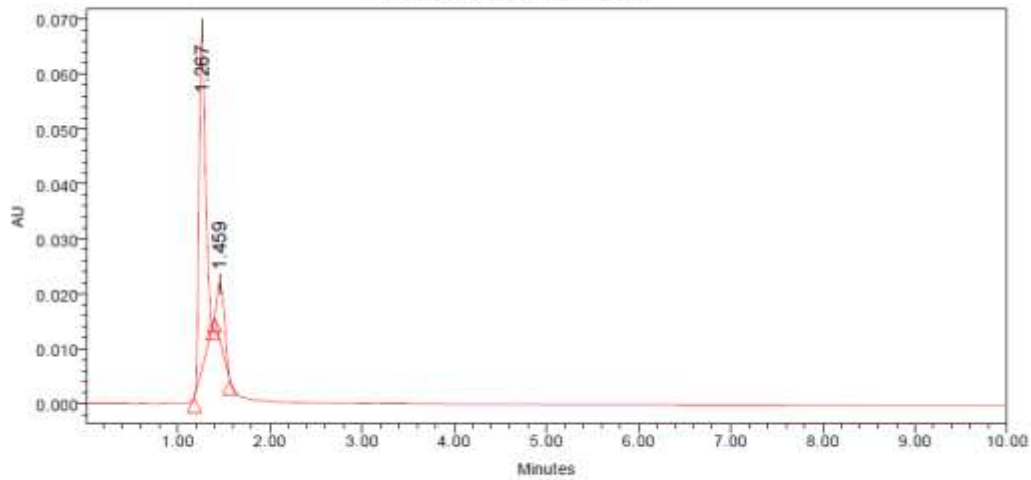
Peak Results

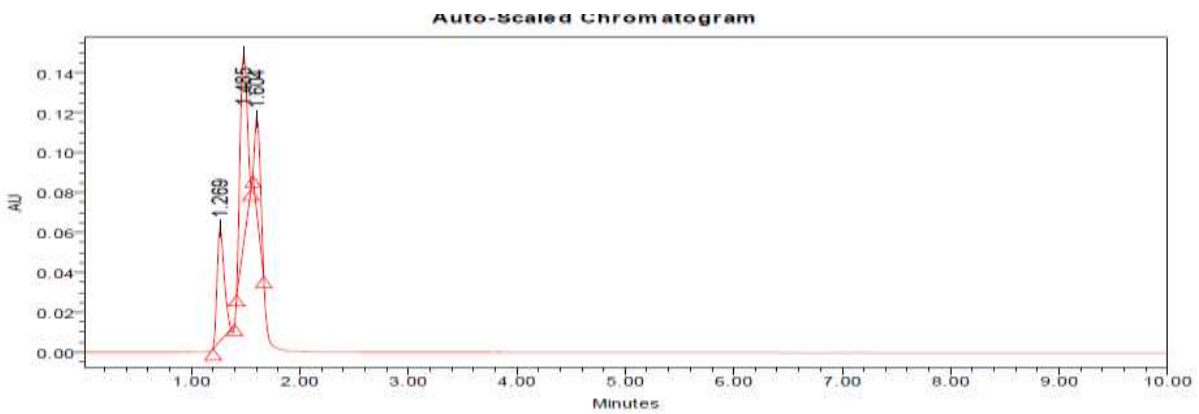|	Name	RT	Area	Height	Amount	Units
1		1.267	295540	61105		

PEGylation Reaction Mixtures

1) Reaction 1

Auto-Scaled Chromatogram

Peak Results

	Name	RT	Area	Height	Amount	Units
1		1.270	254089	55245		
2		1.484	174045	39686		
3		1.602	61853	16204		

2) Reaction 2

Auto-Scaled Chromatogram

Peak Results

	Name	RT	Area	Height	Amount	Units
1		1.269	261272	56371		
2		1.485	423362	94018		
3		1.604	177288	47422		

3) Reaction 3

Auto-Scaled Chromatogram
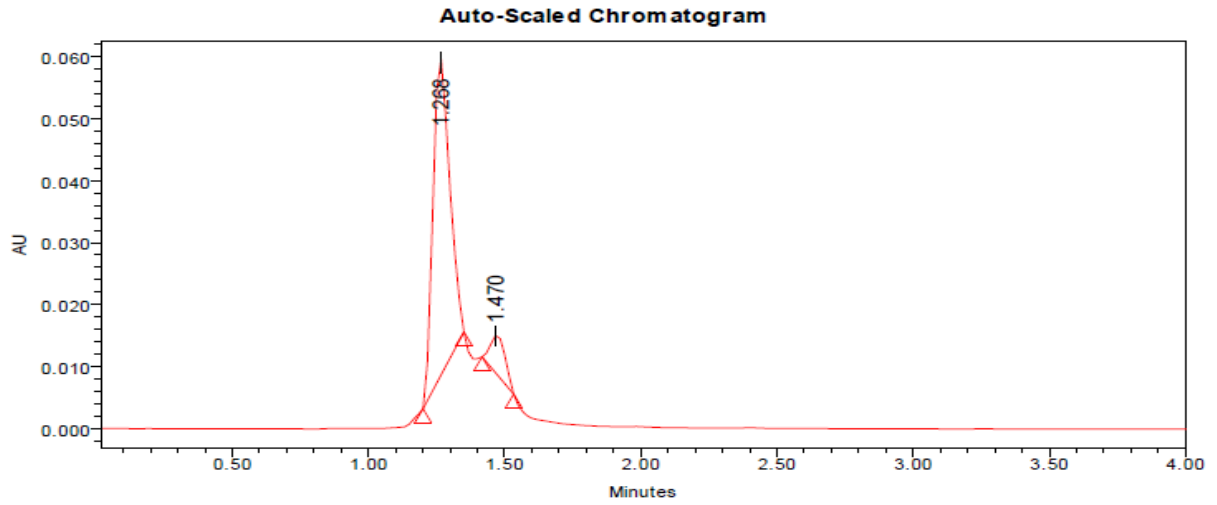
Peak Results

	Name	RT	Area	Height	Amount	Units
1		1.268	219228	49964		
2		1.470	24493	6103		

4) Reaction 4

Auto-Scaled Chromatogram
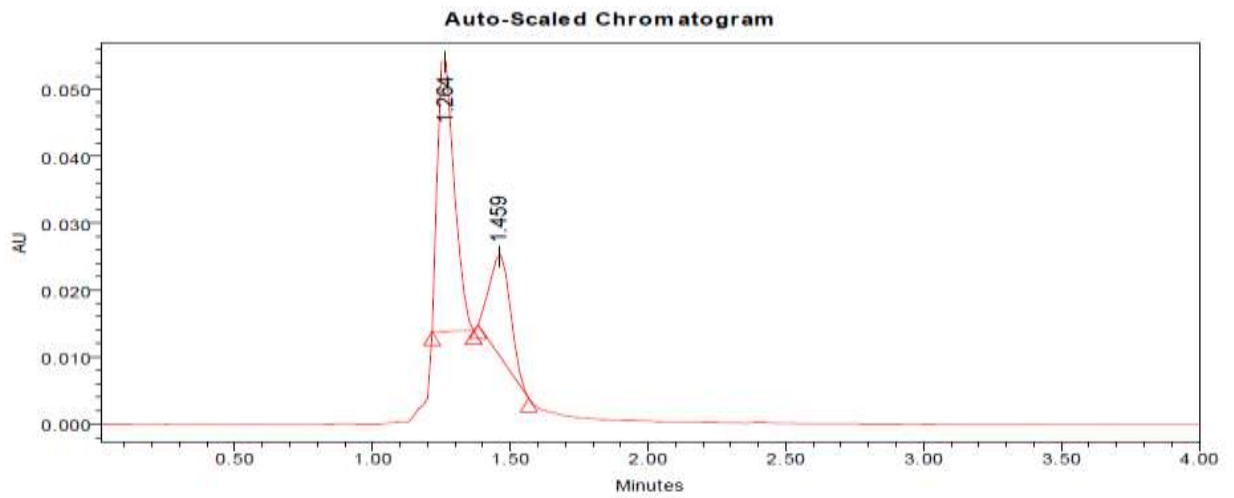
Peak Results

	Name	RT	Area	Height	Amount	Units
1		1.264	170694	40200		
2		1.459	80496	15070		

5) Reaction 5

Auto-Scaled Chromatogram
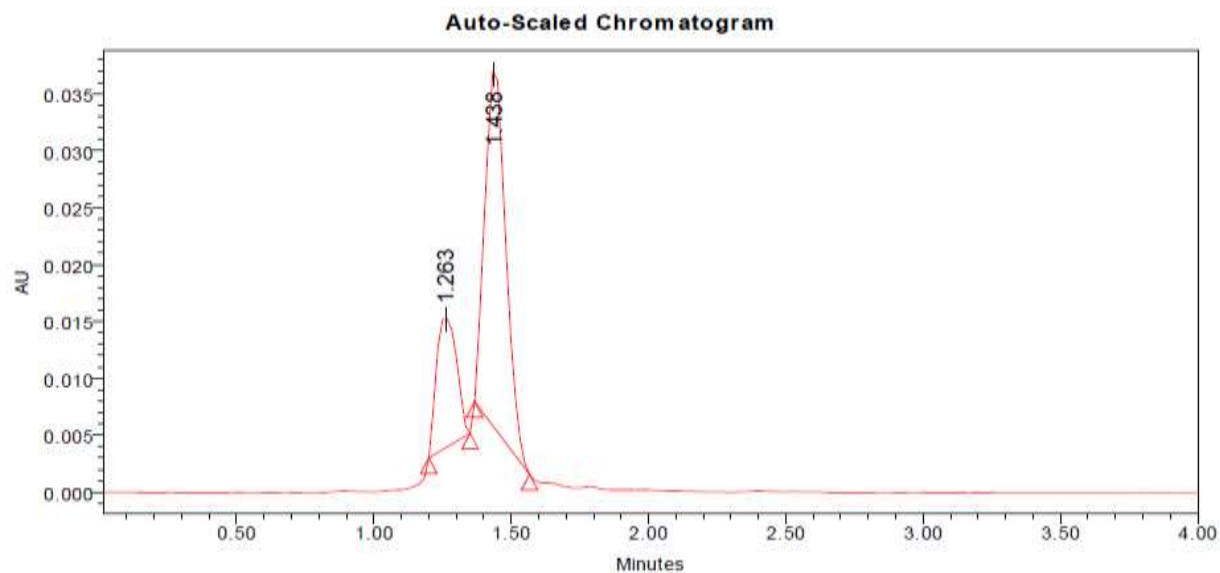
Peak Results

	Name	RT	Area	Height	Amount	Units
1		1.263	58393	11787		
2		1.438	167355	31180		

6) Reaction 6

Auto-Scaled Chromatogram
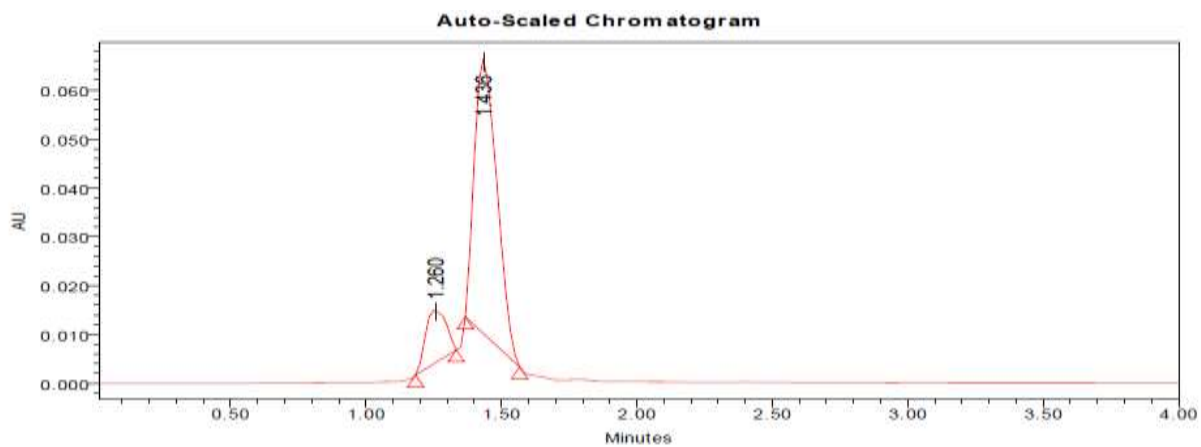
Peak Results

	Name	RT	Area	Height	Amount	Units
1		1.260	56586	10938		
2		1.436	327144	56071		

7) Reaction 7

Auto-Scaled Chromatogram
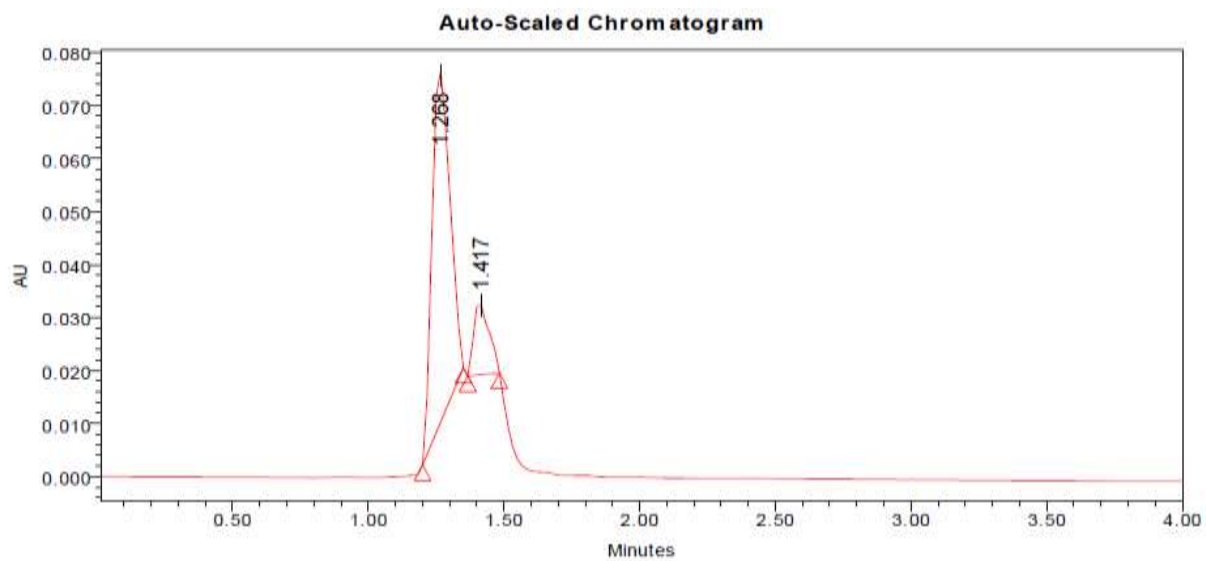
Peak Results

	Name	RT	Area	Height	Amount	Units
1		1.268	305230	65683		
2		1.417	53363	12958		

8) Reaction 8

Auto-Scaled Chromatogram
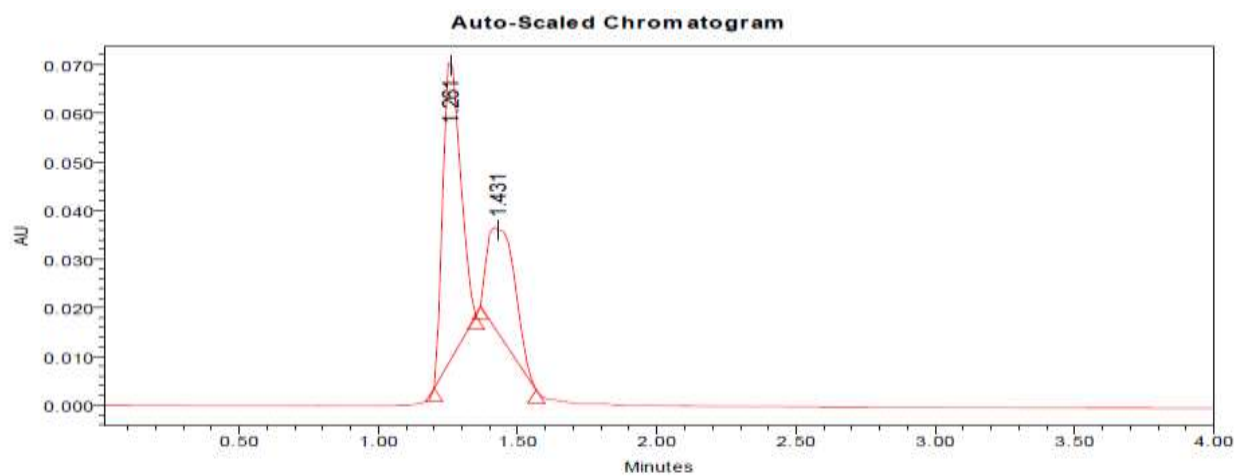
Peak Results

	Name	RT	Area	Height	Amount	Units
1		1.261	272305	61678		
2		1.431	155037	21947		

9) Reaction 9

Auto-Scaled Chromatogram
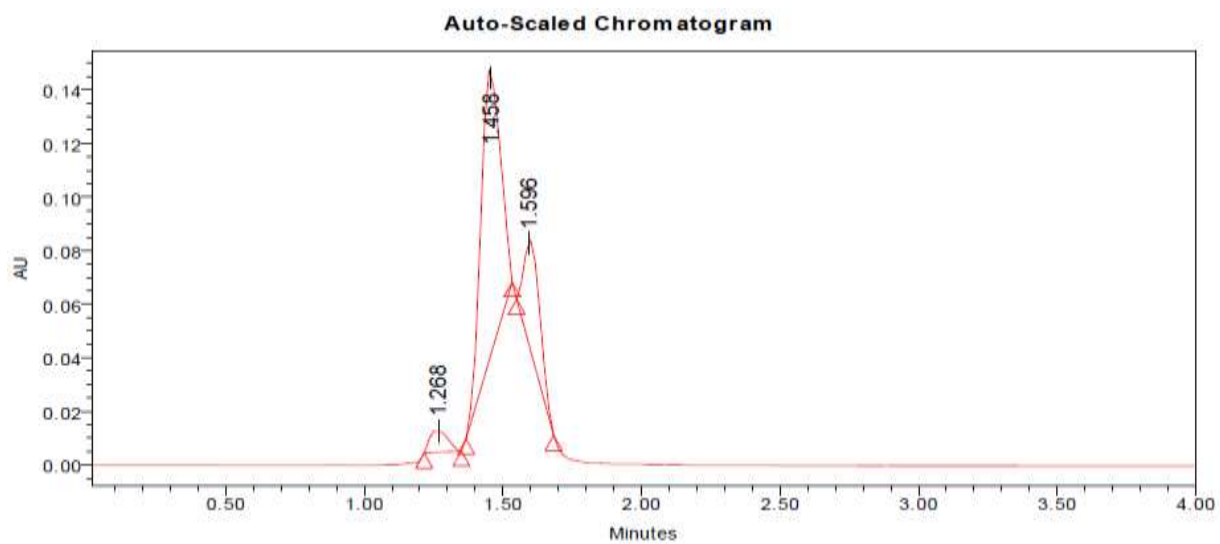
Peak Results

	Name	RT	Area	Height	Amount	Units
1		1.268	36822	8231		
2		1.458	523338	106457		
3		1.596	166007	39068		

10) Reaction 10

Auto-Scaled Chromatogram
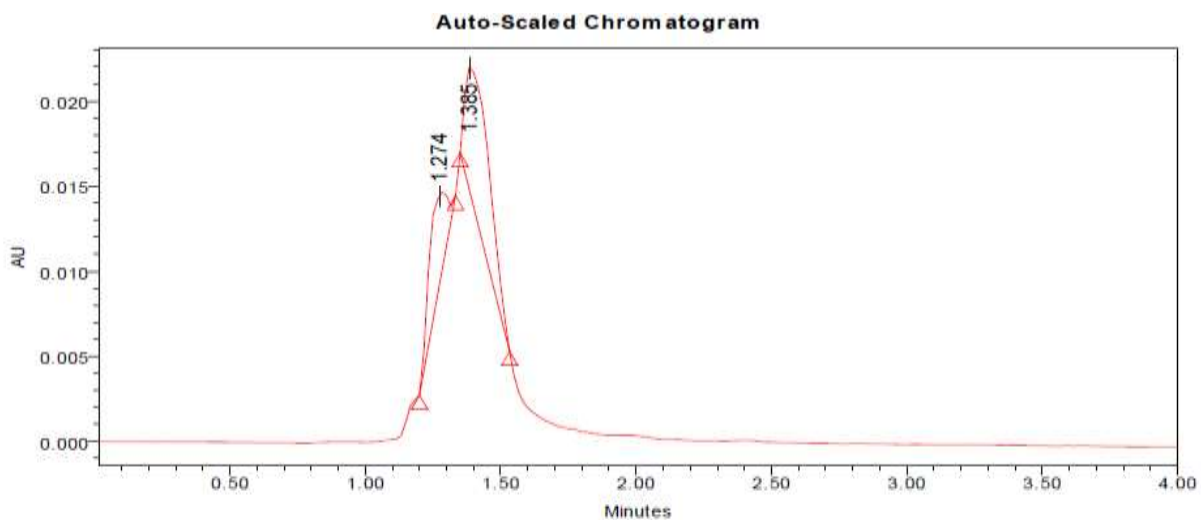
Peak Results

	Name	RT	Area	Height	Amount	Units
1		1.274	25807	5512		
2		1.385	52768	7261		

11) Reaction 11

Auto-Scaled Chromatogram
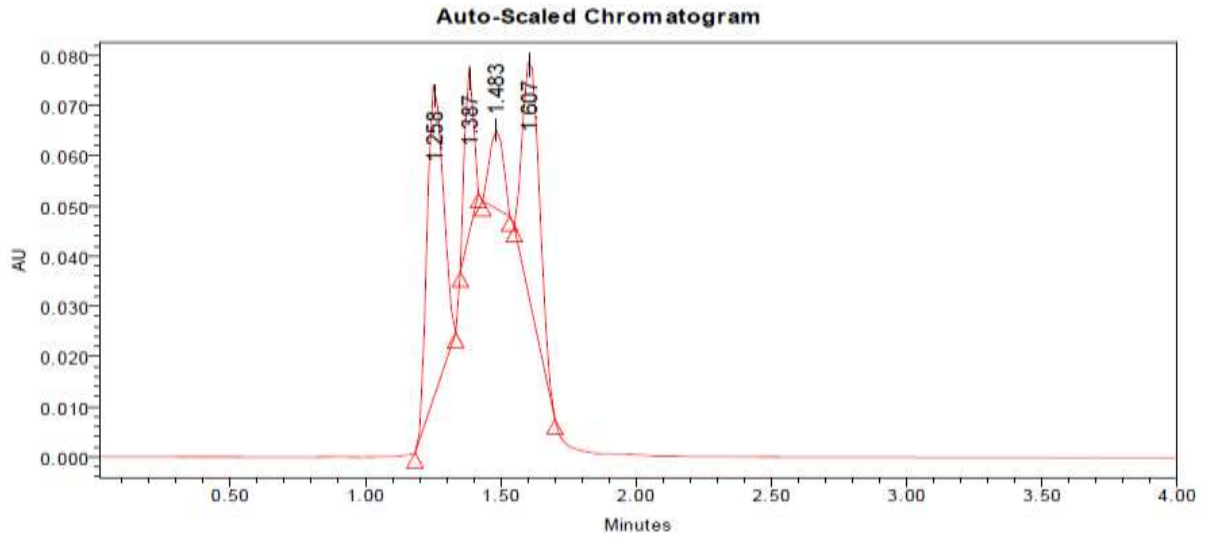
Peak Results

	Name	RT	Area	Height	Amount	Units
1		1.258	254786	61419		
2		1.387	74076	29645		
3		1.483	55533	15572		
4		1.607	210398	47366		

12) Reaction 12

Auto-Scaled Chromatogram
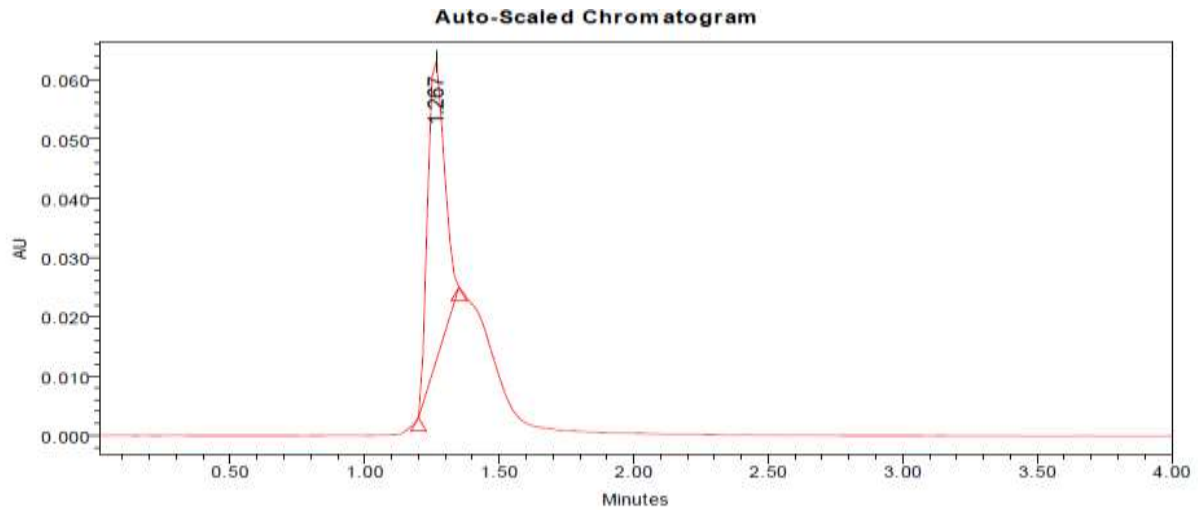
Peak Results

	Name	RT	Area	Height	Amount	Units
1		1.267	220168	50161		

228

13) Reaction 13

Auto-Scaled Chromatogram
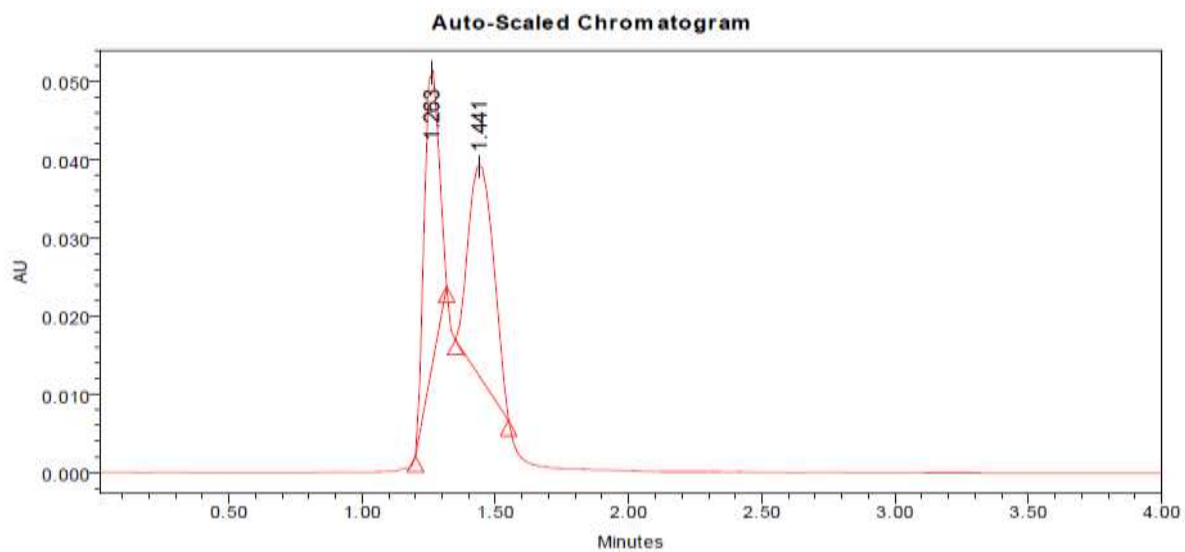
Peak Results

	Name	RT	Area	Height	Amount	Units
1		1.263	144978	37898		
2		1.441	179799	27156		

14) Reaction 14

Auto-Scaled Chromatogram
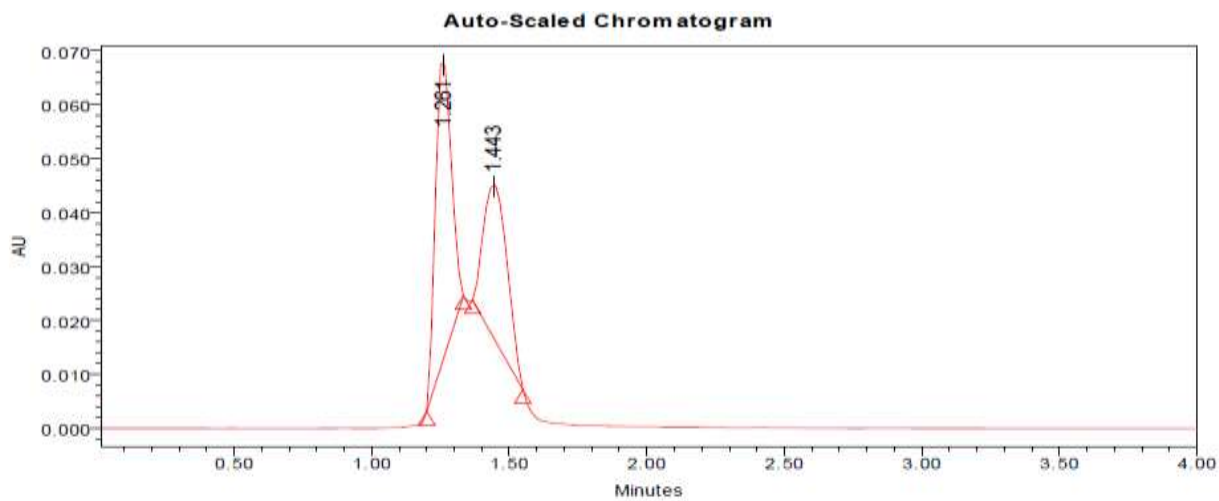
Peak Results

	Name	RT	Area	Height	Amount	Units
1		1.261	224110	55665		
2		1.443	173395	28535		

229

15) Reaction 15

Auto-Scaled Chromatogram
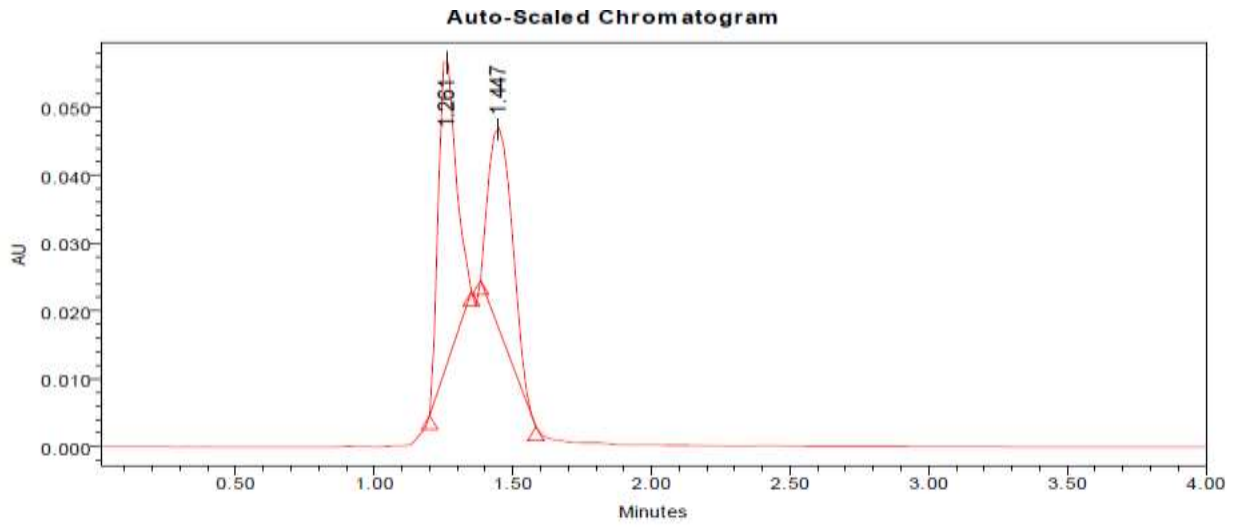
Peak Results

	Name	RT	Area	Height	Amount	Units
1		1.261	201471	45569		
2		1.447	178056	29386		

16) Reaction 16

Auto-Scaled Chromatogram
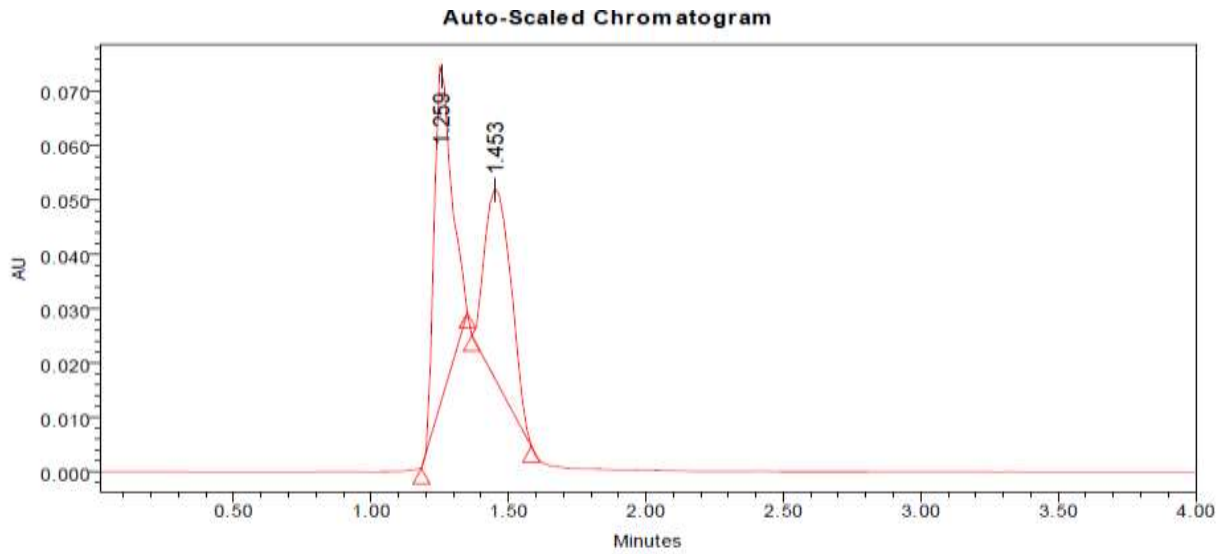
Peak Results

	Name	RT	Area	Height	Amount	Units
1		1.259	271361	61112		
2		1.453	238252	34995		

17) Reaction 17

Auto-Scaled Chromatogram
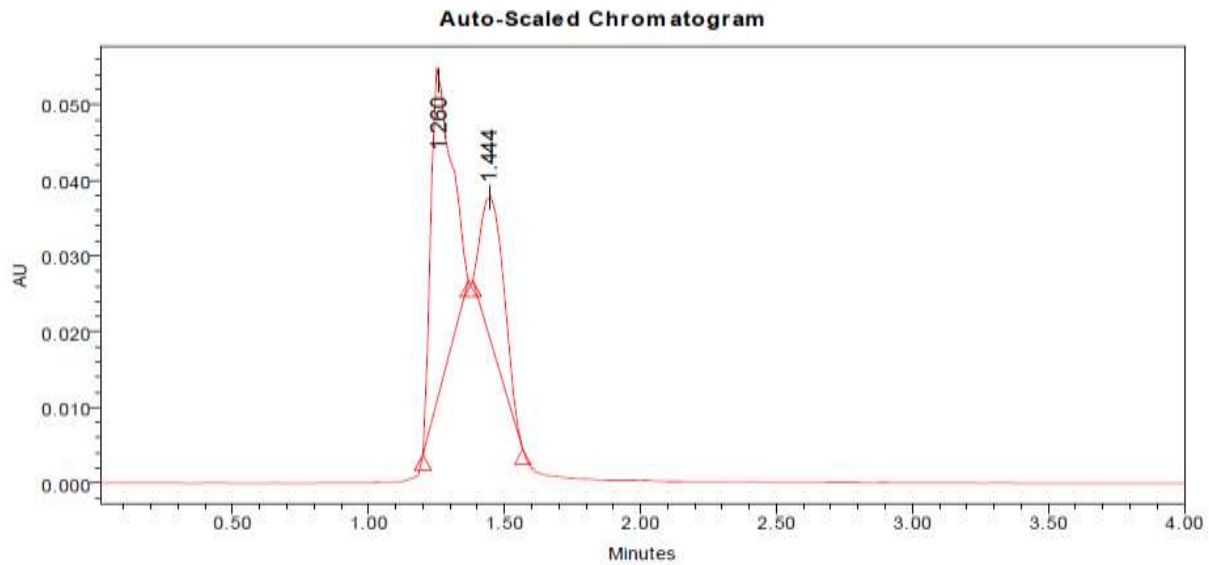
Peak Results

	Name	RT	Area	Height	Amount	Units
1		1.260	224409	43194		
2		1.444	113305	18612		

Calibration of protein standards for conjugate purification using SE-HPLC
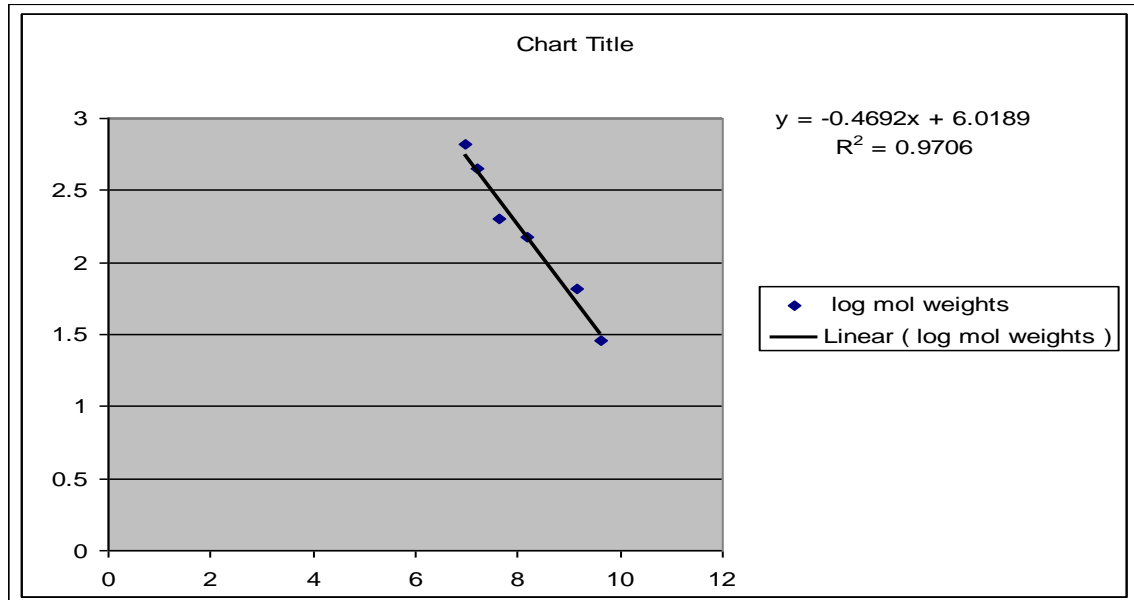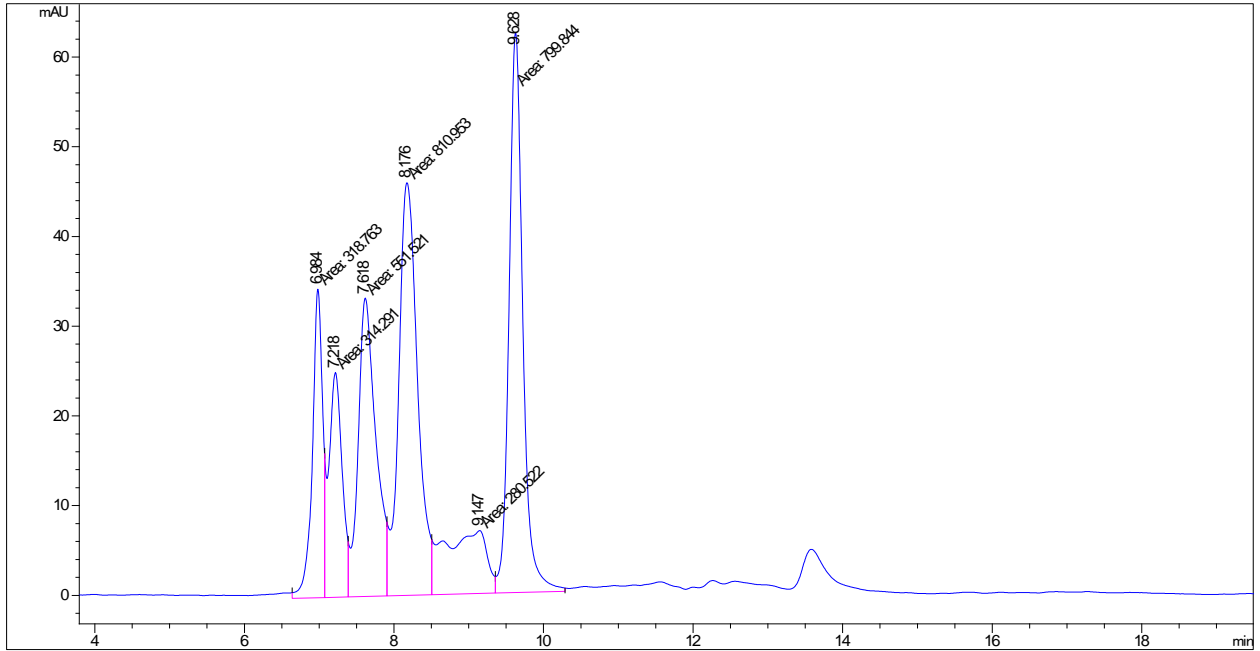
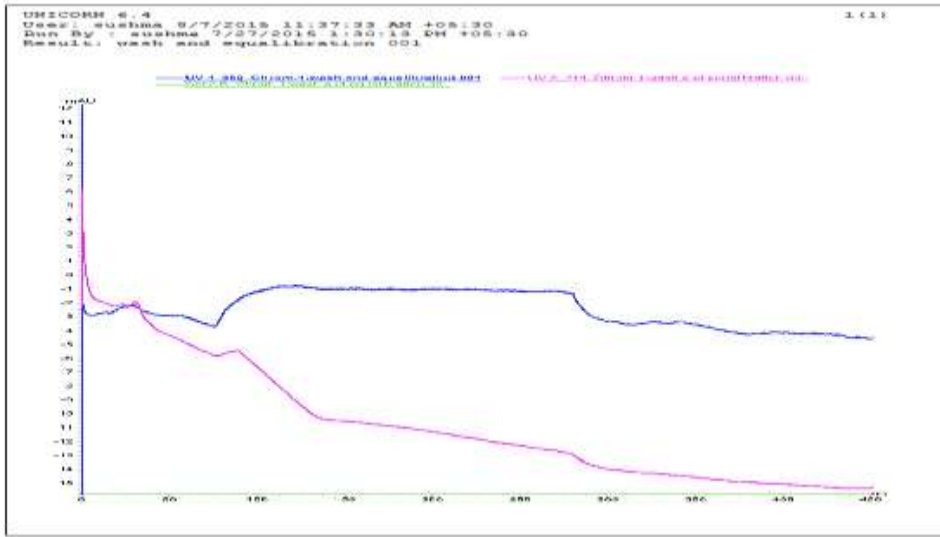**Table: Retention time data of standard proteins used for SE-HPLC**

Peak number	Time	Area	Height	Width	Symmetry
1	6.984	318.8	34.5	0.154	1.154
2	7.218	314.3	25.1	0.2089	0.912
3	7.618	551.5	33.3	0.276	0.672
4	8.176	811	46	0.2936	0.791
5	9.147	280.5	7	0.667	4.292
6	9.628	799.8	62.5	0.2132	0.889

Time	mol weights	log mol weights
6.984	669	2.825426
7.218	443	2.646404
7.618	200	2.30103
8.176	150	2.176091
9.147	66	1.819544
9.628	29	1.462398

Calibration of protein standards for conjugate purification using SE-FPLC

Blank

Standard run
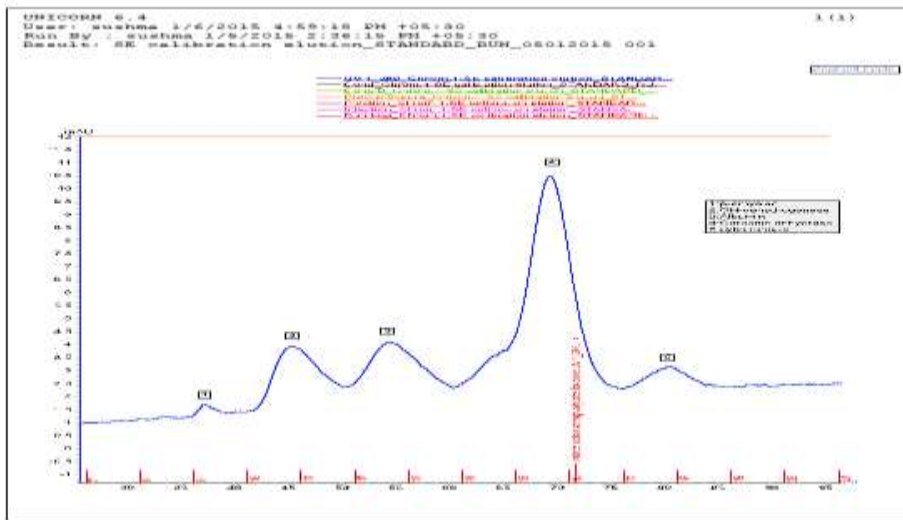
Table: Retention volume data of standard proteins

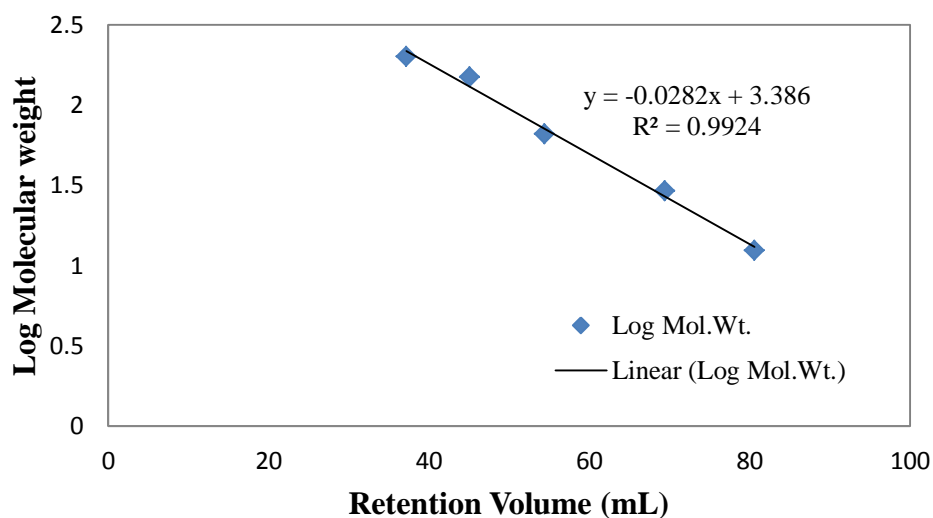|	Standards	Molecular Weight	Retention time	Log Mol.Wt.
1	β-amylase	200	37.17	2.301029996
2	OH dehydrogenase	150	45.06	2.176091259
3	Albumin	66	54.44	1.819543936
4	Carbonic anhydrase	29	69.43	1.462397998
5	Cytochrome c	12.4	80.58	1.093421685

Figure: Retention volume data of standard proteins

LIST OF PAPERS BASED ON THIS RESEARCH WORK

International Journal

Nanda, P., JagadeeshBabu, P. E. (2016). "Studies on the Site-specific PEGylation Induced Interferences Instigated in Uricase Quantification Using the Bradford Method." *International Journal of Peptide Research and Therapeutics*, *22*(3), 399-406. (Springer Link, IF: 0.905)

Nanda, P., JagadeeshBabu, P. E., & Raju, J. R. (2016). "Production and Optimization of Site-Specific monoPEGylated Uricase Conjugates Using mPEG-Maleimide Through RP–HPLC Methodology." *Journal of Pharmaceutical Innovation*, *11*(4), 279-288. (Springer, IF 1.603)

Nanda, P., JagadeeshBabu, P. E., Gupta, P., & Prasad, A. G. (2016). "Development of a spectrophotometric biphasic assay for the estimation of mPEG-maleimide in thiol PEGylation reaction mixtures." *Chemical Engineering Communications*, *203*(11), 1464-1472. (Taylor and Francis, IF: 1.433)

Conferences

Nanda, P. and JagadeeshBabu, P.E. (2015). "Synthesis of site-specifically PEGylated Uricase conjugates with improved pharmaceutical properties for the treatment of hyperuricemia." 4th International Engineering Symposium - IES 2015, Kumamoto University, Japan, March 7, 2015.

Nanda, P. and JagadeeshBabu, P.E. (2016). "Solid Phase PEGylation of Uricase". *International Conference on Recent Trends in Engineering and Material Sciences,* March 17-19, 2016, *Materials Today Proceedings* (Elsevier), JNU, Jaipur, India.

Papers communicated

Nanda, P., JagadeeshBabu, P.E., Shenoy, P, J., Sayeli, V.K. and Pai, S. "Characterization and immunological evaluation of site-specific PEGylated uricase from *Bacillus fastidious*." *International Journal of Peptide Research and Therapeutics* (under review)

Nanda, P., JagadeeshBabu, P.E., Shenoy, P, G., Raju, J.R., Nayak, S., Abraham, K. V., Sayeli, V.K., Pai, S. "N-Terminal PEGylated Urate oxidase". *The Protein Journal.* (Under Review)

Manuscripts under preparation

Nanda, P., and JagadeeshBabu, P.E. "Solid Phase PEGylation: An improved PEGylation strategy".

Nanda, P., JagadeeshBabu, P.E. and Prasad, A.G. "Evolution of PEGylated uricase for the treatment of hyperuricemia and gout: the past, present, and future."

BIO-DATA

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Declaration:

I hereby declare that the above-written particulars are true to the best of my knowledge.

Place: Surathkal, India   (   )

Date: **POOJA NANDA**