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The Development of Biological Therapeutics

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The Development of Biological Therapeutics

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Abbreviations

BCX - Bacillus circulans xylanase

EDTA - Ethylenediaminetetraacetic Acid

FPLC – Fast Performance Liquid Chromatography

IPTG - Isopropyl-Beta-D-Thiogalactoside

mAB – monoclonal antibody

mqH_2O - water which has been distilled and fed through an ion exchange cartridge

NaH_2PO_4 - Sodium Phosphate

NaOAc – Sodium Acetate

PEG – Poly-ethylene glycol residue

sCT – Salmon Calcitonin

SDS PAGE - Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

TCEP - Tris(2-carboxyethyl)phosphine

Abstract

The development of biological therapeutics has advanced medicine dramatically in the 20th century. Protein-based drugs are now commonly used in treatment of disease. Technologies to improve the pharmacokinetic properties of these drugs are at the cutting edge of research within the pharmaceutical industry. I have evaluated a novel thiol-selective specific linker (PermaLink™, Glythera Ltd) for the attachment of chemical groups such as polyethylene glycol (PEG) to cysteine via a stable thio-ether bond.

Proteins are often PEGylated to improve their serum half-life, reduce their immunogenicity and prevent renal clearance by increasing their overall size. The linkers which attach these PEG molecules to a protein are an essential part of this modification as these affect where the molecule is attached and consequently whether the protein stays biologically active. In this study, I have compared PermaLink™-PEG with commercially available maleimide-PEG for the attachment of PEG groups to proteins.

Initially I established a protocol to reduce the test protein prior to reaction with PermaLink™-PEG or maleimide-PEG. Agarose resin-linked Tris(2-carboxyethyl) phosphine (TCEP) was used to reduce cysteines prior to the addition of thiol-reactive compounds. Using this reduction approach, I observed that PermaLink™-PEG demonstrated an increased apparent cystiene selectively compared to maleimide-PEG. PermaLink™-PEG attached the predicted number of PEG molecules based on the number of available cysteines while non-specific multi-pegylation was observed with maleimide-PEG.

Based on my results I propose that PermaLink™-PEG selectively targets cysteine thiol groups compared to maleimide-PEG. Overall I propose that PermaLink™ technology could be used to develop new therapeutic proteins with reduced non-specific PEGylation.

Contents

Chapter 1.0 Introduction

1.1 Biologics as therapeutics

Medicine has advanced from the early 20th century with the development of biological therapeutics; ranging from small biological molecules, gene therapy and proteins. Initially, the treatment of diabetes with insulin after its discovery in 1921 modelled the way for treatment of disease with physiological replacements.

1.1.1 DNA Based Therapeutics

Understanding the DNA molecule has allowed researchers to investigate the potential of DNA-based drugs in therapeutic medicine. Gene silencing, gene replacement, mRNA destruction and target protein antisense inhibition are all achievable using DNA therapeutics (Patil *et al.*, 2005). The major advantage is their exquisite specificity for their target which can be reached at very early stages of disease. The interpretation of the human genome and the relative ease at which genomes can be sequenced also gives rise to tailor-made medicine for the individual, making the fight against disease more likely to succeed (Shastry, 2006).

Plasmid based DNA drugs contain transgenes which are delivered to cells and utilise the cell's transcriptional machinery to produce a protein which is lacking or dysfunctional in a disease state (Uherek & Wels, 2000). DNA vaccines also use this mechanism of action, triggering an immune response through introduction of genes encoding antigens for specific pathogens (Johnston *et al.*, 2002). Similarly, gene suicide therapy is used to transfect malignant cells with chemosensitized genes which produce a pro-drug; on treatment with a chemotherapeutic agent only those targeted malignant cells are killed (Denny, 2003). The first FDA approved gene therapy protocol was in 1998 with a cure of adenosine deaminase deficiency (Anderson, 1998). Since then many more have been approved and in 2003, the Chinese FDA approved a gene therapy treatment for replacement of the p53 gene in head and neck squamous cell carcinoma (Patil *et al.*, 2005).

Oligonucleotides are short single stranded DNA segments which can selectively inhibit protein expression by blocking translation (Crooke, 1999). The design of the oligonucleotide is critical for clinical efficacy and modifications to its length, backbone and secondary conformation can affect this. The first oligonucleotide anti-sense based drug approved was in 1998, formivirsen sodium (Vitravene), to treat the cytomegalovirus retinitis in AIDS patients (Crooke, 1998). Alicaforsen, is an inhibitor of Intercellular Adhesion Molecule-1 (ICAM-1) for treatment of ulcerative colitis is being investigated in clinical trials (van Deventer *et al*., 2004).

Ribozymes are RNA molecules which target specific mRNAs for destruction (Lilley, 2005). The two major types of ribozymes which have been studies are the hairpin and hammerhead (Stull *et al.*, 1995), although new ribozymes structures have also been characterised (Lilley, 2005). They allow endogenous targeting of mRNA transcripts from ubiquitous RNases making them unstable *in vivo*. They can be used for knockout gene therapy in cancer cells where particular proteins have been implicated; epidermal growth factor receptor gene was targeted by ribozymes exhibiting successful inhibition of growth in tumour cells (Yamazaki *et al.*, 1998). DNAzymes are mechanistically the same as RNAzymes but are more biologically stable (Sheng *et al.*, 2007). Although with vast therapeutic potential, they have yet to be developed into therapeutic molecules.

Aptamers are small single-stranded or double-stranded nucleic acid segments that can directly interact with proteins (Famulok *et al.*, 2000). They are favoured over antibodies therapeutically because they are non-immunogenic, highly specific and more stable in the body (Jayesena, 1999). Endogenous expression of an RNA aptamer targeting the HIV-1 reverse transcriptase enzyme showed promising results in an *in vivo* setting, where virus replication was reduced by 95% (Chaloin *et al.*, 2002). In 2004, the US FDA approved an anti-VEGF aptamer for use in treatment of age-related macular degeneration (AMD), Pegatanib (Eugene *et al.*, 2006).

Small interfering RNAs (siRNAs) are short, double-stranded RNA molecules which are usually about 21-23 base pairs long; they work by down regulating or blocking the expression of a protein by binding to its target mRNA strand (Soutschek *et al.*, 2004).

The hardest challenge in therapeutic treatment with siRNAs is the delivery, as with other DNA-based drugs, they must pass through the plasma membrane to have effect. siRNAs possess a lesser challenge in this respect however, as they do not have to pass through the nuclear membrane like plasmid based therapeutics – their mechanism of action takes place in the cytosol (Patil D., 2006). siRNA therapies are being extensively investigated in treatment of cancer and attempts to control over active genes and cell signalling pathways. The silencing of polo-like kinase (PLK)-1 by siRNAs has proved successful in inducing apoptosis and impairment of mitosis machinery in human prostate cancer cells (Reagen-Shaw & Ahmad, 2005).

DNA-based drugs are fast emerging onto the biological therapeutic market, however, delivery systems and confidence in their long-term stability and safety is an essential validation needed for the FDA to approve more of these drugs.

1.2 Proteins for Therapeutics

The use of proteins as therapeutics has increased substantially since the introduction of the first US FDA approved human recombinant protein, insulin, in 1982 (Clark *et al.*, 1982). They have a diverse cross-section of roles across the body and have a large number of benefits associated with their use as therapeutics. With the incidence of alternative splicing of genes being more apparent, as well as post-translational modifications of proteins, increased numbers of functionally distinct proteins are present in the body (Leader *et al.*, 2008). This increasingly diverse range of proteins, as well as a better understanding of their functioning, highlighted the opportunity of utilising proteins for the potential suppression of disease states, and exploiting them pharmacologically.

Proteins are good pharmaceutical targets (Leader *et al.*, 2008) because they are highly specific with very complex functions, resulting in less adverse effects from interference with off-target biological processes which often happens with small molecule drugs. They are also well tolerated by the body, with less immunogenic effects as a consequence of treatment. They can also provide effective treatment for

disease states which result from a deleted or mutated gene, providing the missing/correct protein in absence of gene therapy being available. The clinical development and FDA approval time is less with protein therapeutics than with small molecules. A study in 2003 showed that the time for clinical development and FDA approval was on average a year faster with 33 protein therapeutics then 294 small molecules from 1980-2002 (Reichert, J. M., 2003). From a financial perspective, this is an attractive quality of investing in drug development. Alongside this, the specificity and complexity of proteins when compared to small molecules allow more extensive patents to be filed to protect them.

1.2.1 Types of protein therapeutics

1.2.1.1 Enzymes and regulatory proteins

In disease states, these proteins are deficient or abnormal within the body and a replacement is required regularly for sufficient treatment, or sometimes only at specific times. Insulin and human growth hormone (HGH) are good examples of these types of therapeutic proteins, where regular administration is required to be of use. The treatment of Haemophillia A with Factor VIII is another common protein therapy treatment which replaces the deficient coagulation factor with the recombinant protein. This allows the correct steps in the coagulation pathway to happen, preventing the congenital bleeding associated with the disease (Nogami *et al.*, 1999).

Particular therapeutic proteins may only be required at certain times during a medical procedure and are not necessarily required to treat a disease state. Follicle stimulating hormone (FSH) is administered to patients undergoing *In Vitro* Fertilisation (IVF) to increase the maturation of developing oocytes available for fertilisation (Out *et al.*, 1997). This is only required at the beginning stage of the IVF cycle and is an important illustration of the use of a therapeutic protein to enhance a natural physiological process.

Similarly, a therapeutic protein can be used to treat certain conditions associated with a disease, or the side effects of drugs used in treatment. Alteplase or tPA is an enzyme which disrupts blood clots and is a standard enzyme used in the treatment of blood clots induced by myocardial infarction (COBOLT, 1997) or strokes (Albers *et al.*, 2000). It works by cleaving plasminogen to plasmin, which then degrades fibrin and thereby lyses fibrin-based clots (Golan *et al.*, 2007). Although tPA is endogenously secreted by endothelial cells lining the walls of the blood vessels, it is often required in much larger amounts to disrupt the blood clot (COBOLT, 1997). Tenecteplase is a genetically modified version of this enzyme, showing greater binding affinity to plasminogin and a longer plasma half-life showing more efficacy at breaking up blood clots (ASSENT-2, 1999).

Foreign proteins which are not naturally expressed in human cells or where they may be desirable can also be administered to treat particular symptoms. Papain is a protease used to degrade proteinaceous debris in wounds, it is purified from the *Carica papaya* (Burke & Golden, 1958). Similarly, collagenase which is obtained from fermentation of Clostridium histolyticum, is used to digest collagen in the necrotic base of wounds (Boxer *et al.*, 1969). Dystonia, a condition which causes involuntary muscle spasms often resulting in affected parts of the body into painful and abnormal positions, is treated with the Botulinium toxin derived from *Clostridium botulinum*. This toxin cleaves SNAP25 at neuromuscular junction disrupting the SNARE complex, this prevents acetylcholine release causing flaccid paralysis (Jankovic & Brin, 1991). Not only is this used therapeutically, but also cosmetically under the trade name Botox, where people have injections of the toxin to remove wrinkles from their face.

1.2.1.2 Cytokines and Monoclonal Antibodies

Through major milestones in research into molecular biology, a new generation of biological agents were highlighted in their potential for treatment of disease. Receptor and antibody based targeting for drugs and progress in understanding

cytokine biology and development of monoclonal antibodies (mAb), revolutionised medicine in the 20th century.

Initial work on the cytokine Tumour Necrosis Factor (TNF) was to investigate its effects on tumour biology. Subsequent discoveries highlighting TNF as an important mediator of the inflammatory response encouraged research into development of neutralising agents (Cerami, 2011). The discovery that erythropoietin (EPO) blocks TNF synthesis lead onto investigation into other cytokine antagonists across the scientific community. In Dr. Cerami's group, EPO derivatives which do not bind to the receptor but are tissue protective have been discovered, and are now in Phase 2 clinical trials (Brines *et al.*, 2004). TNF itself is associated with many major inflammatory diseases, such as rheumatoid arthritis (RA), psoriasis and inflammatory bowel disease (IBD) (Bradley, 2008). Its diverse range of signalling pathways and cellular responses have highlighted TNFs role in a variety of disease. A monoclonal antibody for TNFα, infliximab, is used in treatment of disease such as RA (Lipsky *et al.*, 2000) and IBD (Present *et al.*, 1999), working by neutralising TNFα in the circulation.

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive lung disease which is characterised by the thickening of the walls of the lungs by excessive fibrotic tissue. This lowers lung capacity and results in unusual gas exchange. There is little definitive etiology of the disease and treatments are often only sporadically effective (ICS, 2000). Traditional treatment has usually been with corticosteroids and cytotoxic agents; however, these may exhibit adverse side effects (Flaherty *et al.*, 2001). Pro-fibrotic cytokines and reduced levels of IF-γ are detected in patients with IPF (Coker & Laurent, 1998); with IF-γ exhibiting several regulatory effects on IPF phenotypes (Raghu G. *et al.*, 2004). However, the placebo-controlled trial performed by Reghu *et al.* (2004) did not show any advantageous effects, although the size and length of treatment could have been a factor. Various cytokines are used in protein therapy, regulating immunoresponses and having protective effects in various bodily processes (Andersson & Tracey, 2010).

By utilising the binding affinity of protein ligand-receptor binding domains, the antigen recognition site of immunoglobulins, or both; protein therapeutics can be synthesised to target specific molecules, guiding the immune system to destroy it (Leader *et al.*, 2008). Monoclonal antibodies can target specific proteins associated with disease, resulting in their removal being organised by the immune system. Although monoclonal antibodies are predominantly used to target inflammatory disease and cancer, they are also used to treat infectious diseases. Patients at high risk of severe respiratory synctial virus (RSV) are given the recombinant monoclonal antibody for the RSV F Protein, palivizumab, which targets the virus for clearance by the immune system (Impact-RSV, 1998). In the randomised, double-blind, placebo trial it was concluded that palivizumab was safe, well-tolerated and an effective treatment for prevention of RSV in young children.

Monoclonal antibodies can also be used therapeutically simply by binding to a cell-surface receptor, thereby preventing its natural ligand binding and inducing a signal transduction pathway. Specific cells can be targeted by the immune system, hence their role in cancer therapy. Patients with B-cell lymphocytic leukaemia are treated with alemtuzumab (Campath), a humanised mAb which targets CD52 antigen on T and B cells. Binding of this mAb to the T and B cells targets them for destruction by either complement activation, antibody-dependant cellular toxicity or apoptosis (Keating *et al.*, 2002); irrespective of which, the treatment has been effective in patients which have failed alternative therapy. Another cell targeted anti-cancer therapy treatment with a mAB is trastuzumab (Herceptin) used to treat breast cancer. Some malignant cells are seen to express high levels of HER2/Neu receptor on their cell surface, this anti-HER2 mAb binds to this receptor helping the cells to be targeted by natural T-killer cells (Slamon *et al.*, 2001); however, this is thought not to be the mAb's only mechanism of action.

Immunoadhesion molecules combine the receptor-ligand/protein binding domain and an immunoglobulin constant domain to improve on the therapeutic potential of mAbs (Ashkenazi and Chamow, 1995). Etanercept is an immunoadhesion used to

target TNF, this combines the TNF receptor and the Fc domain of IgG1 in order to bind to TNF and simultaneously target it for destruction by the immune system (Mease *et al.*, 2000). Alefacept (Amevive) is a dimeric immunoadhesion molecule which binds with CD2 on the surface of lymphocytes thereby inhibiting interaction with LFA3 (important for activation of T lymphocytes in psoriasis) (Krueger *et al.*, 2002); simultaneously it target the T lymphocytes for destruction by Natural Killer cells.

1.2.1.3 Other Protein Therapeutics – vaccines and protein diagnostics.

Disease prevention is the ultimate goal in modern medicine. Vaccines which contain heat-killed or attenuated forms of a foreign pathogen provide the immune system with a chance to prepare itself for invasion (Leader *et al.*, 2008). However, these vaccines contain unavoidable risk in causing infection or adverse reactions (Poland *et al.*, 2009). Through genetic engineering, the immunogenic but non-pathogenic part of the corresponding protein can be injected, providing the immunogenic imprint but none of the risk of toxicity. Hepatitis B vaccine is now successfully administered utilising this method of production. A non-pathogenic, recombinant, hepatitis B surface antigen (HBsAg) protein is injected providing significant immunity in the large majority of individuals (Szmuness *et al.*, 1980). Similarly, the HPV vaccine (Shi *et al.*, 2007)) and a vaccine containing a non-pathogenic lipoprotein to protect against Lyme Disease (Sigal *et al.*, 1998), follow the same principal.

Some proteins are used in diagnostics and play a key role in disease detection. Purified, recombinant proteins are administered and results both *in vitro* and *in vivo* are monitored, subsequently leading to the choice of definitive treatment (Leader *et al.*, 2008). Growth hormone releasing hormone (GHRH) stimulate cells in the pituitary gland to release growth hormone (GH), it can help determine whether it is pituitary secretion which is defective in patients with a clinical sign of growth hormone deficiency (Ghigo *et al.*, 1996). Proteins can also be used as imaging agents; in cancer therapy, it is helpful at early stages of disease where treatment can be most effective. Satumomab pendetide (OncoScint) is an indium-111-labelled mAb

specific for tumour associated glycoprotein (TAG-72), this emits gamma radio-nucleotides for detection of sites of malignant disease (Maguire *et al.*, 1993). In vitro detection also uses proteins for standard screening and confirmatory tests, a major one of which is Human Immunodeficiency Virus (HIV). In this case, mAbs are used in Western blot and ELISA assays and are specific to the antigens presented through the course of HIV infection (Urnovitz *et al.*, 1997).

Protein therapeutics are a playing an increasingly important role in modern therapeutic medicine. They have a range of properties and their production is becoming cheaper and more efficient, as well as potential for modification increasing their efficacy in the body. Recombinant human proteins make up the largest group of FDA approved biotechnology medicine (Leader *et al.*, 2008) and they can be used in conjunction with small molecule drugs to provide synergistic benefits to a treatment. It is undoubted that the field of protein therapeutics will grow dramatically in future years.

1.2.2 Production of therapeutic proteins

The method by which these proteins are produced is dependent on the cost of production and/or the extent of post-translational modification required, which some expression systems cannot offer. Very few are isolated from their native source, pancreatic enzymes from hog and pig pancreas (Staff *et al.*, 1984), but more commonly, recombinant DNA technology is used and the protein is isolated from a wide range of organisms. Bacterial (Terpe, 2006), yeast (Gerngross, 2004), insect (Caron *et al.*, 1990) and mammalian cells (Wurm, 2004) are used. The benefits of recombinant technology have broadened the capabilities of therapeutic proteins; they allow more efficient production, a much lower incidence of immunological adverse effects, there is a reduction in exposure to animal or human disease and the option to modify the protein for improved activity (Leader *et al.*, 2008).

Transgenic plants and animals are also used to produce fully refolded and functional proteins. For example, human somatotropin was successfully produced in a soluble,

biologically active, disulfide-bonded form. It was also isolated to the chloroplasts by transplastomic transformation technology which not only contained the protein for purification purposes but also apparently prevented transmission of transgenes through pollen (Staub *et al.*, 2000).

Transgenic animals have been used as novel 'bioreactors' in production of recombinant therapeutic proteins. Shortly, recombinant antithrombin III will be launched as ATryn for prophylactic treatment of patients with congenital antithrombin deficiency (FDA approved in 2006). The protein is raised in goats and purified from their milk and is identical to the human amino acid sequence but differs in its glycosylation profile (Neimann and Kues, 2007). However, the major hurdle in production of protein therapeutics this way remains the complex post-translational modifications (O- and N- linked glycosylation, phosphorylation and sulphation for example) which can initiate an immune response, although the success of the above mentioned drug, for example, exhibits its potential in the feasibility of is production.

1.2.3 Clearance of protein/peptide drugs

With all the benefits of proteins' as therapeutics, there are disadvantages regarding the clearance of the molecule from the body. The desired effect of the therapeutic is ongoing and a continuous outcome seen from administration is looked for. As with any naturally produced protein in the body, endogenous clearance mechanisms are in place to 'switch off' a signaling pathway or an effect.

The way in which a protein or peptide is removed from circulation is dependent on its size, charge and confirmation (Brenner *et al.*, 1978 & Deen *et al.*, 1979). The main pathway for protein removal is through ultrafiltration in the kidneys (Rabkin & Dahl, 1993); the glomerular capillaries are restricted by a double barrier, which are not just size selective but also affected by the charge of the molecule (Deen et al, 1979). The protein or peptide can also be later reabsorbed by the proximal tubules for re-circulation or degradation. The effective molecular weight cut off of the glomerular

membrane is 70KDa (Knauf *et al.*, 1988), where the membrane witholds proteins larger then albumin. It was noted here that the clearance rate through filtration above 70KDa slowed dramatically, although it did not drop to zero, suggesting another mechanism for clearance.

It is at this stage receptor mediated clearance is thought to play a role. Layton *et al.*, (1989),investigating the pharmokinetics of G-CSF on clinical trial, administered subcutaneous injections to determine the half-life. At higher doses this increased, indicating one mechanism becoming saturated at certain dosage levels. Similarly, after monitoring serum levels of G-CSF after continuous administration, a sudden drop in levels was seen in the fourth and fifth day, denoting the induction of an alternate clearance mechanism. Also, G-CSF levels did not decrease while patients were still neutropenic; showing that levels of neutrophils increased as G-CSF decreased, highlighting the potential negative feedback mechanism involved in neutrophil homeostasis.

1.3 Chemical Modification of Proteins for Therapeutics

To prevent these clearance mechanisms from rendering the protein with little value therapeutically, modifications of the protein are made to try and improve its pharmokinetic properties. These modifications target efficacy, stability, specificity and immunogenicity. The stability of a protein may be enhanced by making their structure more robust against attack from proteases or changes in temperature and pH. Modifications to the residues in the protein's binding site may improve its binding affinity for its receptor, improving its efficacy. Efforts to mask its immunogenic properties by incorporation of a chemical or post-translational modification, as well as fusion partners are also common in therapeutic protein modifications.

1.3.1 Fusion Proteins

Low plasma-half life and decreased circulating time is a common problem with therapeutic proteins. Attachment of these proteins to partners which improve its

clearance rate has been achieved by genetically fusing the therapeutic to another molecule. TNF ligands are of increasing interest therapeutically because of their connection with stimulating the immune system and apoptosis inducing properties (Wajant H. *et al.*, 2002), however, their plasma half life is very low. Soluble variants of this family of ligands have been genetically fused to human serum albumin (HSA), producing fusion proteins with similar bioactivity of TNF ligands less HSA. Half life was seen to increase from 1 – 16 hours after subcutaneous injection. Bioactivity remained higher at both 6 and 24 hours, compared to control (Müller N *et al.*, 2010).

The therapeutic protein is often bound to a carrier protein such as transferrin or albumin. Investigation into the pharmacokinetic (PK) properties of these fusion proteins is limited, despite their rapid development within the pharmaceutical industry. A recent study looked into these properties and developed a pharmacokinetic model that can be transposed onto other fusion proteins. By linking Growth Hormone (GH) to Transferrin (Tr) via different peptide linkers, different binding affinities and plasma half-life was detected. This allowed investigation into how the bifunctional fusion protein would be recycled; the subsequent binding of GH and then Tr being essential in the balance between degradation and recycling (Chen X. *et al.*, 2011). This model was further validated by testing the fusion protein Granulocyte colony stimulating factor (G-CSF) and Tr.

Proteins are also fused to human immunoglobulins (IG) to attempt to increase their plasma half life. The Fc and C_H domains of IG_1 and IG_4 were expressed in monkey COS cells and their bioactivity was measured using standard assays to determine G-CSF activity. The G-CSF/IGFc fusion proteins were purified as disulphide-linked homodimers and on a molar basis their activity was the same as G-CSF alone in *in vitro* bioassays. However, purified G-CSF/IGFc fusion protein's bioactivity were 3-4 fold slower than unmodified G-CSF. The conjugate protein displayed a slower plasma clearance rate and stimulated production of white blood cells which were longer lasting then G-CSF alone (Cox G. N. *et al.*, 2004); these factors perhaps detracting from its decrease in bioactivity. The enhanced heamopoetic properties of this fusion

protein demonstrate the potential of immunoglobulin domain fusion to a therapeutic protein.

The neonatal Fc receptor (FcRn) is expressed on the pulmonary epithelial cells of both human and non-human primates, aiding transport of immunoglobulins across the epithelial cells. This receptor became an interesting target for potential assistance in delivering therapeutics to the lungs using fusion proteins. Erythropoietin fused to the Fc domain of IG_1 was successfully delivered to the lungs and its biological affects seen via Phase 1 clinical trials, acting through the FcRn mediated transport pathway (Dumont JA *et al.*, 2005). A second generation of these fusion molecules were developed and improved pharmacokinetic properties were seen; these fusion proteins were monomeric with respect to the therapeutic but dimeric with respect to the Fc region. Both the half-life and the bioavailability of the therapeutic are enhanced by these Fc fusion monomers (Dunmont JA *et al.*, 2006). However a recent study has shown that these Fc fusion proteins actually have a relatively low affinity for the FcRn, indicating there may be other critical factors involved in maintaining its plasma half-life (Suzuki T. *et al.*, 2010). Further investigation indicated the structural affect the fusion protein may have on the receptor binding domain.

1.3.2 Rational Design and Protein Engineering

The expansion of knowledge pertaining to properties of individual amino acids, conserved domains and the interaction of a protein/ligand with its receptor, has allowed a rational thought process into engineering of a protein to enhance its therapeutic efficacy. A protein's affinity for its receptor, its stability and immunogenicity can be altered by mutagenesis and protein engineering (Marshall *et al.*, 2003). The therapeutic protein is exposed to a variety of stresses which can result in degradation or unfolding. Single residue mutagenesis can prevent some of these features, for example free cysteine substitution which prevents aggregation and dimersation of the protein and therefore exhibits a longer shelf-life (Arakawa T. *et al.*, 1993).

The protein human Growth Hormone (hGH) is widely used therapeutically for the treatment of pediatric hypopituitary dwarfism and in children suffering from low levels of hGH. However, its stability is low in solution and as it has to be administered subcutaneously several times a week, efforts into optimising its stability and half-life were attempted. By using Protein Design Automation® (PDA™), computational analysis designed variants of the protein containing from six to ten mutations. These protein variants were seen to be active in cell proliferation studies and were stable at 16^{o}C higher than the wild type protein (Filkov AV. et al, 2002). Similarly, mutations of G-CSF were designed via computational analysis and the mutant exhibited enhanced thermostability and 5-10 fold increase in shelf-life, whilst maintaining its biological activity (Luo P. et al, 2002).

Protecting a protein from degradation by proteases can also dramatically improve a therapeutic's pharmacokinetic properties. Mutations can be introduced to the specific site on the protein which is known to be more susceptible to proteolysis, these are often seen to be flexible loop regions on a protein and there are attempts at decreasing flexibility (Marshall *et al.*, 2003). Patients with hemophilia A require regular treatment with coagulation factors, several of these factors within the coagulation pathway rely on proteolytic cleavage for both activation and inactivation. Researchers re-designed Factor VIII (FVIII) making it less susceptible to inactivation by cleavage by deleting residue 794-1689 so that the A2 domain is covalently attached to the light chain. Further mutations at specific inactivation sites resulted in a single chain protein which has maximum activity after single cleavage activation (Pipe & Kaufman, 1997).

The use of biological knowledge to generate protein mutants which can contend with endocytic trafficking is another useful tool employed by rational design. Usually, on binding to the cell surface, the receptor and the ligand are endocytosed by the cell and the ligand is either recycled to the cell surface after being released by the receptor during this process; or degraded by the lysosomes along with the receptor (Smythe & Warren, 1991). The pH inside the endosome is often much lower than at the cell surface plasma and as ligand-receptor binding is pH dependent, some ligands

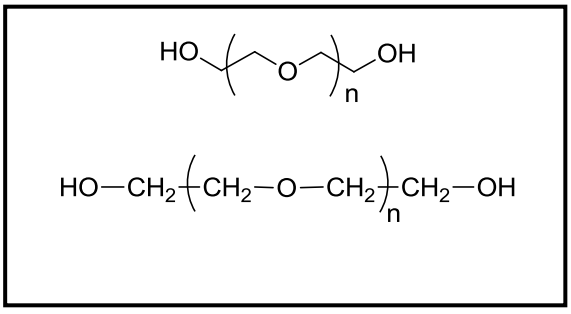
are released before degradation. By mutating residues at the receptor ligand binding site, a molecular switch can be generated which holds strong association with the receptor at the cell surface but less so within the endosome. This was successfully achieved with G-CSF where histidine residues were mutated into the protein which remain neutral at the cell surface but become positively charged within the low pH of the endosome, reducing electrodstatic attraction between receptor-ligand, allowing it to be released (Sarkar *et al.*, 2002)

Glycosylation is a natural prost-translational modification which has a variety of functions such as cell adhesion, self/non-self recognition, molecular trafficking and clearance, receptor activation and endocytosis (Ohtsubu & Marth, 2006). In regards to improving pharmacokinetic properties of a protein, increased glycosylation sites can be engineered to give a longer plasma half-life and stronger binding affinity *in vivo*. Erythropoietin (EPO) which controls the production of red blood cells was engineered to contain an additional 1-4 glycosylation sites. This increased elimination half-life, activity and functional time when compared to recombinent human EPO (Su *et al.*, 2010).

1.4 PEGs and PEGd therapeutics

Poly-ethylene glycol (PEG) residues are polymers of ethylene oxide. Their overall molecular weight determines its physiochemical properties when attached to another molecule.

Figure 1.4.1 – PEG molecule



PEG molecules are generally regarded as stable and non-biodegradable, although there have been reports which show degradation by oxidative enzymes: oxidation of terminal alcohol groups followed by terminal ether cleavage by alcohol and aldehyde dehydrogenases (Kawai, 2002). Similarly, cytochrome P-450 dependent oxidases have also been shown to degrade PEGs (Beranova *et al.*, 1990). This degradation however, is molecular weight dependent and larger PEG molecules which are used for therapeutic purposes are often at least 20KDa in size.

PEGylation is the process of attaching a PEG to a larger molecule, a therapeutic protein for example. The properties gained after attaching these residues to a therapeutic are highly beneficial in terms of its pharmacology. It is now a commonly used method of post-production modification of therapeutic proteins (and other biological molecules) with a view to enhance their biomedical efficacy in the body. By attaching them to proteins, this is achieved by several mechanisms: prevention of renal clearance by increasing the overall size of the molecule, reduced immunogenicity and degradation by proteolysis and increased serum half-life. All of these attributes result in a smaller dosage frequency which can be more economically and patient friendly.

1.4.2 PEGylated Therapeutics

PEGylated therapeutics are not limited to one type of molecule. They have been used to improve the pharmacokinetics of DNA based therapeutics, drug delivery systems and proteins. Evolutionarily, our biology has developed to protect us from contaminating DNA and foreign invaders making the passage through the cellular barrier very difficult (Patil & Burgess, 2003). Various drug delivery systems have been developed to try and aid this passage: liposomes, virosomes, protein scaffolds and nanoparticles.

1.4.2.1 Liposomes

Liposomes were discovered from self-forming enclosed lipid bi-layers upon hydration; they are now formulated to improve drug delivery, reduce toxicity and help accumulation of a drug at target sites. Lipophillic drugs are encorporated almost completely by the lipid bi-layer, whilst hydrophilic drugs are located exclusively in the aqueous compartment. Liposomes are initially recognised by the mononuclear phagaocyte system (MPS) and removed from circulation (Scherphof *et al.*, 1985); this can be utilised accordingly when targeting antiparastitic and antimicrobial infections in the MPS (Basu & Lala, 2004). However, when the target for drug delivery goes beyond the MPS, problems occur when trying to prevent their rapid clearance from the circulation.

Although they are biologically inert and weakly immunogenic, they are quickly removed from circulation either via the MPS, after the binding of opsonins such as immunoglobulins and fibronectin or through the binding of complement components (Patel, 1992). PEGylated liposomes or stealth liposomes were introduced to improve its circulating half-life and are mostly used in the delivery of hydrophillic drug molecules involved in cancer therapy, such as doxorubicin (Samad *et al.*, 2007). The PEG molecule can be incorporated onto the lipid surface by several mechanisms but most commonly by a cross-linked lipid i.e. PEG-distearoylphosphatidylethanolamine (DSPE).

The most significant properties gained by attaching a PEG molecule to a liposome are a reduced uptake by the MPS and the PEG chains help prevent aggregation of the vesicles improving the stability of formulations (Immordino *et al.*, 2006). It has also been noted that the PEG molecules bound to the lipid membranes prevent opsonins and other plasma proteins binding to the surface and therefore reduce immunogeneicity and removal by the MPS (Blume & Cevc, 1993); although other studies have shown this may not be the case (Johnstone *et al.*, 2001).

PEGylated liposomal doxorubicin (PLD) (DOXIL/ Caelyx) was the first and currently the only PEGylated liposomal formulation to be approved in both the USA and Europe for treatment of Kaposi's sarcoma (Krown *et al.*, 2004). The plasma half-life of the drug is very slow, exhibiting a biexponential curve of 1.5 and 45 hours (median values), compared to 0.2 hours for the free drug. Nearly 100% of the drug detected in the plasma after injection was the lipid-encapsulated form, with a very slow clearance rate of approximately 0.1L/hour. It also reduces the dose-related side effects which are a consequence of administration of the free drug.

Cisplatin is a drug used for treatment of ovarian cancer, although this treatment is limited due to its nephrotoxicity (Arany & Safirstein, 2003) and gastrointestinal intolerance, among others (Boulikas & Vougiouka, 2004). This drug was encapsulated in a PEGylated lysosome, preventing the drug being freely exposed to non-cancerous tissue, resulting in less adverse side effects. Phase I clinical trials began in 1995 mild side-effects were seen; nausea and vomiting and at does above ≥ 320mg/m^2 muscle weakness (Meerum *et al.*, 2002). In a Phase II clinical study, there were no unexpected adverse affects, although there were concerns related to high lipid doses and prolonged accumulation of platinum in the body (Seetharamui *et al.*, 2010).

1.4.2.2 PEGylated Protein Therapeutics

PEGylation is non-toxic, non-immunogenic and is 'generally recognised as safe' by the US Food and Drug Administration (FDA) (Pasut & Veronese, 2007). It implements many of the desirable improvements to pharmacokinetic properties of a therapeutic drug and has therefore rapidly escalated in its employment when developing new drugs. First attempts at PEGylating a protein was in the 1970s, where improved immunogenic and pharmacokinetic properties were seen on both bovine serum albumin (BSA) and bovine liver catalase by Abuchowski *et al.*, (1977). In 1990 the FDA approved the first PEGylated drug to be put on the market, a PEGylated version of adenosine deaminase (Adagen®) for treatment of patients with Severe Combined Immunodeficiency disease (SCID). Since then nine more have been approved and marketed, including four 'blockbuster' drugs – see table X (Jevsevar S. *et al.*, 2010).

The widely acknowledged and success of current PEGylated pharmaceutics has allowed up and coming PEGylated drugs to be fast tracked into various stages of clinical trials. BiogenIdec Inc. received a FastTrack from the FDA for its PEGylated IFN-β1a (for the treatment of multiple sclerosis) at the beginning of July 2009 (Baker DP., 2006). The well established process and successful results from PEGylating pharmaceuticals mean that there will always be an increasing number of PEGylated drugs on the market. Conjugation of PEG to protein results in significant changes to the proteins physiochemical characteristics. The most obvious property gained from attaching a PEG molecule to a protein is the increase in the overall size and hydrodynamic volume of the protein. However, the main draw back of this being its low bioactivity *in vitro,* most likely due to steric hindrance of the protein being able to interact with its receptor. However, this is compensated by its bioavailability through its increase in elimination half-life (Fishburn C.S., 2008).

1.4.2.3 Other Types of PEGylated Therapeutics

PEGylation is not limited to attachment to a protein; it is also used to enhance drug distribution by attachment to various drug-delivery systems such as protein scaffolds and nanobodies. Protein scaffolds demonstrate further development of binding structures for delivery of proteins in biopharmaceutical drug design. They provide an alternative to antibodies, which are no longer classed as the only receptor proteins available in biotechnology and medicine. They also provide more practical advantages when compared to antibodies; they have a higher production yield in microbial expression systems and elevated stability, also providing wide scope for intellectual property (Gebauer & Skerra, 2009). They are also small in size making them efficient in tissue penetration and are therapeutically effective to both intracellular and extracellular targets (Nuttall S. D., 2008). However, this consequently gives them a short plasma half-life, and they are therefore often PEGylated.

A type of protein scaffold, Andenectins™, are a new class of therapeutic protein; they are highly specific and have a high affinity with their relevant targets. Based on

the 10th Fibronectin III domain; they are structurally similar to antibody variable domains, they have favourable biochemical properties and are abundant in the blood plasma – verifying that it is not toxic and immunogenic (Lipovšek D., 2011). CT-322, a PEGylated Andenectin was designed to inhibit Vascular Endothelial Growth Factor Receptor (VEGF R)-2. Using surface plasmon resonance studies, CT-322 was showed to be specific to VEGFR-2 at high affinity (K_D 11nM), but did not bind to VEGFR-1 or VEGFR-3 at very high concentrations, up to 100nM (MamLuk *et al.*,2010). CT-322 also inhibited growth of human tumor xenograft models of colon carcinoma and glioblastoma; similarly, enhancing anti-tumour activity of the chemotherapy agent temsirolimus in the colon carcinoma model.

A single-domain antibody fragment, or a Nanobody®, was first discovered naturally expressed in camelids (Hamers-Casterman *et al.*, 1993). These were an important finding in development of antibody based drugs in pharmaceuticals; nanobodies bind antigens without requiring domain pairing. These are also easier to express in microbrial expression systems and do not loose affinity and stability like antibody fragments have proven to do (Ward *et al.*, 1989). Although most research into the biological application of these single-domain antibody fragments have taken place on those sourced from camelids, they were later discovered in cartilaginous fish (Greenberg *et al.*, 1995). Some advantages of these Nanodies® compared to monoclonal antibodies are depicted in Table X ([1]Hamsen & De Haard, 2007) -

Again, because of the size of these single-chain antibody fragments, chemical modification by PEGylation can be utilized to increase its plasma-half life. An example of which was the PEGylation of the foot and mouth disease virus (FMDV) virus ([2]Harmsen *et al.*, 2007). The single-chain antibodies were raised in immunised llamas using phage display and were tested *in vitro* (monolayers of secondary swine kidney cells) and *in vivo* (guinea pigs). The results of the study were contradicting; the FMDV PEGylated nanobody showed more neutralisation efficiency in vitro, but convalescent guinea pig serum proved more effective *in vivo*. The results indicated

more emphasis on the importance of opsonophagocytosis of FMDV for *in vivo* protection.

1.5 Linkers

PEGs are commercially available in a variety of forms, with the capacity of utilising different chemistries to attach to the molecule of choice. The conjugation requires the reaction between the functional group on the target molecule and the reactive group which has been activated on one or both ends of the PEG molecule (Roberts *et al.*, 2002). These activated groups or linkers are vital to the conjugation of the PEG to the protein and have to be highly reactive and often target-site specific to be a successful constituent in the reaction process.

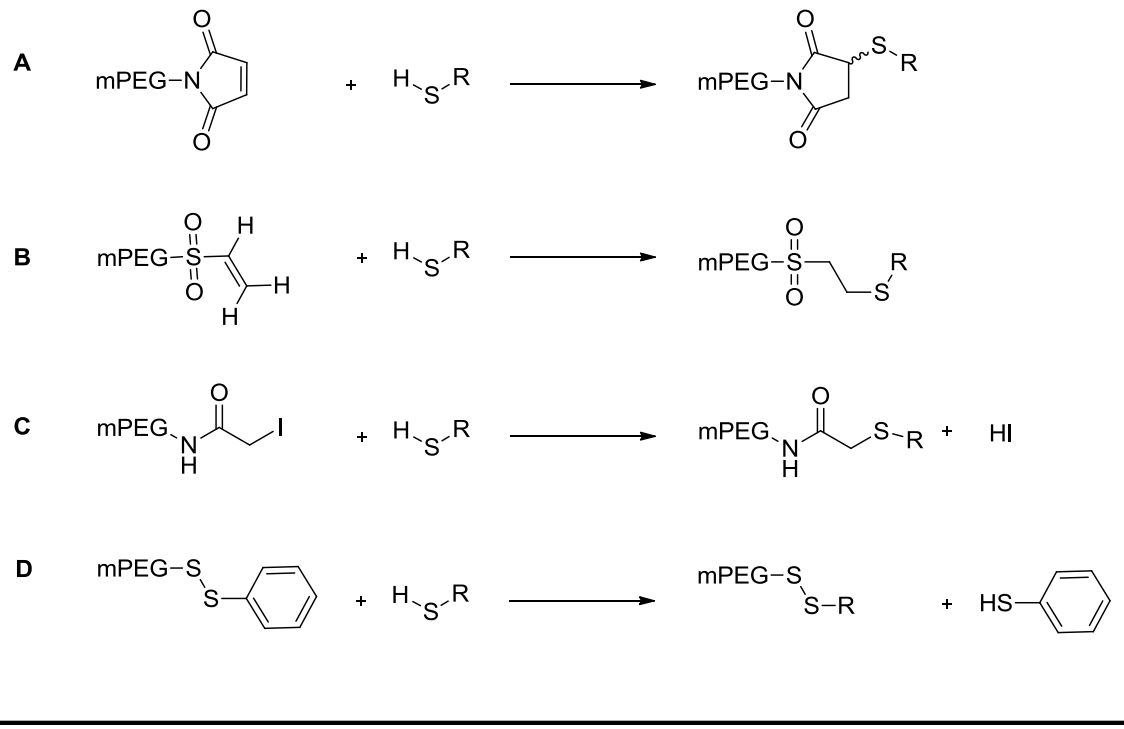
Many different linkers have been developed and there have been substantial advancements in the chemistry surrounding PEGylation of proteins. Their chemistry can target various amino acids and both N-terminal amino group and C-terminal carboxylic acids. The most common of which however, target the lysine residue on proteins which is the most abundant in the protein structure and can make up to 10% of the total amino acid content in a protein. This provides ample target sites for PEGylation which often results in multi-PEGylated proteins. The extent of PEGylation has an effect on its pharmacological properties; typically, the larger the number of PEGs attached the longer its circulation half-life and reduced immunogenicity (Clark *et al.*, 1996).

1.5.1 Thiol Reactive Linkers

A major development was thiol or cysteine specific PEGylation. Cysteine residues are the most infrequent on the protein surface (Exarchos K *et al.*, 2011) and it is also the only amino acid with a free thiol as its active functional group which further ensures the PEGylation specificity. PEG linkers have been developed to coincide with this chemistry, such as PEG-maleimide, PEG-vinylsulphone, PEG-iodoacetamide and PEG-orthopyridyl disulphide. Of these linkers maleimide reacts quickest with the free thiol and is the most commonly used linker in the production of PEGylated

therapeutic proteins. However, there are disadvantages concerning maleimide stability and it also exhibits multi-PEGylation (non-specifc and off-target) which results in lack homogeneity.

Figure 1.5.1 – Thiol Reactive Linkers (Veronese & Pasut, 2005)



Thiol Reactive Linkers. 1) PEG-Maleimide, 2) PEG- vinyl sulfone, 3) PEG- iodoacetoamide, 4) PEG-orthopyridyl disulfide

1.5.2 Maleimide

To ensure homogeneity in the production of PEGylated proteins, site specific targeting of an amino acid residue is a desirable characteristic of the linker attaching the PEG to the protein/peptide. Maleimide is one of several linker technologies developed which is cysteine specific and reacts extremely quickly with thiol groups.

A significant milestones in PEGylation chemistry was the modification of interleukin – 2 (IL-2), an important therapeutic in treating patients with depleted immune system. The protein was genetically modified to contain a cysteine residue in its natural glycosylation site and a maleimide linked PEG was used for conjugation (Goodson &

Katre, 1990). Here, they retained bioactivity of the cytokine but enhanced its serum half life by attaching the PEG molecule.

Maleimide's many advantages in conjugation chemistry, such as speed and reaction under acidic conditions, are also met by some disadvantages. Although the maleimide – thiol bond is stable, in water it is seen to undergo ring opening or addition of water across the double bond. Slow cleavage of one of the amide linkages is also seen over time (Roberts et a., 2002).

The specificity of maleimide is also questionable, as multi-PEGylation is detected in a reaction mixture where only one cysteine residue is available for conjugation (data shown in results). However, maleimide is the most commonly used thiol specific linker technology in conjugating proteins/peptides to PEG molecules, and throughout this research project, maleimide is used as a control.

PermaLink™ Technologies (see figure 5.1.3.1) have been developed by an independent company, Glythera Limited. They have quickly advanced in linker reactivity and specificity and have shown improved reaction efficacy when compared to maleimide PEGs. Throughout this project PermaLink™ technology has been used and tested, with maleimide as the control.

1.6 Summary of Project Aims

The aims of this project were to attempt to covalently attach novel PermaLink-PEG molecules to a therapeutic target, either a peptide or model protein. Comparisons could be made to current commonly used thiol-linker technology i.e. maleimide-PEGs, which have been used as a control throughout these studies. After optimising the methods with smaller PEG molecules, the effect of increasing the molecular weight of the PEGs (for therapeutic effect) will be investigated.

1.7 Conjugation Targets

1.7.1 Model - Bacillus circulans xylanase (BCX)

Xylanase is a small glycoprotein of approximately 22KDa in size and its natural function is the breakdown of hemicellulose in plant cell walls. It is a member of the glucanase enzyme family which are characterized by their ability to break down various xylans to produce short-chain xylo-oligosaccharides. It has a variety of commercial applications such as bio-bleaching of paper pulp, improvement of animal feeds and enhancement of fermenting processes (such as silage). For the purposes of this project however, it has been used as a model protein for PEGylation optimisation. It has been extensively characterised through X-ray crystallography and NMR spectroscopy assignments giving a thorough example of its three-dimensional structure (Wakarchuk *et al.*, 1994). Its enzymatic activity would also allow easy analysis of its bioactivity and whether it has been hindered by PEGylation.

1.7.2 Target Proteins with Therapeutic Potential - Calcitonin (CT)

Calcitonin is a 32 amino acid (3418 Da) peptide containing a disulphide bridge which is formed between the two cysteine residues at position 1 and 7 (Breimer *et al.*, 1988). Its primary function is to reduce levels of calcium in the blood by inhibiting bone resorption (Chambers & Magnus, 1982). Within the calcitonin gene family there are four known genes (CALC I – CALC IV), however, only CALC I produces calcitonin. The other genes produce structurally and functionally similar peptides such as calcitonin gene related peptide (CGRP) and amylin (Figure X – Masi & Brandi, 2007). However, these similar peptides are less potent in inducing hypocalcemia in blood plasma levels. However, salmon calcitonin (sCT), sharing only 50% sequence similarity to human calcitonin has a much greater potency than human calcitonin in most biological assays (Houssami *et al.*, 1995; Breimer *et al.*, 1988).

1.7.2.1 Roles of Calcitonin

Calcitonin is usually produced by the thyroid gland, although there is evidence showing non-thyroidal production of calcitonin. For example, Davis *et al.*, (1989),

showed calcitonin being produced by the prostate gland. Similarly, Fischer *et al.*, (1983), showed calcitonin production in the central nervous system. Various roles of calcitonin have been determined.

17.2.2 Calcitonin in bone

Osteoclasts are the primary target for calcitonin. It acts directly on calcitonin receptors to inhibit bone resorption by inducing contraction and inhibits osteoclast motility (Chambers *et al.*, 1991), followed by a more gradual retraction of osteoblasts. cAMP and Ca^{2+} are secondary messengers of the aforementioned affects are GPCR mediated. Inhibiting the release of acid phosphatases by the osteoblasts, important in cell signalling pathways, is another effect of calcitonin in bone.

1.7.2.3 Kidney

The kidney is the site for calcitonin degradation. Calcitonin receptors in the kidney have a high affinity for calcitonin and influences the renal handling of electrolytes (Clifton-Bligh *et al.*, 1980). It also increases the excretion of calcium and phosphate. Therapeutically, in patients with hypercalcemia and bone metaplasia, calcitonin decreases levels of calcium in the blood plasma due mainly to prevention of renal tubular reabsorption (Pondel, M., 2000).

1.7.2.4 Central Nervous System

The calcitonin receptor is found in various parts of the brain, the hypothalmic floor being the most sensitive site (de Beaurepaire & Freed, 1987[1]). Hypothalmic injections of calcitonin are also found to decrease gastric secretion; the sites where calcitonin decrease locomotive activity and decrease food intake appear to coincide (de Beaurepaire & Freed, 1987[2]). High densities of the salmon calcitonin (sCT) receptor have been found by autoradiographic techniques (Henke *et al.*, 1983). In humans it has been shown that large doses of sCT decrease levels of testosterone, LH and FSH, indicating action at the hypothalamic level (Mulder H., 1993).

1.7.3 Salmon Calcitonin

Salmon calcitonin (sCT) is the most widely used source of calcitonin for clinical use because of its increased potency and improved analgesic properties (Chesnut *et al.*, 2008). It is commercially available in an injectable form or as a nasal spray; formulations of sCT which would be orally available are also being developed. In this case, the sCT is combined with a caprylic acid derivative to protect it from intestinal degradation; its bioavailability and biological efficacy were successful in a clinical trial with healthy volunteers (Buclin *et al.*, 2002). However, approved for use in the USA in 1995, sCT-nasal spray (sCT-NS) is currently the most widely used formulation due to its efficacy, tolerability and convenience for daily, long-term administration (Chesnut *et al.*, 2000).

In a single dose study, the bone reabsorption marker serum C-telopeptide of type 1 collagen (serum-CTX-1) showed maximum expression an hour after administration of sCT-NS (Zikan & Stepan, 2000). Advantageously, the reversibility of osteoblast inhibition by sCT has been detected; this study showed that bone reabsorption markers returned to baseline levels three months after treatment discontinuation (Overgaard *et al.*, 1990).

sCT has shown to improve bone mineral density (BMD) in postmenopausal women with established osteoporosis (Overgaard *et al.*, 1992). This study also noted a statistically significant reduction in overall bone fracture in these women compared to a placebo. sCT provides an alternative treatment for sufferers of Pagents disease, who do not tolerate or have secondary resistance to bisphosphonates (Siris, 1999). It has been noted however, that as sCT has affinity for other cell types such as liver, kidney and lung (Masi & Brandi, 2007); as a therapeutic, some of its efficacy may be lost due to off-target effects. A study looked at combining sCT with bisphosphonate to help specifically target osteoclast cells. This conjugate exhibited significantly greater affinity for bone mineral over unmodified sCT, representing a new class of antiresorptive drug (Bhandari *et al.*, 2010).

1.7.3.1 sCT - Therapeutic Potential in Other Disease

sCT has been indicated in the suppression of cancer cell lines. Mitogen-activated protein kinases (MAPKs) are implicated in the proliferation and survival of various human tumours (Johnson & Lapadat, 2002). In a prostate cancer cells and calcitonin was seen to inhibit the phosphorylation of the MAPK extracellular signal-related kinase (ERK1/2) (Segawa *et al.*, 2001). Similarly, in MDA-MB-435 and T47D human breast carcinoma cell lines, ERK1/2 phosphorylation was inhibited by treatment with calcitonin (Nakamura *et al.*, 2007).

1.7.3.2 PEGylated sCT

Low molecular weight peptides can show more improved pharmacokinetic properties when PEGylated than proteins. This is because their half life is substantially increased after PEGylation, because usually, they are subject to rapid elimination by renal clearance (Youn *et al.*, 2007). Similarly, they exhibit distinct biological activity and stability according to PEGylation sites because they have more specific active sites then proteins (Youn *et al.*, 2004). They are also more easily targeted for site-specific PEGylation; different methods of PEGylation can also be utilised for easier conjugation, such as solid-phase PEGylation (Lu & Felix, 1994).

sCT does not require the disulphide bridge to be bioactive (Orlowski *et al.*, 1987), which means that the two cysteine residues 1 and 7 can be targeted for PEGylation for thiol specific conjugation. sCT has previously been PEGylated by maleimide PEG conjugates at both cysteine 1 and 7 (Cheng *et al.*, 2006). The Mal-sCT conjugate presented a stable helical structure in aqueous solutions, with the potential for aggregation at concentrations higher then 11μM. It also proved to have better cellular uptake and improved enzymatic stability, however, its stability was inadequate at allowing oral delivery. Attempts had therefore been made to improve on this advantageous, pharmacokinetic property by also attaching a lipid molecule to sCT; this was seen to improve its resistance to enzyme degradation but did not enhance its biological efficacy (Cheng & Lim, 2009).

Alternative sites for PEGylation of sCT have previously been reported as lysine^{11} and lysine^{18}. The lysine^{18} conjugate, using a 2 KDa PEG, showed the highest bioactivity using cAMP assays on T47D cells, as well as increased resistance to enzymatic degradation. (Youn *et al.*, 2006). It was also found to have a similar membrane permeability to non-PEGylated sCT and has a reduced liver accumulation. In attempts to further improve stability and half-life, a comb-shaped PEG was conjugated to sCT at cysteine 1 (Ryan *et al.*, 2008). It was noted in this study that the comb-shaped PEG conjugate improved serum half-life and the AUC of serum sCT over values achieved with sCT alone and PEG-sCT.

Chapter 2 .0 Aims

The aims of this research project were: -

1. To attempt to covalently attach a PEG molecule using novel linker technology to a therapeutic protein or peptide.
2. To investigate the effects of increasing the size of the PEG molecule on the reaction and the reactivity of the PEG-linkers.

In doing so a direct comparison of this novel linker technology could be made to current commonly used maleimide-PEGs. The novel linkers were developed by Glythera Ltd. (figure 5.1.3.1) to demonstrate more specificity to thiol groups than commonly used maleimide-PEGs. Maleimide has previously reported to react with lysine residues which are commonly found on the surface of proteins and peptides (Sharpless & Flavin, 1966). However, benefits in speed of reactions are favoured by industry.

In order for a therapeutic protein/peptide to be improved by PEGylation, the PEG molecule must be large enough for the pharmacokinetic properties of the therapeutic protein to be enhanced. Properties such as reduced renal clearance, reduced immunogenicity and increased serum half-life (Jevsevar S. *et al.*, 2010) are all desirable properties when seeking to improve drug efficacy. However, by increasing the size of the PEG molecule the overall reactivity of the linker used to attach it to the therapeutic protein is reduced (Veronese, 2001). In this study, the effect of increasing the size of the PEG molecule on reactivity of the linkers are compared.

Chapter 3.0 - Methods

3.1 Reagents and Buffers

3.1.1 Table of Reagents

Reagent	Supplier
NaOAc	The Sigma-Aldrich Chemical Co., Dorset, UK
EDTA	Fisher Scientific., Loughborough, UK
Tris	Fisher Scientific., Loughborough, UK
Sorbitol	VWR Jencons, Leicestershire, UK
Urea	Fisher Scientific., Loughborough, UK
NaH_2PO_4	Fisher Scientific., Loughborough, UK
Imidazole	Fisher Scientific., Loughborough, UK
Tris(2-carboxyethyl)phosphine HCL TCEP (solution)	The Sigma-Aldrich Chemical Co., Dorset, UK
TCEP Resin	Thermo Scientific. Nortumberland, UK
Ellman's Reagent	The Sigma-Aldrich Chemical Co., Dorset, UK
Coomassie	Fisher Scientific., Loughborough, UK
Methanol	The Sigma-Aldrich Chemical Co., Dorset, UK
Ethanol	The Sigma-Aldrich Chemical Co., Dorset, UK
Bromophenol Blue	Fisher Scientific., Loughborough, UK
2-Mercaptoethanol	Fisher Scientific., Loughborough, UK
IPTG	Fisher Scientific., Loughborough, UK
Ampicillin	Fisher Scientific., Loughborough, UK
Kanamycin	Fisher Scientific., Loughborough, UK
LB	Fisher Scientific., Loughborough, UK
Cellulose Dialysis Membrane (10 KDa molecular weight cut off)	Fisher Scientific., Loughborough, UK
Salmon calcitonin (CT)	Warwick Polymers, Warwick, UK
NuPAGE® Tris-Acetate SDS Running Buffer	Novex®, Bracknell, UK
Maleimide-PEG (Various MW)	Thermo Scientific. Nortumberland, UK

3.1.2 Polyacrylimide SDS PAGE Gel Buffers and Solutions

Buffer	Components
2 x SDS loading dye	2.5 mL 0.5M Tris-HCl, pH6.8 2 mL Glycerol 4 mL 10% (w/v) SDS 0.5 mL 0.1% (w/v) Bromo phenol Blue Then make up to 10 mL with H_2O *(for denaturing loading dye, add Beta-Mercaptoethanol to make a 5% concentration)*
Coomassie Stain	0.25 g Coomassie Brilliant Blue R-250 100 mL Ethanol 100 mL H_2O 25 mL Acetic Acid then make up to 250 mL with H_2O
Coomassie Destain Solution	450 mL mqH_2O 450 mL Methanol 100 mL Acetic Acid
PEG Dye	Solution A: Dissolve 0.5 g bismuth nitrate in 20 mL of 20% acetic acid Solution B: 5 mL of a 40% KI solution in water Procedure: Before use, mix 20 mL solution A, 5 mL solution B and 70 mL water

3.1. 3 BCX S22C Expression and Purification Buffers

Buffer	Components	pH
Lysis Buffer	40mM NaOAc 20mM Imidazole	8
FPLC Buffer A	40mM NaOAc	8

	20mM Imidazole	
FPLC Buffer B	40mM NaOAc 500mM Imidazole	8
Protein Purification Buffer A	40mM NaOAc 0.12M Sorbitol	4.5
Protein Purification Buffer B	40mM NaOAc 0.12M Sorbitol 1M NaCl	4.5

Concentration can be measured using ∑4.08 = 1mg/mL at 280nm

3.1.4 Reaction Buffers

Buffer	Components	pH
Reaction Buffer Method 1, 2 and 3 for BCX S22C	20mM NaOAc 1mM EDTA	Various
Reaction Buffer for sCT	20mM Tris-Cl 1mM EDTA	8

3.2 Solutions

Solutions were made up dissolving the components in mqH_2O, NaOH or HCl were used to achieve the desired pH. This was measured using a Hanna pH210 Microprocessor pH meter and calibrated between pH 4-7 or pH 7-10, dependent on the buffer.

Buffers which were being used in anaerobic conditions were de-oxygenated by bubbling nitrogen through them.

3.3 Aseptic Techniques

Standard aseptic techniques were used throughout microbiology work. Solutions were autoclaved at 121^{o}C for 20 mins and all work was done by a Bunsen burner.

3.4 Protein Expression and Purification

3.4.1 BCX S22

3.4.1.1 Expression

The BCX S22C mutant (Supplied by Dr A. Watts, University of Bath) was transformed into an *E. coli* expression system (Wakarchuk *et al.*, 1994). Expression plasmids were transformed into *E. coli* BL21 (DE3) pLysS Competent Cells and grown in LB Miller media supplemented with 100 µg/mL ampicillin at 37 ˚C in a shaking incubator at 200 r.p.m. (Thermoscientific MaxQ 400). A starter culture (20 mL) was used to inoculate 1 L of the same LB media and grown at 37 ˚C until OD_{600} = 1.2 was achieved where optical density was measured using spectrophotometer (Unicam Helios γ machine, Thermo Scientific) with the cell suspension placed in a plastic 1 mL cuvette. The culture was cooled to 18 ˚C, protein expression was induced with 1 mM IPTG and cells harvested 12 - 14 hrs later. Cells were collected by centrifugation using a Bechman J2-MC centrifuge, JA-10 rotor at 7500 r.p.m., 9950g for 30 mins at 4^{o}C and resuspended in chilled 1 M Tris-HCl buffer pH 8.0. The cells were then re-centrifuged as above and stored at -20 ^{o}C.

3.4.1.2 Lysis of Cells

Cells were incubated with lysis buffer (5 mL buffer/gram weight of cells) at 4 ˚C for approximately 5 mins until the cell pellet was resuspended in the lysis buffer and the suspension was homogenous in appearance. Total volume ranged from 20 mL to 80 mL depending on the weight of the cell pellet. In the current study the resuspended *E. coli* cells were lysed by using a Constant cell disruption system with 30.1 MPa where the cells were maintained on ice. The lysed cell suspension was centrifuged using a Bechman J2-MC centrifuge, JA-14 rotor at 8000 r.p.m., 9820g for 25 min at 4 ˚C. The crude cell extract supernatant was collected and filtered using a 0.88 µm filter (Milipore) to remove cell debris.

3.4.1.3 His Tag Purification using Nickel (Ni^{2+}) Column

The BCX protein was purified using a HisTrap™ FF Ni^{2+} column (GE Healthcare) followed by elution with an imidazole gradient to compete with histidine binding to the Ni^{2+} column (Voges & Jap, 1998). Fast protein liquid chromatography (FPLC) and absorbance at 280 nm was used to detect protein eluted from the Ni^{2+} column.

A sample of crude lysed cell supernatant was initially collected for subsequent analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). The Ni^{2+} column (HisTrap™, GE Heathcare) was pre-equilibrated with FLPC buffer A containing 20 mM Imidazole. The crude cell extract was loaded onto the pre-equilibrated Ni^{2+} column that was attached to a ÄKTA FPLC (GE Healthcare) for protein detect at 280 nm. The Ni^{2+} bound protein was eluted with a linear gradient of 20 mM to 500 mM imidazole using FPLC buffer A and B described over 150 mL (Table 3.1.3). Fractions (0.5 mL) were collected and stored at 4 ˚C for subsequent analysis (Figure 4.1.1.3).

The combined BCX fractions (BCX MW 22 kDa) eluted from the Ni^{2+} column were concentrated using a centrifugal 10 kDa MW cut-off 15 mL Amicon Ultra concentrator in 20 min cycles using a Beckman CS-15R, S4180 rotor at 4200 r.p.m., 1381g at 4 ^{o}C. This was repeated until a single concentrated protein sample with a maximum volume of 30 mL remained. The total crude cell lysate proteins were compared to the eluted proteins from the Ni2+ column by SDS PAGE analysis where protein was detected using Coomassie Brilliant Blue R-250 staining (see 3.2.1.5 for further details). BCX has a predicted MW of 22 kDa and it was clear that the concentrated eluted protein sample still contained additional proteins that are predicted to not be the target BCX protein. As such a second purification step was performed.

3.4.1.4 Further Purification

At this point, as previously mentioned, the concentrated protein sample was still impure and required further purification to extract the 22 kDa BCX protein. The

protein sample was further purified using a 1 mL HiTrap™ SP FF cation-exchange column (GE Heathcare) equilibrated with protein purification buffer A using the FPLC. This column work at a range of pH values, specifically at a lower pH as is desired with BCX S22C's low isoelectric point. A linear salt gradient (0 – 1M NaCl) over 150 mL was used to elute the protein using Protein Purification Buffer A and B, this gradient promotes slow elution off the column and therefore separation of any contaminants. Eluted protein was detected at 280 nm absorbance values where a distinct peak was detected, illustrated in figure 4.1.1.3(C)., and fractions analysed by SDS-PAGE (figure 4.1.1.3(D)).

3.4.1.5 SDS PAGE and Coomassie Brilliant Blue R-250 Staining

Two approaches were used to detect proteins in this study, FPLC detection of proteins using absorbance at 280 nm and Coomassie Brilliant Blue R-250 staining of proteins resolved by SDS-PAGE. Coomassie Brilliant Blue R-250 binds to basic amino acid residues and can be used to detect proteins or peptides within a SDS-PAGE gel (Westermeier, 2006). Importantly Coomassie Blue staining has a limit of detection and varies dependent on localised protein concentration (Neuhoff V. *et al.*, 1990) and as such both FPLC 280 nm absorbance and Coomassie Brilliant Blue R-250 staining to detect proteins/peptides was used in this study. Protein detection was required for (i) expression/purification of proteins and (ii) linker modification of proteins/peptides leading to a change in molecular weight.

Protein samples were prepared by mixing 5 mL of protein solution and 5 mL of 2 x SDS-PAGE sample buffer. Samples for denaturing SDS-PAGE were heated at 95 ^{o}C for 5 mins before loading onto the gel.

Protein samples (8 µL) and Fisher Molecular Weight Standards (11, 17, 24, 33, 40, 55, 72, 100, 135, 170 KDa) were loaded onto a SDS PAGE gradient gel (4-12% NuPAGE® Novex Bis-Tris gel). The loaded gel was immersed in NuPAGE running buffer and a constant voltage of 180 V applied across the gel for approximately 50 mins. Proteins were stained in the SDS PAGE gel using Coomassie Brilliant Blue R-250 stain by

immersing the gel in the stain (approx volume 15 mL), covering the chamber in cling film followed by microwaving (Proline Microchef, ST22) for 30 s followed by incubation at ambient room temperature on a Stuart mini-orbital shaker for 10 mins. The stain was removed and the gel immersed in destaining solution and incubated at room temperature until the protein bands were visible and the background stain was removed. The destain solution was replaced 3 times over an 30 min period to achieve acceptable destaining and visualization of Coomassie Brilliant Blue R-250 stained bands.

3.5 Conjugation Chemistry for BCX S22C

The aim of this study was to compare the reactivity of a range of novel cysteine-reactive linkers, PermaLink PEG conjugates with commercially available Maleimide PEG. The PermaLink compounds were synthesized and provided by Mr T. Katner and Dr A.G. Watts (Glythera Ltd and University of Bath, UK). Structures for Linker 7 (PL7), Linker 11 (PL11) and Linker 12 (PL12) can be found in the results chapter 5. Three approaches were investigated for reduction of target proteins or peptides followed by reaction with maleimide or PermaLink.

A number of commercial reductants are available for the reduction of cysteine thiols prior to functionalization with cysteine reactive compounds such as maleimide. In this study I choose to use Tris(2-carboxyethyl)phosphine HCL (TCEP) to selectively reduce cysteines in proteins and peptides. This rationale was based on the evidence that (1) TCEP rapidly reduces disulfides in water under acidic conditions at room temperature (Burns *et al.*, 1991), (2) TCEP is resistant to air oxidation unlike other reductants such as Dithiothreitol (DTT) (Getz *et al.*, 1999), (3) TCEP is nonreactive towards functional groups other than cysteine and (4) its presence in the reaction mix is 'predicted' to effect conjugation to a lesser extent with other thiol reactive compounds such as maleimide or iodoacetamide (Getz *et al.*, 1999). In this study water-soluble crystalline TCEP HCl and TCEP covalently bonded to a beaded agarose resin (Thermo Scientific).

3.5.1 Method 1 – Remove TCEP by dialysis

3.5.1.1 TCEP Reduction and Removal by Dialysis

In the first approach to TCEP reduction of cysteines, soluble TCEP was reacted with the BCX target protein and excess reductant removed by dialysis. Purified BCX protein (1 – 2 mg/mL) was reacted with 1 mM TCEP HCl at room temperature for 3 h in an anaerobic chamber (100 % N_2) (miniMAX Anaerobic Workstation) under varied pH conditions (pH 5.5, 7.0 and 8.0) as described in results. TCEP was then removed from the reaction by dialysis. A length of dialysis tubing was cut and left soaking in degassed buffer inside the anaerobic chamber during protein reduction. The prepared cellulose dialysis membrane (10kDa MW cut off, Fisher Scientific) was tied at one end, filled with the BCX TCEP reaction mix (up to 1 mL) and excess air was expelled by squeezing the tubing followed by tying the open end of the tubing. The filled dialysis tube was immersed in 500 mL dialysis buffer (20mM NaOAc + 1mM EDTA, pH varied on experiment) and stirred at room temperature for 40 mins (see figure 3.3.1.2). Dialysis buffer was removed, replaced with an equal volume of fresh dialysis buffer and stirred for a further 40 mins. This procedure was repeated a total of four times to dialyse the BCX protein and remove TCEP from the reaction mix.

3.5.1.2 Reaction with thiol-reactive PEG linker

Once this was complete, the dialysed protein solution is removed from the dialysis tubing (figure 3.3.1.2) and reacted with the PEG-linker (molar equivalents varied dependent on experiment) in reaction buffer. Reduced BCX and PEG-linker were reacted for 12-16 h on a rotorting stand in an anaerobic chamber at ambient room temperature. The reactions were analysed by FPLC and/or SDS PAGE to determine the extent of PEGylation by evaluation of (i) molecular weight shifts and (ii) staining with a PEG detection dye.

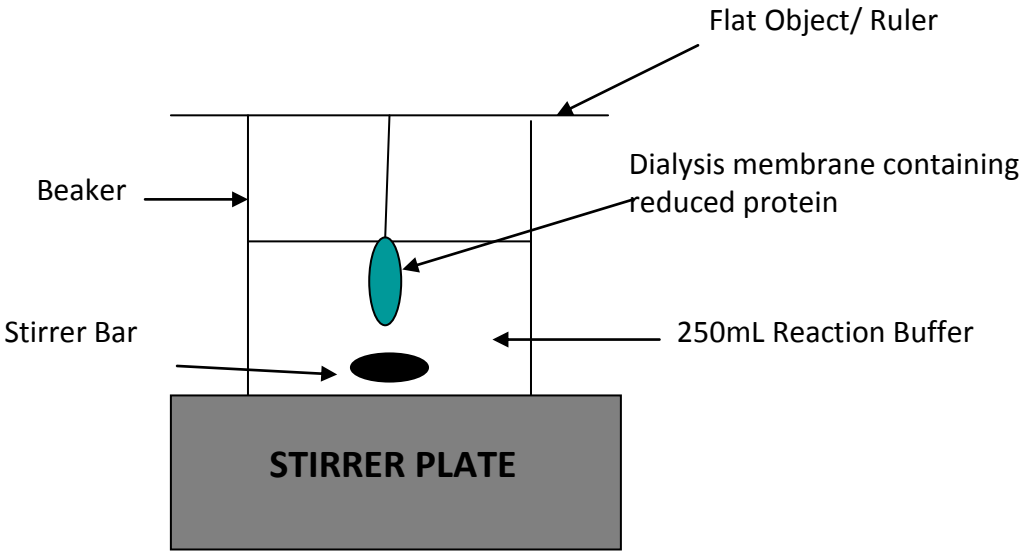


Figure 3.5.1.2 Diagram illustrating the removal of TCEP by dialysis.

3.5.2 Method 2 – Leave TCEP in solution

3.5.2.1 *TCEP Reduction*

As previously mentioned, TCEP is reported to have minimal effects on the cysteine reactivity of other thiol reactive compounds such as maleimide or iodoacetamide. (Getz *et al.*, 1999). In method 2, the thiol reactive conjugate was added in the presence of TCEP in atmospheric oxygen at room temperature. Purified BCX S22C (1-2 mg/mL) was reduced for 2 h with 1.7 molar equivalents of TCEP in 20 mM NaOAc + 1 mM EDTA, (pH dependent on experiment as stated, ambient room temperature).

3.5.2.2 Reaction with thiol reactive PEG-linker

After TCEP reduction, the reduced protein was reacted with 2 – 5 molar equivalents of PEG linker in reaction buffer, and the reaction was left to shake overnight in atmospheric oxygen conditions at ambient room temperature. The reactions were analysed by FPLC and/or SDS PAGE to determine the extent of PEGylation.

3.5.3 Method 3 – TCEP on immobilised resin

3.5.3.1 Preparing the TCEP resin and protein/peptide reduction

Results from Methods 1 and 2 suggested that the presence of TCEP adversely alters the reaction of PermaLink with cysteine residues in the target protein or peptide (see results sections 5.2.3). As a result I also tested a TCEP resin to facilitate efficient removal of TCEP from the reaction mix without the requirement of dialysis or gel filtration. First the sealed immobilised TCEP resin had to be prepared before it was ready to be used to reduce the protein. A volume of the resin slurry (equivalent to the volume of the protein solution – no more then 250 µL) was centrifuged using a HermLe Z160 M centrifuge for 1 min at 4900 r.p.m, 1342g at room temperature and then the supernatant was removed. The resin was then resuspended via vortexing with an equivalent volume of 20mM NaOAc + 1mM EDTA, (pH dependent on experiment) and centrifuged as above. Once all the supernatant is carefully removed, an equivalent volume of protein or peptide solution (0.5 - 1 mg/mL in 20mM NaOAc + 1mM EDTA) was added to the resin and vortexed. The protein/peptide and TCEP resin reduction mix is left shaking for 2 h at ambient room temperature.

3.3.3.2 Reaction with thiol reactive linker

After the reduction with the immobilised TCEP resin, the protein-resin solution was centrifuged for 1 min as above to pellet the TCEP resin. The resulting supernatant contains the reduced protein. This was quickly transferred into a solution containing the PEG-linker (2-5 molar equivalents) in atmospheric oxygen conditions and this is left to react overnight (12 - 14 h) on the shaker. The reactions are analysed by FPLC and/or SDS PAGE to determine the extent of PEGylation.

3.5.4 PEG staining

To determine where conjugation of the linker-PEG to the peptide/protein had been achieved, the SDS PAGE could also be stained with a PEG dye (Dragendorff reagent). After coomassie staining, as outlined in section 3.2.1.5, the SDS PAGE was covered in the PEG dye and left on the shaker for 2-3 mins. The dye was then removed (it can

be recycled) and the bands are resolved in mqH_2O. The areas where PEG is present are stained an orange-brown colour, where when corresponded to areas of Coomassie staining, confirmation of conjugation is achieved.

3. 5.5 Ellmans Reaction

Ellman's Reagent or DTNB [5,5'-Dithiobis(2-nitrobenzoic acid)] solution is used to quantifiy the amount of free thiols in a protein or peptide (Riddles *et al.*, 1979). The absorbance of DTNM was measured using a UV spectrophotometer (Unicam Helios γ machine, Thermo Scientific) at the wavelength 412 nm (A_{412}) where samples were placed in a plastic cuvette. The control background signal was determined using mqH_2O and set as the zero reading. The reaction mix buffer absorbance A_{412} was determined and compared to the A_{412} measured for the reaction mix containing test proteins. The reaction buffer contained: 840 µL mqH_2O, 100 µL 1M Tris (pH 8.0), 50 µL DNTB solution, 10 µL protein sample (0.5 – 1 mg/mL) or mqH_2O for blank. Each sample was well vortexed before being placed into a cuvette and A_{412} absorbance read. To calculate the number of free thiols the following equation is used -

Absorbance of sample = (Total Volume/sample volume) x (Absorbance read at 412nm)

Free Thiols (Molar) = Absorbance of sample)/13600 (Ellman's coefficient)

Equation taken from Ellman's Reagent Instruction Manual supplied with the product from Thermo Scientific, Pierce Biotechnology.

3.5.6 Densitometry

In order to collate the information from multiple Coomassie Brilliant Blue R-250 stained SDS PAGE, I decided to perform densitometry measurements and summarize the data in the form of a histogram. However it is recognized that densitometry has several fundamental limitations that can introduce variation and errors in the quantification of Coomassie Brilliant Blue R-250 labelled proteins by SDS PAGE. Three steps are required for quantification: image acquisition, selection of bands and computed aided density measurements. It is important to ensure that (i) continuity in image acquisition is maintained, (ii) awareness that proteins may present different

modifications leading to alterations in Coomassie Brilliant Blue R-250 staining and (iii) different software used may use different alogorithms to quantify a protein band (Gassmann *et al*., 2009).

To minimize these variations and limit errors in quantification, samples were run in the same SDS PAGE gel for each individual experiment and scanned under the same conitions for all experiments. The Coomassie Brilliant Blue R-250 stained SDS PAGE was sealed in a plastic wallet and digitally scanned using a Canon Lide 70 scanner and Adobe Photoshop 7.0 software at 150 dpi where the image was stored as a JPEG file. Each scanned image was then analysed using ImageJ software (Public domain software developed by National Institutes of Health, USA). Three measurements were taken from each protein lane, (1) the background signal, (2) predicted PEG'd protein signal and (3) predicted unreacted protein signal. The total protein signal was the sum of all the density measurements of all the bands in the protein lane minus the background signal.
The percent PEGylated protein was calculated by dividing the PEGd protein (-background signal) by the total protein signal.
Data illustrated in a histogram as the mean with the standard error of the mean (SEM using Microsoft Office Excel). Comparisons were made to compare the extent of conjugation between methods, pH and maleimide to PermaLink linkers.

Data were represented by a histogram with the data reported as the mean ± S.E.M (n=3 unless stated other wise) where values were obtained from independent experiments. Where assessed, significance was determined using a paired t-test on Microsoft Excel 2007 software.

3.5.7 NMR Analysis

Small reactions were set up between the PermaLink linker and TCEP in 10 mM NaOAc at pH7 which was then monitored by NMR.

Chapter 4.0 Target Proteins and Competitive Technologies

4.1 Protein Expression and Purification

4.1.1 BCX S22C

4.1.1.1 Introduction

BCX is used as a model protein in these experiments because of its similar properties to other therapeutic proteins. The expression of the *BCX* enzyme has long used *E.coli* as a suitable expression system. However, the natural gene of this enzyme does not express well in *E.coli* (Yang *et al.*, 1989). The potential applications of this enzyme in industry make it a desirable target for genetic mutation to ensure its stability when used in manufacturing. In 1993, Sung *et al.*, produced a gene for BCX which imitated the frequency of degenerate codons in *E.coli*. This also contained multiple restriction sites for future mutagenesis studies and was modified for direct expression in *E.coli.* The development of this mutant marked a steep improvement in levels of expression, up to 300mg, when compared to previous expression of the gene. This development was cemented by the purification of a functionally active enzyme from the expression.

Various mutants of BCX have been produced in the proceeding years, many of which involve the thermostabilisation of the enzyme by attempts at introducing a disulphide bridge (Wakarchuk *et al.*, 1994). The production of the BCX S22C mutant was initiated to produce a model neoglycoprotein. Substituting the serine residue at position 22, a cysteine was introduced (Muellgger *et al.*, 2006 – supplementary information).

All mutants of BCX, follow the expression protocol laid out in Sung *et al.*, (1993), produced high yields of the enzyme. For the purpose of this investigation, the BCX S22C mutant had been further mutated to express a his tag for purification purposes. This was then expressed in *E.coli* strain BL21 DE3, successfully producing high yields of the protein – see figure 4.1.1.3.

4.1.1.2 Summary of Findings

Optimum conditions were found to express the protein including a) lowering the temperature after induction of expression and b) cooling the culture before addition of IPTG. The two step purification of the protein ensured the sample was uncontaminated; however, the longer the process of purification the more chance there is of loss of yield.

4.1.1.3 Results

4.1.1.3.1 Expression

BCX S22C was expressed using *E.coli* BL21 DE3 with optimal protein production detected when expression is induced at 18 ^{o}C with IPTG. In figure 4.1.1.3 (A) expression of proteins are visualised using SDS PAGE alongside total lysate (TL) and the flow through (FT), after the first stage of purification (see figure 4.1.1.3.B), where protein of the predicted molecular weight for BCX S22C is indicated in the figure.

4.1.1.3.2 Purification

There are two stages in the purification of BCX S22C. Firstly, affinity purification from the total lysate using a Ni^{2+} column to bind to the BCX His tag, followed by elution using an imidazole gradient (20 – 500mM) to compete for Ni^{2+} binding - see figure 4.1.1.3 (C). Here, the cell pellet is lysed in buffer A containing 20mM imidazole to equilibrate with the starting buffer of the FPLC. The column is also equilibrated to prevent any non-specific binding and to ensure the lysate has affinity to the resin in the column. The protein always elutes at about 40% buffer B, so about 200mM imidazole concentration. A steep increase in absorbance is detected at this stage as the protein is eluted off the column (Figure 4.1.1.3 (D).

Fractions eluted off the column were combined and concentrated where the average concentration yielded from the expression was between 2-4 mg/mL. The purified BCX S22C was used for subsequent thiol conjugation experiments.

Figure 4.1.1.3 –BCX S22C Expression

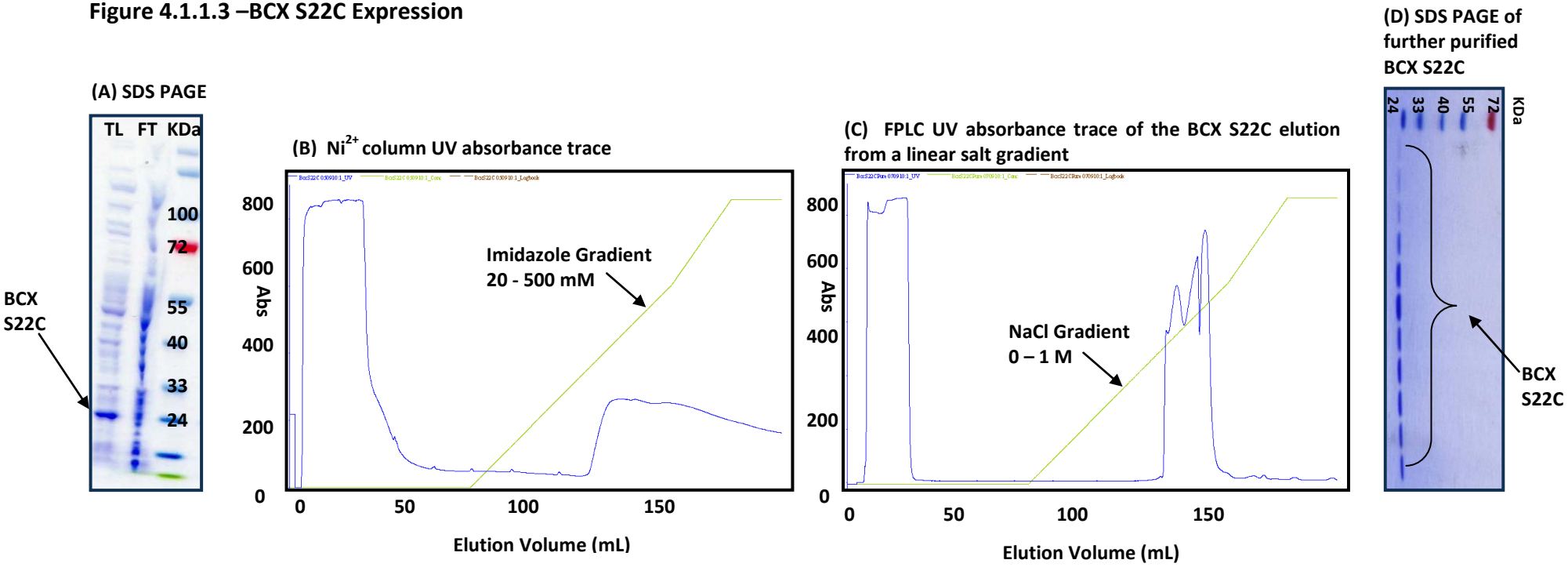


Figure 4.1.1.3 – The protein is expressed at 18°C overnight using 100 µg/µl IPTG. In **(A)** the flow through (FT) from the first step of purification can be seen, alongside the collected total lysate (TL) where higher levels of BCX S22C expression can be seen. Cells are lysed using a cell disruption system and purified using a nickel column on an Akta FPLC machine using a linear imidazole gradient **(B)**. The fractions are collected and further purified using an ion exchange column and a salt gradient **(C)**. Fractions collected from the second purification are run on an SDS PAGE and the protein bands produced are checked they are of the correct molecular weight for BCX S22C. **(D)**

4.1.2 Salmon Calcitonin (sCT)

4.1.2.1 Introduction

sCT has a variety of roles in the body and is an important therapeutic for different types of bone disease (Chestnut III *et al.*, 2008)). It is naturally produced as a hormone precursor which is post-translationally processed in the endoplasmic reticulum before secretion (Takahashi *et al.*, 1997). There are several advantages in harvesting a peptide from the extracellular matrix; more simplified purification process, increased chance of correct refolding and post-translational processing and you elicit less stress response from the host cell (Ray *et al.*, 2002).

Problems have arisen as gram-negative *E. coli*, which contain both an inner and outer membrane, making it difficult for the peptide to be secreted (Nikaido, 1996) in expression systems. Attempts have been made to make the membrane more permeable (Atlan *et al.*, 1986) or implementing osmotic stress to release the protein after cell growth (Blackwell & Horan, 1991). However, some of these methods result in a fragile host cell, making generated target peptides more susceptible to degradation by proteases. Despite this, peptides that can be harvested from the culture media are still highly desired and research is ongoing into making this process more efficient (Ray *et al.*, 2002).

The sCT used in these studies was provided by Warwick Polymers as a dry power stored at 4^{o}C. A known mass of sCT was dissolved in reaction buffer (see methods) and reacted with the relevant PEG. It was important in this instance to check the purified product and find out where it elutes on the FPLC as the peptide would potentially be too small to detect on an SDS PAGE.

4.1.2.2 Summary of Key Findings

The sCT was eluted off a linear salt gradient in one distinct peak at approximately 40% Buffer B.

4.1.2.3 Results

As seen in Figure 4.1.2.3, the sCT is eluted off the column at about 40% Buffer B, where a minimum of 0.5mg of sCT needed to be used to detect an absorbance on the FPLC.

Figure 4.1.2.3 – Purified sCT

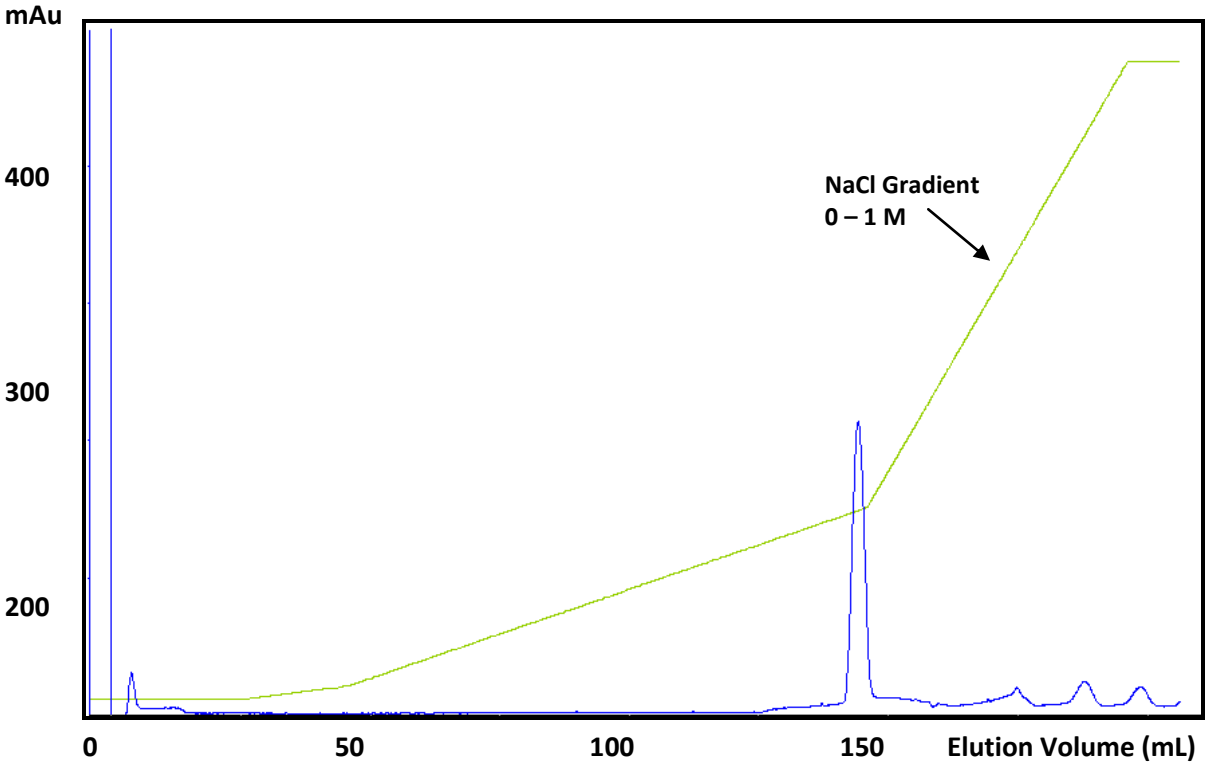


Figure 4.1.2.3 – Approximately 1mg of sCT (molecular weight 3200 Daltons) was eluted off the Akta FPLC using a linear salt gradient; Buffer A – 10mM NaOAc, pH4.5 and Buffer B – 10mM NaOAc + 1M NaCl, pH 4.5 were used to create the gradient. One distinct peak is detected at approximately 40% buffer B.

4.2 Conjugation of proteins/peptides to maleimide-PEG

4.2.1 BCX S22C

4.2.1.1 Introduction

BCX S22C works successfully as a model protein in these studies because it is a well characterised protein of approximately the same size as a cytokine (a commonly used therapeutic) and its three dimensional structure has been established by X-ray crystallography and NMR spectroscopy (Wakarchuk *et al.*, 1994), potentially providing a means of analysis of conjugation. Similarly, it is xylanase enzyme, its natural function is the breakdown of hemicellulose in plant cell walls, therefore its activity after conjugation can be analysed.

TCEP has long been used as a convenient phosphine for reduction of disulphides in water (Burns *et al.*, 1990). It is known to be very stable in both acidic and basic solutions, contrary to dithiothreitol (DTT) which rapidly reoxidises above pH7.5. Similarly, it is still active at either end of the pH range, effectively reducing thiols at very low or very high pH (Han & Han, 1994). TCEP was chosen in this instance as it had previously been noted to allow maleimide attachment to myosin in its presence, whereas DTT had not and the conjugation was inhibited (Getz *et al.*, 1999). Similarly, after Ni^{2+} column purification, contaminating Ni^{2+} ions do not affect the stability of TCEP by oxidation as they do with DTT.

Maleimide is one of the most commonly used linker technology for thiol specific conjugation (Roberts *et al.*, 2002). It is fast and stable in most reaction conditions, and shows specific conjugation at more acidic pH (Pasut & Veronese, 2007). It has been used to conjugate PEGs to therapeutic proteins and has been used in commercially available biopharmaceuticals for some time. However, some problems with maleimide such as stability and specificity have arisen. Maleimide can also react with amino groups on lysine residues which are common on a protein surface (Sharpless & Flavin, 1966), resulting in heterogenous products from protein conjugation. However, this is often a consequence of reaction conditions which can

be manipulated. Ring unfolding can also lead to the production of a heterogenous products even if malemide reacts with only the required cysteine.

As maleimide is commonly used for cysteine conjugation of PEG molecules to proteins or peptides (Roberts *et al.*, 2002), we have used maleimide-PEG as the standard control to compare the rate and specificity of PermaLink-PEG reactions. Two approaches have been used to evaluate the PEGylation of proteins or peptides. First, we have separated proteins by SDS PAGE and labelled for protein (Coomassie Brilliant Blue R-250) and PEG (Dragendorff reagent). Standard densitometry measurements have been used to assess the intensity of the bands (Schmidt *et al.*, 1987). Using a second method, FPLC can be used to separate unPEGylated from PEGylated proteins where UV absorbance measurements are used to determine the amount of protein in each fraction. Similarly, the FPLC trace used to separate the larger molecular weight PEG reaction mixtures and are visually representative of the extent of reaction. The FPLC separated fractions can be analysed by SDS PAGE where PEGylation can be confirmed by (i) the shift in molecular weight and (ii) a reaction with the PEG specific dyes..

4.2.1.2 Summary of key findings

- Using BCX as a model protein, maleimide conjugated to 2KDa PEG or 20KDa PEG is highly reactive at an optimum pH of 7.
- The reactivity of maleimide-PEG with the target protein is optimal if the reducing agent TCEP is removed before the addition of the maleimide PEG for conjugation. After comparing the range of methodologies, I used immobilized TCEP resin to reduce the target cysteine as this could be efficiently removed from the target protein or peptide.
- The reaction of BCX S22C with Maleimide-2KDa PEG leads to multi-PEGylation of the target protein suggesting a reaction with non-cysteine amino acids.
- Maleimide-20KDa PEG efficiently reacts with BCX S22C to generate one PEGylated product detected by FPLC and SDS PAGE.

4.2.1.3 Results

4.2.1.3.1 BCX S22C Controls with TCEP

The free cysteine residue in the mutated protein facilitates disulphide bridge formation and therefore dimer formation. BCX S22C monomer and dimers can be detected in a sample of purified BCX S22C using FPLC and a non-reducing SDS PAGE. By FPLC analysis, the peaks of absorbance are predicted to equate to the monomer and dimer of the protein (figure 4.2.1.3.1). The protein is stored and run through the FPLC at pH4, the BCX S22C monomer elutes off the column at about 40% buffer B (20mM NaOAc, 1mM EDTA, 1M NaCl) and the dimer at about 50% buffer B; that is 400mM and 500mM NaCl respectively.

Figure 4.2.1.3.1 – BCX Controls

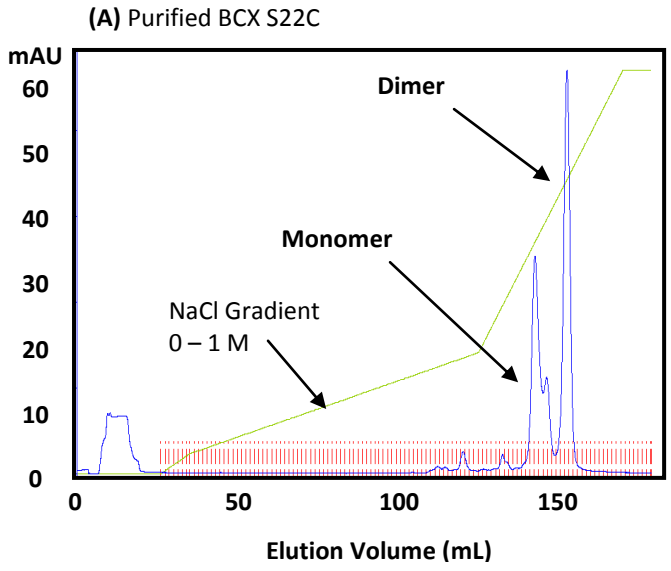


Figure 4.2.1.3.1(A) –0.5mg of BCX S22C was run through the FPLC with a linear salt gradient from 0 – 1M NaCl. **(A)** You see two distinct peaks which equate to the monomer and dimer which the protein naturally forms due to the cysteine residues.

In order to break the disulphide bond and allow the thiol group on the cysteine residue free for conjugation, the protein must be reduced. The target protein is incubated with a molar excess of a reducing agent TCEP. In this case, 1.7 molar equivalents of TCEP (1 hour at ambient room temperature) was used to reduce BCX S22C. The reduction of the protein is clearly seen in figure 4.2.1.3.1 (B), where there is a shift and an increase in the amount of monomer to dimer. It could be considered that in the time taken to get the mixture onto the FPLC and the running of the mixture through the FPLC, a certain amount of reoxidation could have taken place.

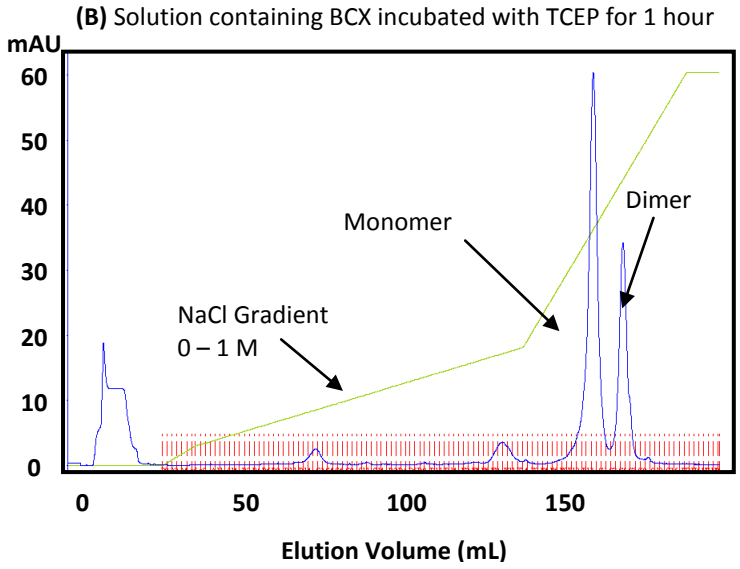


Figure 4.2.1.3.1 (B) After the 0.5mg of BCXS22C is incubated with TCEP solution (1.7 molar equivalents) for 60 minutes you see a shift in the peaks and the amount of monomer increases relative to the dimer. The small peaks seen eluted off the column before the protein are probably minor impurities, they were not detected on an SDS PAGE and there absorbance value detected via the FPLC meant they were negligible.

4.2.1.3.2 Reactivity of BCX S22C with maleimide 2KDa PEG

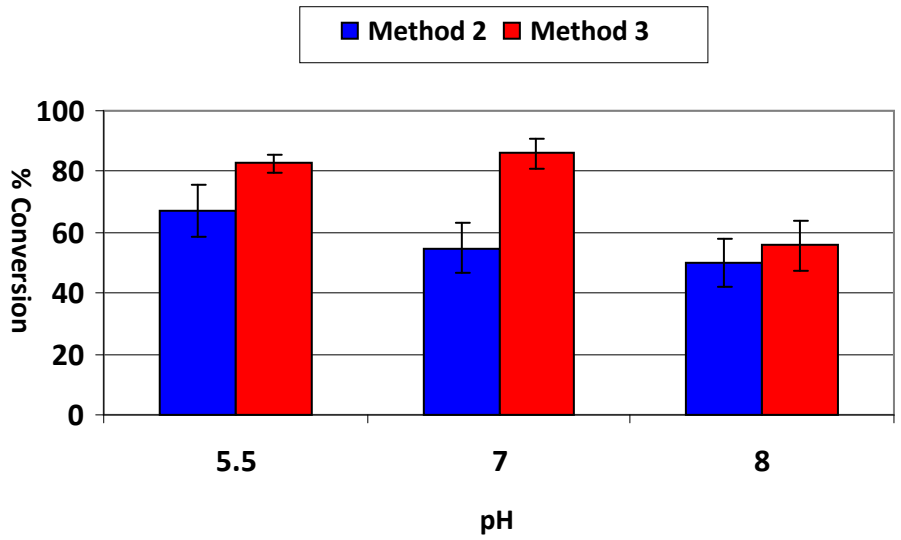
In a series of control experiments, the reactivity of maleimide 2KDa PEG with BCX S22C was investigated by altering the pH and reducing conditions.

I compared the amount of monoPEGylation in the continued presence of TCEP (Method 2) versus removing TCEP from the reaction before the addition of maleimide-PEG (Method 3). Also, varying the pH of the reaction to find its optimum was important as the optimum pH of the reaction is often dependent on the pH at which the protein is most stable. Leaving the TCEP in the solution (Method 2) as seen in figure 4.2.1.3.2(A & B) decreases the degree of protein PEGylation detected. Although a high amount of conjugation is seen, the presence of TCEP clearly has an effect with its optimum pH 5.5 only achieving 67 % ± 8.7 % (*n* = 3 ± S. E. M) conversion determined by densitometry measurements of SDS PAGE analysis. Unreacted and PEGylated protein were detected at the reaction pH 5.5 using Method 2 (4.2.1.3.2B, n=3)

Next the effect of removing TCEP from the reaction mixture by using an immobilised TCEP resin (Method 3) was investigated. At all three pH's tested the conversion rate was improved compared to Method 2. However, pH7 was its optimum achieved with 86 % ± 4.8 % (*n* = 3 ± S. E. M) maleimide-PEG BCXS22C conjugation. However, SDS PAGE analysis reveals that BCX S22C is multiPEGylated under these reaction conditions (pH7, method 3) indicating non-specific binding to non-cysteine residues (figure 4.2.1.3.2C).

Figure 4.2.1.3.2 – BCX S22C and Maleimide 2KDa PEGs

(A) Graph Displaying the Percent Conversion of Maleimide Conjugation with BCX S22C (mean±S.E.M), n=3 (independent samples)



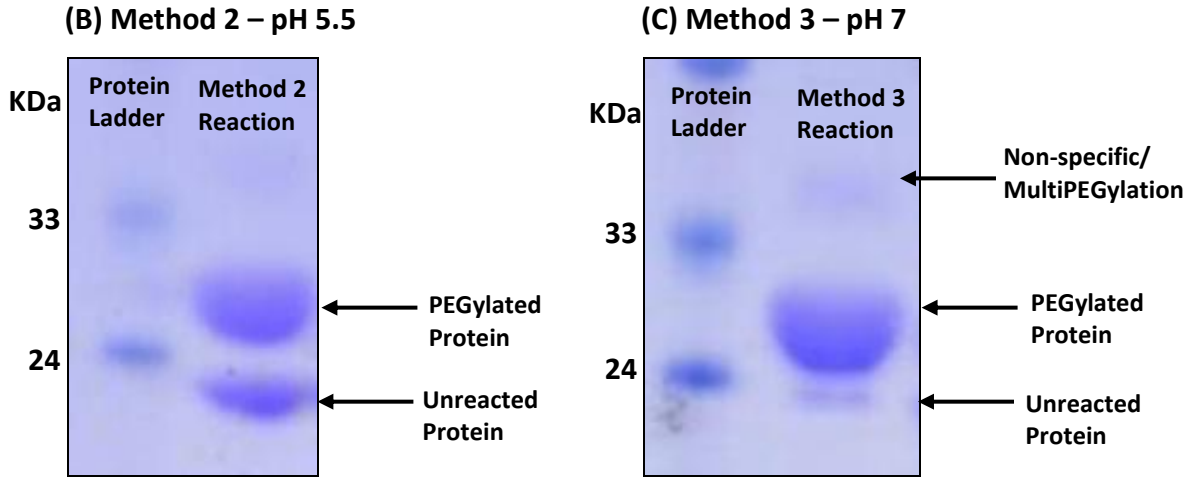


Figure 4.2.1.3.2 – A known concentration of BCX S22C was reacted with 5 equivalents of Mal-PEG at pH5.5, 7 and 8 using methods 1 and 2. **(A)** Shows the difference in extent of reaction, where method 3 is clearly favoured over leaving TCEP in the solution, mean ± S.E.M. (n=3 independent samples). **(B)** shows an example of the SDS PAGE using the optimum pH for method 2. **(C)** shows the high conversion rate using method 3 at its optimum pH, however, non-specific and multi-PEGylation is also seen at this pH.

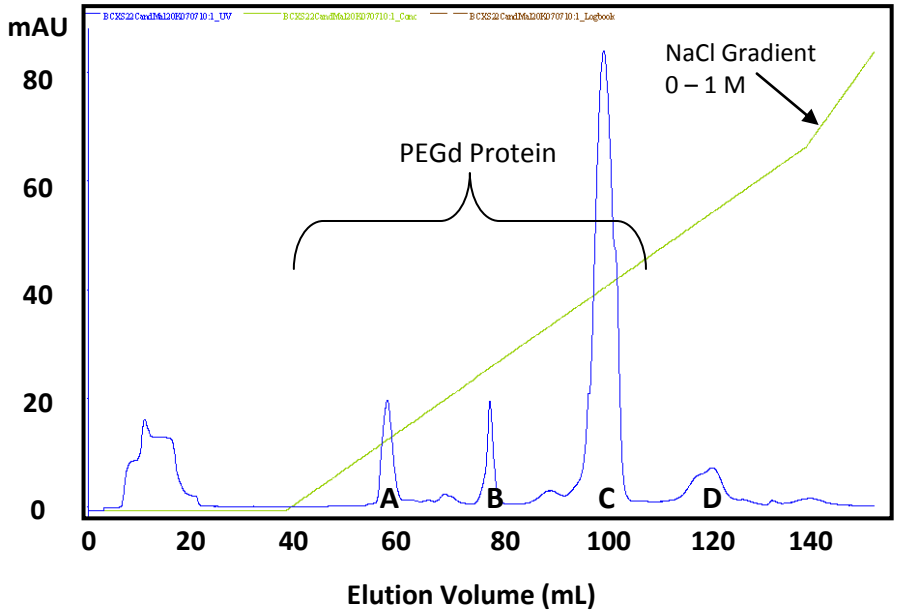
4.2.1.3.3 BCX S22C and maleimide 20KDa PEG

For the PEG attached to have an effect on a protein's pharmacokinetic properties it must increase the protein's molecular weight substantially (Jevesar *et al.*, 2010). By attaching a 20KDa PEG the overall weight of a BCX S22C and Mal-PEG conjugate would be approximately 42KDa; however, the migration of the PEG through the SDS PAGE means it does not fall at exactly this molecular weight.

In the presence of TCEP (Method 2), the majority of protein is PEGylated, visually determined by the lack of unreacted BCX S22C (seen on the SDS PAGE gel, figure 4.2.1.3.3.1B). However, the PEGylated species is eluting in various places along the salt gradient (figure 4.2.1.3.3.1A).

Figure 4.2.1.3.3.1 - BCX S22C and Maleimide 20KDa PEG – Method

(A) UV trace of reaction mixture from FPLC



The SDS PAGE gel highlights that all the PEGylated species are of the same molecular weight (figure 4.2.1.3.3.1B), indicating the attachment of one PEG molecule.

(B) SDS PAGE of fractions obtained from FPLC, gel also stained with PEG Dye

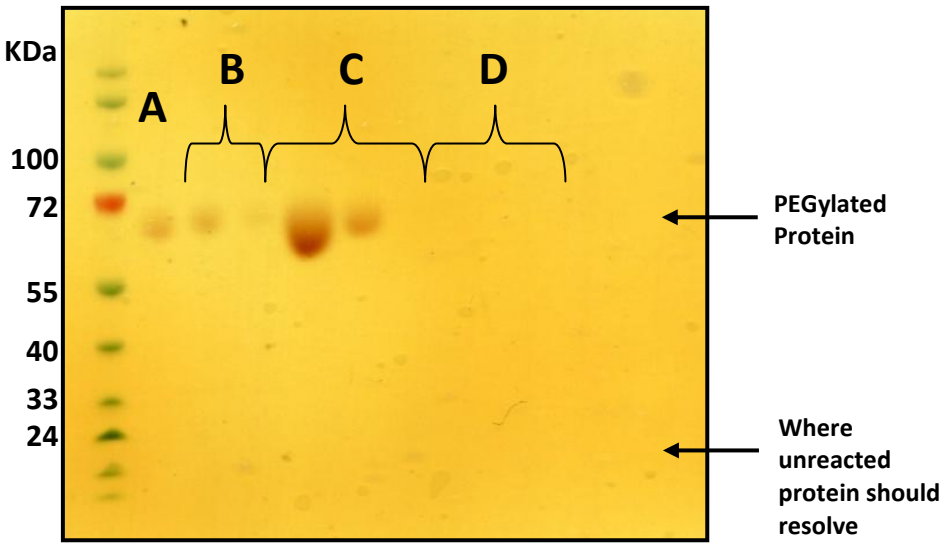


Figure 4.2.1.3.3.1 – BCX S22C was reduced at 2mg/mL with 1.7 molar equivalents TCEP for 2 hours at pH7. It was then reacted with 2 equivalents of Maleimide P20K overnight. **(A)** FPLC Trace obtained from the reaction mixture. **(B)** reducing gel of FPLC fractions ranging over all peaks from the FPLC. There is unusual separation of PEGylated fractions seen, perhaps suggesting non-specific PEGylation.

As seen with the lower molecular weight PEGs, the presence of TCEP may be having an effect on the PEGylation process. After investigation into TCEPs presence in the reaction, another method where TCEP is removed was developed (method 3). A vast improvement is noted in PEGylation efficiency (Figure 4.2.1.3.3.2).

Figure 4.2.1.3.3.2 – BCX S22C and Maleimide 20KDa PEG – Method 3

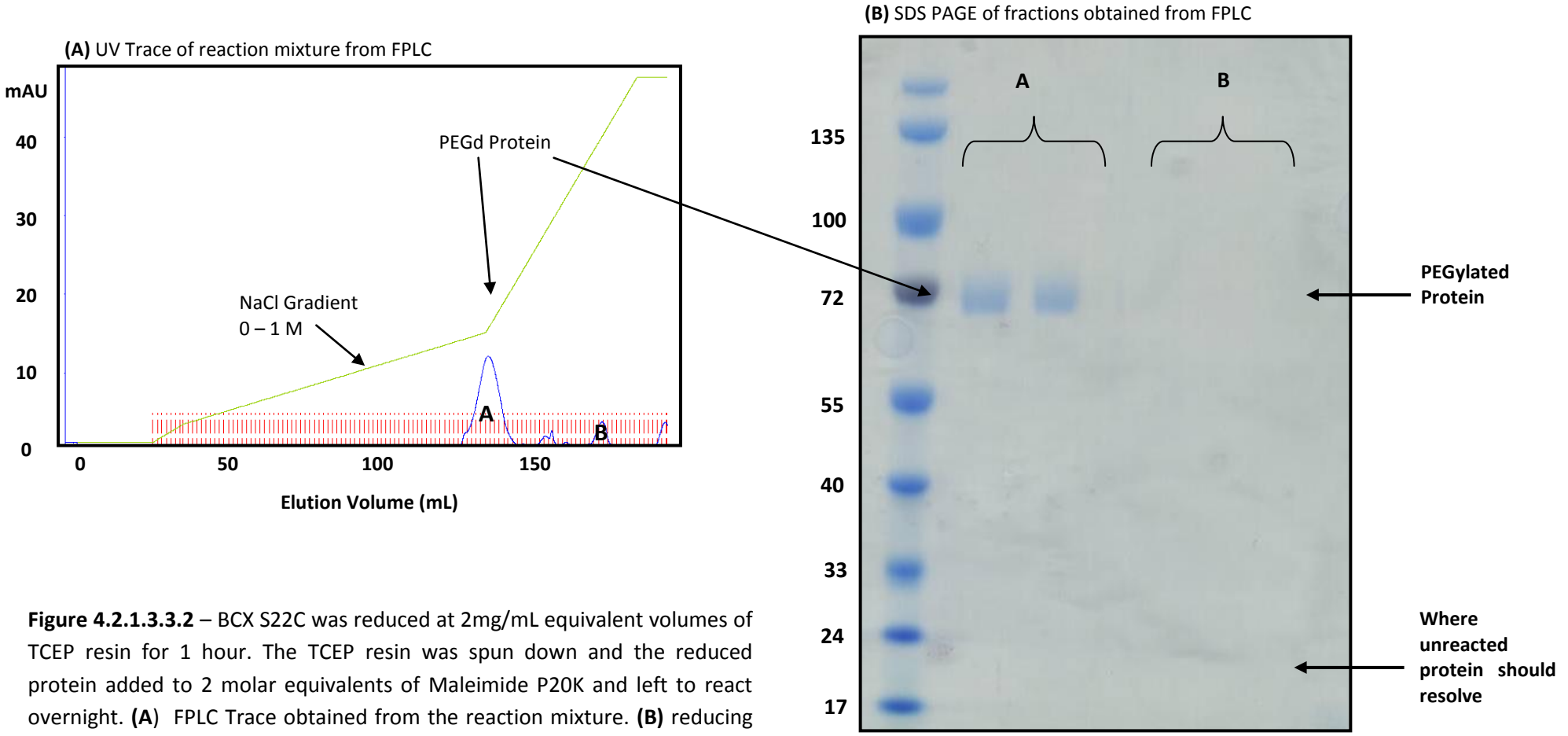


Figure 4.2.1.3.3.2 – BCX S22C was reduced at 2mg/mL equivalent volumes of TCEP resin for 1 hour. The TCEP resin was spun down and the reduced protein added to 2 molar equivalents of Maleimide P20K and left to react overnight. **(A)** FPLC Trace obtained from the reaction mixture. **(B)** reducing gel of FPLC fractions ranging over all peaks from the FPLC.

4.2.2 sCT

4.2.2.1 Introduction

Although sCT only shares 50% sequence homology with human calcitonin, it is the mostly widely used source of calcitonin used for clinical therapy (Chesnut *et al.*, 2008). This is because sCT exhibits more potency and analgesic properties then human calcitonin in most biological assays (Houssami *et al.*, 1995; Breimer *et al.*, 1988). sCT's variety of clinical uses make it a desirable target for processes which increase its therapeutic potential in the body. Established as one of the first antiresorptive osteoporosis therapies, sCT has exhibited efficacy and a positive safety profile over the last thirty years. It is currently used to treat metabolic bone disease such as osteoporosis and Paget's disease and also indications into treatment of osteoarthritis (Chesnut III C. *et al.*, 2008).

Various efforts towards increasing its therapeutic potential and efficacy have been investigated. Cetin *et al.* (2008), successfully biotinylated sCT and evaluated its hypocalcemic effects in rats. They noted an increase in sCT permeability through Caco-2 cell monolayers and the consequent sustained reduction of calcium ions in the blood plasma. These data indicate the oral bioavailability of conjugated sCT and the potential clinical application.

Similarly, conjugating sCT to maleimide PEGs has previously been attempted. The sCT was multi-PEGylated at both cysteine resides (position 1 and 7) and again at either lysine residue 11 or 17 (Cheng & Ling, 2009). When comparing the different conjugates, increased peptide PEGylation was found to correlate with increased stability in rodent intestinal fluids. However, when injected into the rat, the extensively PEGylated conjugate exhibited no enhanced efficacy (or was inactive) when compared to just one Mal-PEG attached to sCT. This indicated that the hormones hypocalcemic activity was not enhanced with its increased stability in the rodent intestinal fluid.

In conclusion, while PEG groups can be successfully attached to sCT to generate a therapeutic there are potential issues with multi-PEGylation using the maleimide technology. My first aim was to functionalise sCT with the maleimide PEG to assess the level of PEGylation under different reaction conditions.

4.2.2.2 Summary of key findings

- The reaction between maleimide 20KDa PEG and sCT is complete in 30 minutes.
- The reaction with Mal 20KDa PEGs produce the mono- and di- PEGylated species, due to a predicted attachment to free thiol groups on cysteine residue 1 and 7. However, on closer analysis, the crude reaction mixture shows multiple PEGylated species indicating off-target, non-specific PEGylation.

4.2.2.3 Results

4.2.2.3.1 Reaction Conditions

Ellman's reagent was used as described in *Methods* to determine an optimum reduction time for the small peptide. At 30 minutes, the absorbance value no longer increased indicating that it had reached its maximum extent of thiol reduction, both with method 2 and method 3. At 2mg/mL the peptide was reduced (using either method 2 or method 3) for 30 minutes. The resulting peptide solution was then reacted with 2 molar equivalents of Maleimide 20KDa PEG overnight on a shaker at ambient room temperature. The 20 KDa PEG conjugate was analysed by FPLC and SDS PAGE as described in the following sections (Section 4.2.2.3.2).

Attempts were also made to attach a 2KDa PEG to sCT however the total predicted molecular weight of sCT-2KDa PEG was 3.4KDa and were not easily resolved with an SDS PAGE.

4.2.2.3.2 sCT and Maleimide 20KDa PEG

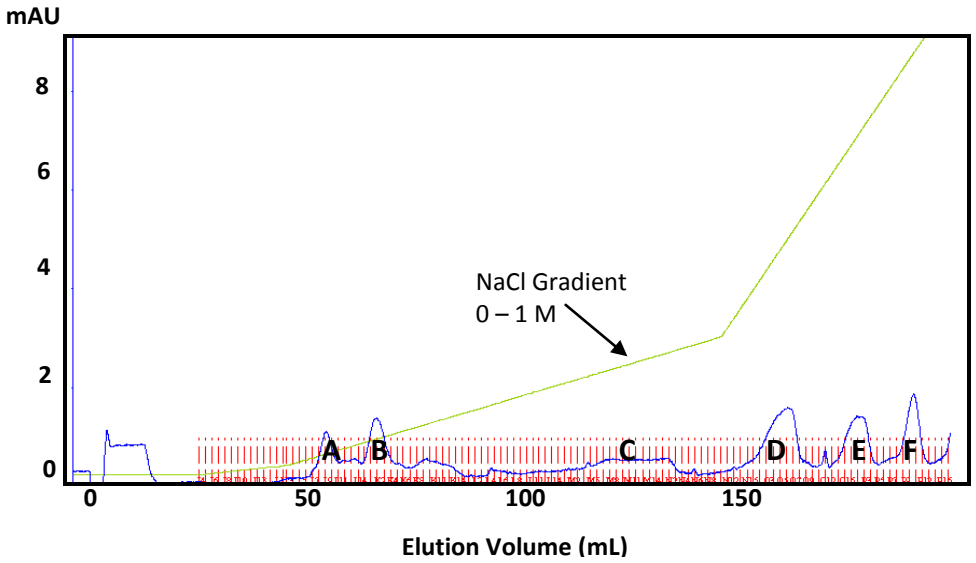
Maleimide 20KDa PEG (2 molar equivalents) was reacted with sCT as previously described using method 2. The products of the reaction were separated by FPLC using Reaction Buffer A and B as explained in the *Methods* section. The FPLC separated products were then analysed by SDS PAGE and the molecular weight compared to the unreacted sCT control (figure 4.2.2.3.2).

When maleimide 20KDa PEG with sCT you would expect a molecular weight of approximately 3200 + 20,000 Da = 23,200Da if one PEG molecule attaches to one cysteine; or 3200 + 40,000 Da = 43,200 Da if it attaches to the two available cysteines (residue 1 and 7). However, it has been noted that due to the size and migration of the PEG molecule through the polyacrylamide gel, the molecular weight associated with the protein ladder is not accurate (Kurfust M. M., 1992). Therefore, with the assumption made that only a thiol specific conjugation takes place with maleimide and therefore only the two cysteine residues are PEGylated, the mono- and di-PEGylated species of sCT can be seen in figure 4.2.2.3.2B in samples A and B.

Both the absorbance peaks on the FPLC and bands on the SDS PAGE are of comparable intensity, indicating that the preference of one cysteine over the other is not a factor (Figure 4.2.2.3.2). If one cysteine was being sterically hindered in some way the maleimide PEG molecule would only be able to access the other cysteine. You would therefore get more of the mono-PEGylated species, rather than the di-PEGylated form. It is also important to mention here that the bands detected with the PEG dye corresponds with Coomassie Brilliant Blue R-250 protein staining on the SDS PAGE, indicating the presence of peptide-conjugated PEG groups resolved by SDS PAGE.

Figure 4.2.2.3.2 – sCT and Malemide 20KDa PEGs – Method 3

(A) UV Trace of reaction mixture from FPLC



(B) SDS PAGE of fractions obtained from the FPLC

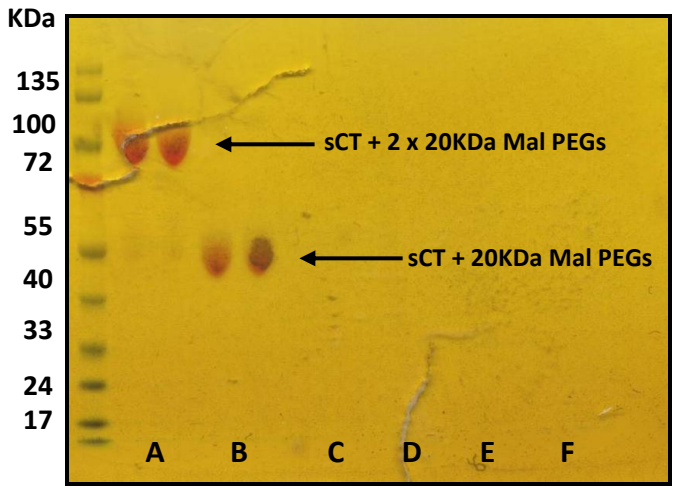


Figure 4.2.2.3.2 - 1mg of sCT was reduced for 30 minutes and reacted with two molar equivalents of linker overnight. The FPLC trace **(A)** exhibits multiple peaks, only two of which show up on the SDS PAGE **(B)** where distinct PEGd protein can be detected at two different molecular weights. Prior to staining with the PEG dye these areas on the SDS PAGE were stained with coommassie, indicating that both PEG and protein are present and stressing that it is not just unreacted PEG.

4.2.2.3.3 Maleimide Crude Reactions

The PEG maleimide sCT reactions products separated via FPLC have a low absorbance value (≤10 mAU), and the samples are diluted as they are eluted from the column. In addition, I could not detect CB or PEG labelling in samples C-F collected from the FPLC separation (Figure 4.2.2.3.2). To ensure that no reaction product is lost or diluted to a level below detection during FPLC purification, the total crude reaction was analysed by SDS PAGE to determine the content of the whole reaction (figure 4.2.2.3.3). Not only are the two dominant PEGylated species present (i.e. A and B) but also multi and non-specific PEGylation can be identified where PEG bands can be seen at higher molecular weights.

Figure 4.2.2.3.3 – Crude Reaction Mixture of sCT and Maleimide 20KDa PEG Method 3

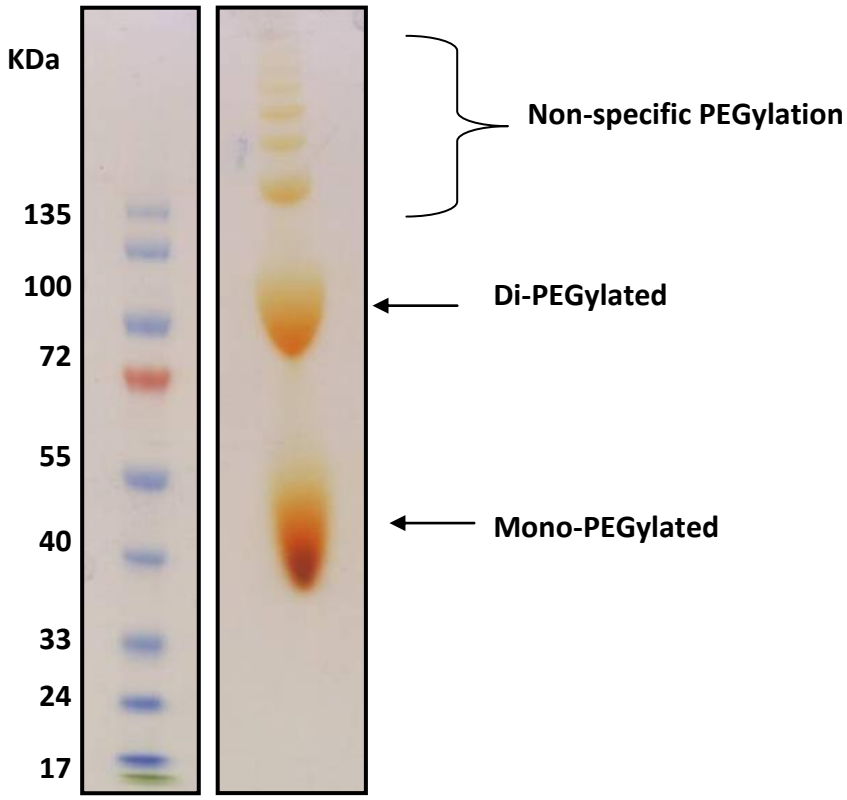


Figure 4.2.2.3.3 – Crude reaction of maleimide 20KDa PEG and sCT. sCT reacted at 2mg/mL with two molar equivalents of PEG. Non-specific PEGylated bands detected, indicating that the maleimide linker is not only reacting with the cysteine residue.

4.3 Discussion

4.3.1 Protein Expression

Expression of the BCX mutant which was modified for direct expression in *E.coli* by Sung *et al.*, (1993), produced high yields of the enzyme - up to 300mg/L. On average, each litre of BCX S22C mutant culture produced only about 4mg of BCX S22C. Clearly, this is not to the same level of yield as the original mutant produced. However, our mutant contained a modified cysteine residue which can form a disulphide bridge and therefore dimers during production (Sigman *et al.*, 2003). This could have lowered the yield as the protein may have formed aggregates. Similarly, the concentration coefficient was not corrected for the potential introduction of disulphides (Wakarchuk *et al.*, 1994), possibly affecting the UV absorbance reading.

Addition of a polyhistidine tag in this BCX S22C mutant allowed purification through a nickel column which has been proven in its efficiency of isolation of recombinant proteins (Cao & Lin, 2009). The initial stage of purifying the protein through the nickel column and an imidazole gradient allowed a notable clearance of unwanted proteins produced alongside the BCX S22C. The protein eluted from the nickel column was then concentrated in the knowledge that it contained the purified target product. As the future use of the purified protein required precise, known concentrations of the reactants it was essential that the product contained no contaminants. This is why it was necessary to further purify the protein by ion exchange chromatography.

4.3.2 BCX and Maleimide Conjugation

It is clear that TCEP present in the solution has an effect on the extent of reaction with both the 2KDa and 20KDa Mal-PEG conjugates. When using the TCEP resin and therefore its removal from the solution, the extent of conjugation increases significantly ($p<0.05$) at pH 7. This result suggested a potentiation of the reaction between TCEP and maleimide that has previously been reported by Shafer *et al.*, (2000) under certain conditions. Therefore by leaving TCEP in solution, especially in excess of the protein and of similar molar equivalents of the PEG, it is likely that

there was an ongoing side reaction between the linker and TCEP. By effectively removing the linker-PEG from solution it is no longer available for conjugation to the free thiol on the protein, hence its low conjugation efficiency.

The next key question would be why, when using the resin and therefore removing the TCEP from solution, the reaction does not go on to 100% conversion. From the graph, pH7 appears the optimum pH for this reaction but the mean value of conversion is only at about 86%. It is probable that during setting up these small scale reactions, that some protein will be reoxidised. The stage where the reduced protein is removed from the TCEP resin allows for this reoxidation, this is because particular care is required when removing the solution from the top of the resin. The potential of reoxidation of the free thiol increases as the time to remove the protein from the resin increases. If the reduced thiol group reoxidises, the linker cannot react with the protein. Similarly, it is assumed that the protein has correctly refolded and therefore the thiol group is available for both reduction and reaction with the linker-PEG. This could be determined with enzymatic activity assays.

To overcome these technical problems, an improved method of reducing the protein with the resin needs to be formulated particularly upon scale up for the commercial generation of therapeutics. The production of biologics is likely to occur on a larger scale that would facilitate the use of TCEP-resin columns under nitrogen to enable the efficient loading of reactants on and off the column with minimal reoxidation.

4.3.3 sCT and Maleimide Conjugation

The distinct separation of the two PEGylated species in the sCT and Mal-20KDa PEG reaction leads to the assumption that the two cysteine residues have been targeted by this thiol specific linker. The reason for the linker preference of one cysteine versus the other is unclear as there is double the molar equivalents of Mal-PEG to react with sCT – both the reduced cysteines have the potential and availability to react with Mal-PEG. However, steric hindrance created by one 20KDa PEG molecule once attached may prevent consequent conjugation to the other available thiol

group. On looking at the crude reaction on an SDS PAGE (Figure 4.2.2.3.3) it can be seen that there are other sites of conjugation which may also play a role in this.

It took several attempts to optimize the sCT peptide concentration required to detect it by FPLC and SDS PAGE. However, by separating out the reaction mixture by FPLC, all samples are diluted on the SDS PAGE and are therefore difficult to detect. The crude reaction, not separated by FPLC, were run directly on SDS PAGE for additional analysis (Figure 4.2.2.3.3)**.** Interestingly, the maleimide reaction mixture exhibited other PEG bands at higher molecular weights. This indicated other PEGylation sites on the sCT peptide than just the cysteine residues, even though maleimide is supposed to be thiol specific. It has been noted before that maleimide can react with lysine residues (Roberts *et al.*, 2002), which are also present on the peptide.

In subsequent experiments when this crude reaction of maleimide-PEG is run next to another thiol-specific PEG-linker (PermaLink), this non-specific PEGylation is not seen (chapter 5 & 6). Malemide is an extremely reactive linker but its disadvantages become apparent with respect to specificity; it is also seen to react with amino groups on lysine residues which are common on a protein surface (Sharpless & Flavin, 1966),. In the next series of experiments, I compared a new generation cysteine reactive linker with the specificity and reactivity of Maleimide PEG as the commercial standard.

Chapter 5.0 Characterisation of PermaLink

5.1 Evaluation of the rate of reaction for different classes of nonfunctionalised PermaLink

5.1.1 Introduction

In the development of PermaLink™ technology, the inital phase of the project investigated the rate of cysteine reactivity for different classes of PermaLink compounds. Initially, the rate of reaction was determined using the linker alone (no PEG attached), this way the effect of attaching a PEG molecule to the linker can also be observed at a later stage.

In these experiments, glutathione was used as a substrate for unfunctionalised PermaLink as this peptide contains only one free thiol group to react with. The rate can be analysed by determining the speed at which the vinyl peaks disappear, which is equivalent to the rate at which the thio-ether bond is formed between the thiol and the vinyl group.

The pKa of each linker was also determined by titration experiments. This is important in helping to determine the potential optimum pH for the reaction. However, this also has to be a balance between the optimum for the linker-PEG and the thiol group/protein; with the stability of the protein being taken into account.

5.1.2 Summary of Results

The reactivity of the linker has improved as the groups attached to the central vinyl pyridine have been modified and moved around. The movement of the vinyl group from position 2 to 4 on the pyridine ring as well as the addition of the methyl group at position 1 helped the electron flow around the ring, making the vinyl group more attractive to attack from the reduced thiol.

5.1.3 Results

5.1.3.1 Linker Development

The development of linkers by Glythera Ltd. has been an ongoing process, figure 5.1.3.1(A) depicts the linkers 7, 11, and 12 which are used throughout these experiments.

Figure 5.1.3.1 (A) – Linkers

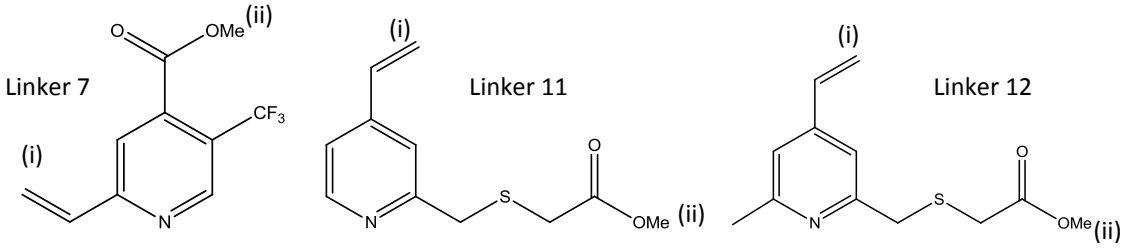


Figure 5.1.3.1 – The Glythera linkers used throughout this work are depicted in figure 5.1.3.1. **(A)**, i.e. linkers 7, 11 and 12. The vinyl groups (i) are where the linker covalently attaches to the reduced thiol group on the protein. The OMe group (ii) is where the PEG molecule is attached to the linker.

The aim in the development of these linkers has been to make the vinyl group as susceptible as possible to the attacking thiol group on the protein. This is achieved by increasing electron flow around the pyridine ring and drawing electrons away from the vinyl group. Attachment of various groups such as CF_3 were tried in linker 7 to improve this property. Similarly, it was found that keeping the PEG molecule away from the vinyl ring helped with reactivity, as can be seen between linker 7 and linker 11. Finally, between linker 11 and 12, the addition of the methyl group was found to improve reactivity.

5.2 Linker Optimisation

5.2.1 Introduction

PermaLink™ technologies have been developed by Glythera limited in attempts to improve thiol specific PEGylation chemistry. It consists of a vinyl-pyridine ring structure with a PEG group attached at various positions of the ring, depending on

the linker (see figure 5.1.3.1). The PEG is attached to the protein by a thio-ether bond; that is by nucleophilic attack of the thiol to the vinyl group. This technology is stable to biological and chemical degradation with predicted improved cysteine-selectivity over maleimide technology.

The exact methodology of conjugation with PermaLink™ PEGs to proteins/peptides needs to be established. In order to comprehensively analyse PermaLink technology and directly compare it to maleimide chemistry, pH, ionic strength of reaction buffer and the length of reaction time have been tested. The pKa of these PermaLink structures vary such that a range of reaction conditions with different pH values have been tested.

Alongside this, whilst testing the range of developing linkers, various methods of reduction were investigated, with TCEP being the main compound used for reduction. TCEP has been found to have advantages over other reducing agents such as DTT, although choice tends to be application specific (Getz *et al.*, 1999). The basis of alternating methods of reduction is to determine whether the presence of TCEP in solution is necessary and feasible. Ideally, the presence of TCEP in the solution would allow for a maintained reduced state of the thiol group, however, as was found in the study by Gertx *et al.*, (1999), although labelling is more efficient without the reductant present. In this study: removing TCEP by dialysis (method 1), leaving TCEP in the solution (method 2) and using TCEP immobilised resin (method 3) was investigated.

5.2.2 Summary of Results

- Highest conversion rates are seen with maleimide PEG using method 1 and 2.
- Leaving TCEP in solution had an overall decrease in PEGylation with all 4 linkers
- TCEP was found to directly react with the PermaLink linkers.

5.2.3 Results

5.2.3.1 Conjugation of Various Linkers using method 1 and 2

Conjugation is detected by SDS PAGE on all four linkers (maleimide; and PermaLink ™ 7, 11 and 12) when TCEP is removed by dialysis (figure 5.2.3.1) at pH 5.5. However, maleimide shows the most extensive conversion with approximately 79(±3.1)% PEGylated protein by densitometry analysis, compared to 62(±4.2)% for linker 12 (n=3). When looking at the non-reducing side of the SDS PAGE, the dimer of BCX S22C is seen at double the molecular weight of the unreacted protein in lanes depicting reactions with PermaLink ™ linker 7, 11 and 12. This shows that during the course of the reaction, in this case overnight, the dimer is slowly being reformed. This is not seen in the lane with the maleimide reaction however, and its presence is less dominant in the PermaLink ™ linkers which have been more recently developed.

Figure 5.2.3.1 BCX S2C Conjugation – Method 1

(A) – SDS PAGE showing both reduced and native reaction mixtures

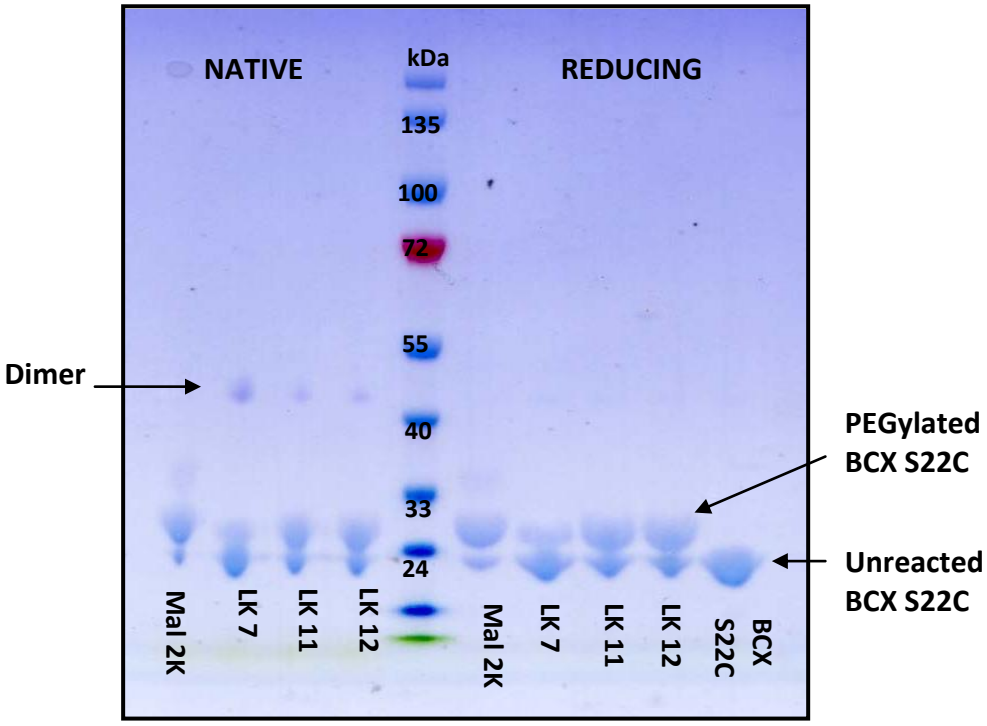


Figure 5.2.3.1 - 5mg/mL BCX S22C was set up using method 1 (see methods section). The resultant reaction mixtures were added to protein loading dye (reducing and non-reducing) and run for 50 minutes at 180V.

Similarly using method 2, leaving TCEP in the solution, conjugation is seen with all four linkers. Again, maleimide shows the highest conversion rate of 67 (±8.7)% at pH5.5 (n=3); compared to 24.66(±6.4)% for linker 7, 38.24(±5.25)% for linker 11 and 43.44(±6.2)% for linker 12 (figure 5.2.3.3). These values are lower then what is seen when TCEP is removed by dialysis which was an unexpected result as the presence of TCEP, helping to maintain the protein in a reduced state, was intended to help conjugation. Alongside this, dimer formation is still detected on the non-reducing side of the SDS PAGE which should not be case when TCEP is present. Notably, when comparing this dimer formation to Method 1, the reverse is seen in amount of dimer formation correlated to the more recently developed linker.

Figure 5.2.3.2 – BCS X22C Conjugation – Method 2

(A) – SDS PAGE showing the reaction mixtures of BCX S22C conjugation when TCEP is left in solution

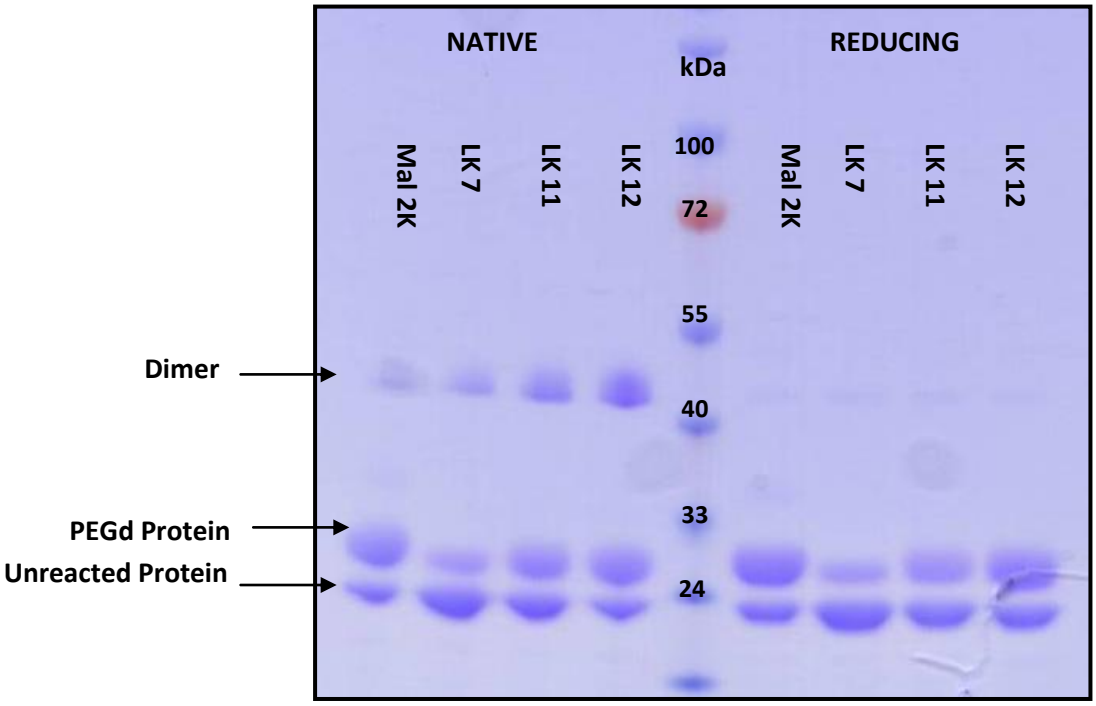


Figure 5.2.3.2 – The SDS PAGE shows the resulting reaction mixtures from the following experiment. 2mg/mL BCX S22C was reduced for 2 hours with 1.7molar equivalents of TCEP in 20mM NaOAc + 1mM EDTA, pH7. Five molar equivalents of the various linkers were then added to the protein solution and the reaction was left to shake overnight. 10ul of reaction mixture was added to 10ul of both reducing (boiled) and non-reducing SDS loading dye and loaded onto the acrylimide gel. The gel was then run for 50 minutes at 180V.

Figure 5.2.3.3 – Quantative comparison of extent of PEGylation using method 2 at variable pH

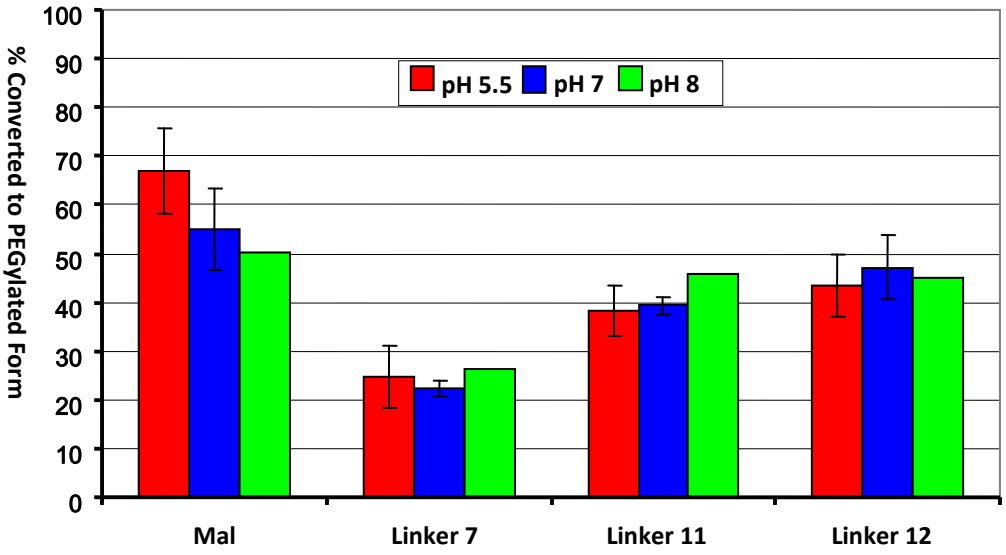


Figure 5.2.3.3 – Densitometry analysis of protein conversion to PEGylated state using various linker-2KDa PEGs, mean±S.E.M, pH 5.5 and 7 (n=3) and pH8 (n=2), independent samples. Maleimide linker clearly shows highest % conversion; however, the extent of conversion is still not at its optimum.

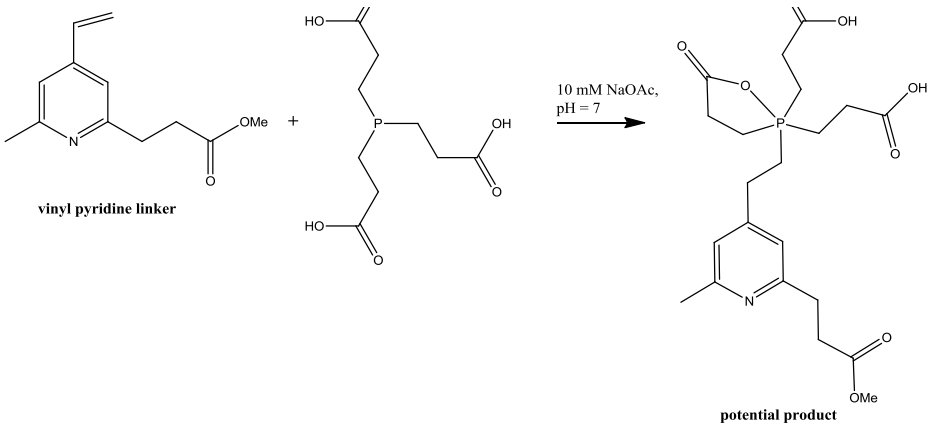
This result with method 2, suggests that TCEP in the reaction decreases Permalink linker reactivity with the thiol group. When TCEP was removed from the reaction a higher conversion rate was seen.

5.2.3.2 Direct TCEP reaction with PermaLink in the absence of a protein/peptide thiol

In light of results from reactions where TCEP was left in solution, investigation into TCEP reactivity with various components of the reaction was undertaken. Reactions were set up between the linker and TCEP and monitored by NMR. As seen in figure 5.2.3.4, the peaks which equate to the vinyl group of the linker disappear over time. This indicates that they are being consumed or degraded by an unexpected side reaction with TCEP. Alongside this, a new peak appears which could equate to the new product. A potential product was hypothesised and later confirmed by the chemists of Glythera Ltd (see Figure 5.3.2.4.A). It can be concluded that TCEP directly reacts with the Permalink linker and therefore inhibiting the conjugation to the actual target cysteine.

Figure 5.3.2.4 – Investigating TCEP

(A) – Reaction between vinyl pyridine linker and TCEP with possible product.



(B) – The disappearance of the vinyl peaks through NMR analysis

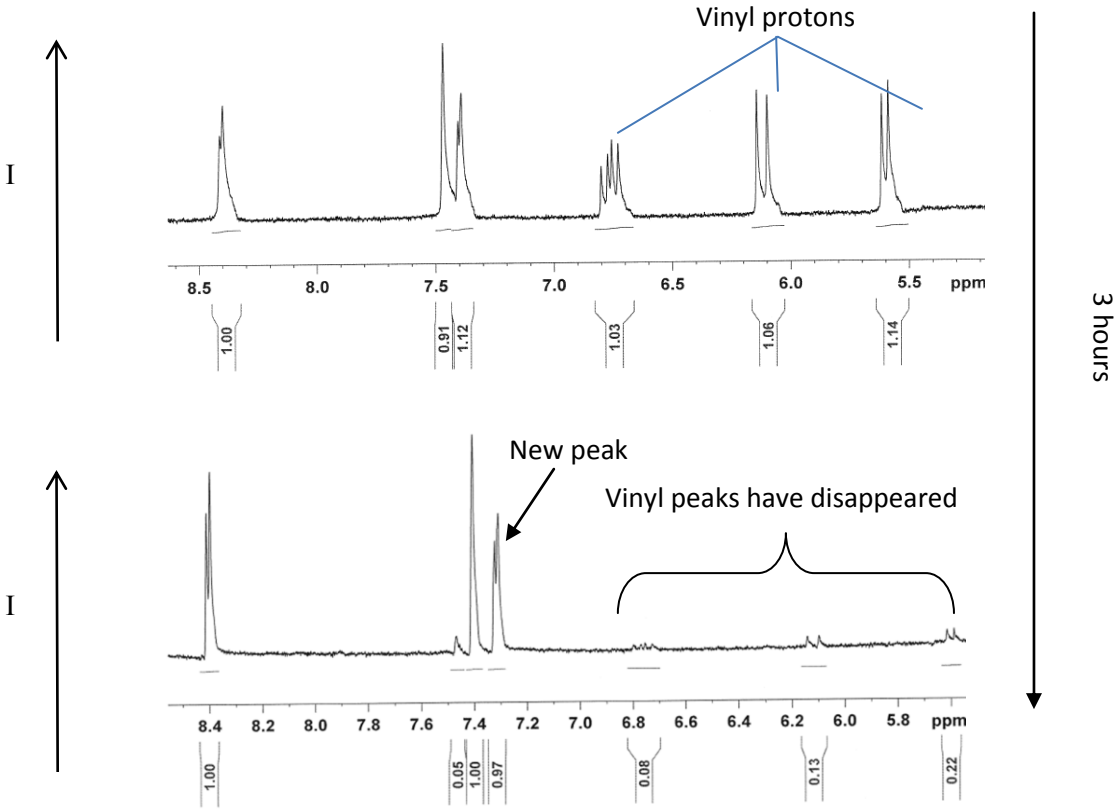


Figure 5.3.2.4 – Data produced and supplied by Terrence Kantner of Glythera Ltd. A reaction with TCEP and the linker was set up in10mM NaOAc at pH7 and was monitored by NMR **(B)**. Here the peaks which equate to the vinyl group of the link disappear over time (seconds), exhibiting some kind of degradation or side reaction. Simultaneously, a new peak appears, highlighting the new potential product **(A)**.

5.2.3.3 Instigation of Method 3

An immobilised TCEP resin would enable the efficient removal of TCEP from the reaction mix prior to the addition Permalink. Based on the results demonstrating a direct reaction of TCEP with Permalink molecules I evaluated Permalink reactivity following reduction using immobilised TCEP.

At pH7, the SDS PAGE comparing all four linkers shows a much improved conjugation results when compared to the other methods (figure 5.2.3.5). As also shown in figure 5.2.3.6A, maleimide and PermaLink™ linker 12, shows near on complete conversion with 86 (±4.8)% and 76 (±7.9)% PEGylated respectively (n=3). At pH5, to directly compare the other methods in this instance, the results were not as good 83 (±2.9)% and 47 (±7.8)% respectively – pH7 was found to be optimum for PermaLink™ technology.

Figure 5.2.3.5 BCX S22C Conjugation – Method 3

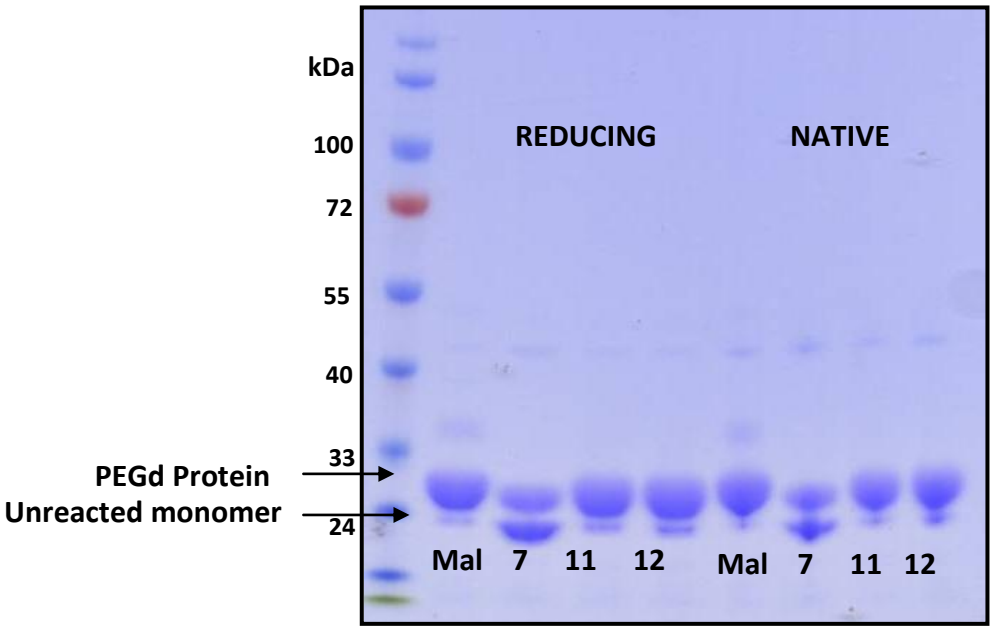


Figure 5.2.3.5 – 4mg/mL BCX S22C was set up using method 3 (as outlined in the methods section). 10ul of reaction mixture was added to 10ul of both reducing (boiled) and non-reducing SDS loading dye and loaded onto the acrylimide gel. The gel was then run for 50 minutes at 180V

5.2.3.4 Comparison of extent PEGylated between method 2 and 3

As well as visually represented in the SDS PAGE, densitometry analysis shows an improvement in extent of PEGylation between method 2 and 3 (Figure 5.2.3.6B) for both PermaLink and maleimide-PEG reactions at pH7. Both maleimide-PEG and Linker 12 show significant improvement in their conversion rate ($p<0.05$); 85 (±4.8)% and 75 (±7.6)% respectively. Linker 7 shows an even further improved conversion rate – from 22 (±1.5)% to 44 (±2.5)%, $p<0005$, $n=3$, which is surprising considering the low reactivity of the linker.

Figure 5.2.3.6 – Quantative analysis of extent of PEGylation using Method 3

(A) - Average % PEGylated at Various pH Using Method 3 – TCEP Resin

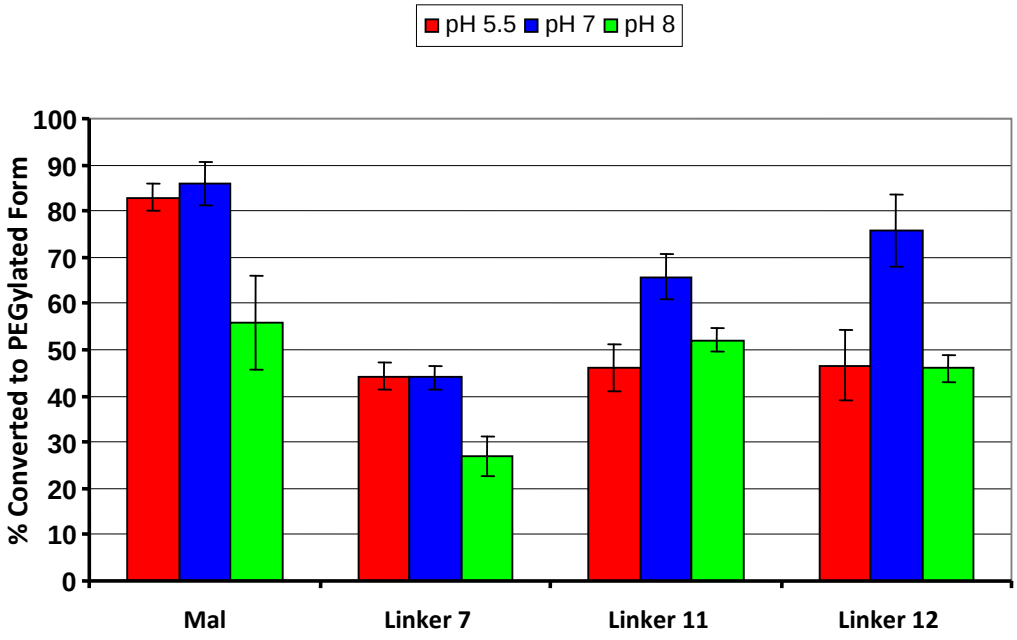


Figure 5.2.3.6 – (A) Densitometry analysis of protein conversion to PEGylated state using various linker-2KDa PEGs, mean±S.E.M (n=3, independent samples). Maleimide linker clearly shows highest % conversion; with pH 7 being optimum for all four linkers.

(B) – Average % PEGylated Comparing Method 2 and 3

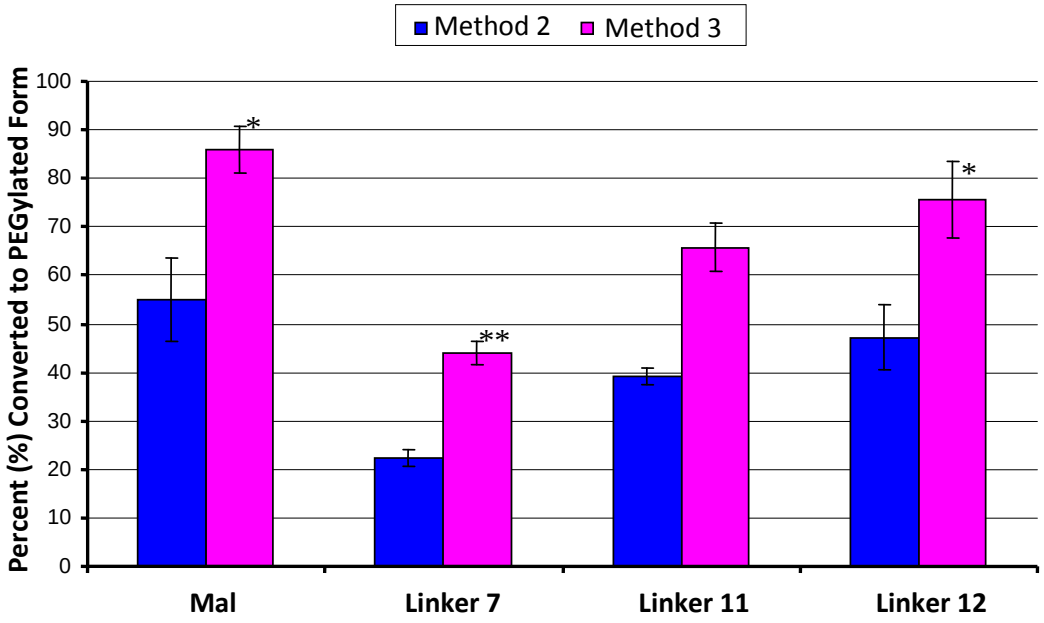


Figure 5.2.3.6 – (B) shows the comparison of method 2 and 3, mean±S.E.M (n=3, independent samples), where a significant increase is seen between conversion rate of each linker using the two methods (paired t-test - *p<0.05 and **p<0.005).

5.3 Discussion

5.2.1 PermaLink Optimisation

The method development process for conjugating PermaLink-PEGs to a protein was an important step in ensuring optimisation of the reaction. Similarly, comparing these various stages of development to maleimide-PEG reactivity was helpful in highlighting the differences in PermaLink technology.

5.2.2 Method 1

Method 1, removing TCEP by dialysis, was a time consuming protocol, harbouring little benefit for PermaLink technology. Maleimide reacted with the reduced thiol on the protein to show a promising conversion rate; 79(±3.1)% PEGylated for maleimide PEG, compared to 62% (±4.2)% for linker 12-PEG (n=3) at pH5.5. A distinct protein band indicating the BCX S22C dimer is also seen in the SDS PAGE (figure 5.2.3.1), in lanes for linker 7, 11 and 12. This suggests that over the course of the reaction, the

reduced thiol group is becoming reoxidised and forming a disulphide bridge with other free thiol groups on other protein molecules. The speed at which this takes place is indicative of the speed of the linker-thiol reactions; with maleimide reacting with the free thiol at a faster rate than PermaLink structures and therefore no dimer formation is detected.

Both the native and reduced sides of the SDS PAGE in figure 5.2.3.1, shows a faint protein band above the main PEGylated protein in the maleimide lane. This indicates that more than one maleimide-PEG molecule has attached to some of the reduced BCX S22C molecules, exhibiting non-specific conjugation. The addition of the mono- and multi-PEGylated BCX S22C maleimide species would highlight a much higher conversion rate. Therefore, It is also worth noting, that the protein was fully reduced at the beginning of the reaction and available for conjugation; further highlighting that the PermaLink-PEGs were distinctly slower than maleimide-PEGs to react.

5.2.3 Method 2

The concept behind leaving TCEP in solution was to maintain the protein in a reduced state, therefore making it continuously available to the linker-PEGs throughout the course of the reaction. However, both maleimide-PEG and PermLink-PEG structures struggled to achieve the predicted high conversion rate: 67 (±8.7)% at pH5.5 (n=3); compared to 24.66(±6.4)% for linker 7, 38.24(±5.25)% for linker 11 and 43.44(±6.2)% for linker 12 (figure 5.2.3.3). An increased amount of dimer formation is also seen in the SDS PAGE compared to method 1 for all three PermaLink structures and maleimide. This is contradictory to the objective of having TCEP present in the reaction mixture; why was TCEP not reducing the protein? This surprising result began the research into TCEP being used in these reactions.

5.2.5 TCEP Investigation

TCEP has long been used as a convenient phosphine for reduction of disulphides in water (Burns *et al.*, 1990). It was successfully reducing the BCX S22C, as discussed and seen in section 4.2.1.3.1, therefore the logical assumption was that the TCEP

was somehow reacting with the linkers. The NMR analysis of the TCEP and vinyl pyridine reaction clearly showed the consumption of the vinyl peaks and the generation of a new product; this was determined in line with a study done by Shafer *et al.*,(2000).

Although the TCEP was incubated with the protein prior to addition of the PEG-linkers, the TCEP is consumed via its side reaction with the linker-PEG. With the free TCEP being mopped up by the molar excess PEG-PermaLink, the protein had little chance of maintaining a reduced state and therefore reacting with any PEG-linkers which were available. This was an important result to have obtained and was essential in attempting to conjugate larger PEG molecules and eventually, therapeutic proteins.

5.2.3.4 Instigation of Method 3

The immobilised TCEP resin was a helpful alternative in the reduction of BCX S22C. The TCEP reduced the protein, but immbolization on a resin allowed removal (which was now known to be essential) to happen much more easily than method 1. The entire reaction could be set up in just over an hour. The results of the reactions are indicative of this; maleimide and PermaLink™ linker 12 show near complete conversion with 86 (±4.8)% and 76 (±7.9)% PEGylated respectively (n=3). As seen with method 1 however, with the maleimide-PEG being in its optimum conditions, multi-PEGylation is detected. At pH7, the determined optimum for the PermaLink structures, this is not seen indicating a higher thiol specificity for Permalink compared to maleimide compounds.

Chapter 6.0 Higher Molecular Weight PEGs for Therapeutics

6.1 Model Protein – BCX S22C

6.1.1 Introduction

Increasing the size of the molecular weight PEG attached to the therapeutic protein will have beneficial effects on its therapeutic availability. A large PEG molecule will firstly prevent clearance through kidney ultrafiltration, not only by increasing its overall size but also by masking its charge and chemical composition (Caliceti & Veronese, 2003). The molecular weight cut off for kidney ultrafiltration is 70KDa, however, if proteins are of a higher molecular weight (or heavily glycosylated for example) they are removed via other pathways like liver uptake and proteolytic digestion. Therefore therapeutic proteins are often designed to be below 70KDa to prevent rapid clearance by these other mechanisms.

As mentioned previously, BCX S22C is an ideal model protein as it is well characterised, both its function and structure (Wakarchuk, 1994). It is also of similar molecular size to most cytokines, a commonly manufactured therapeutic. In these experiments, PermaLink structure PL-LK-12 (figure 5.1.3.1(A)) with a 20KDa PEG is used (PermaLink12-P20K), being the most developed linker available from Glythera Ltd. at that time.

6.1.2 Summary of Results

- PermaLink12-P20K successfully conjugates to BCX S22C using method 3
- PermaLink12-P20K is less reactive with BCX S22C than PermaLink12-P2K

6.1.3 Results

6.1.3.1 BCX S22C and PermaLink12-P20K and P2K

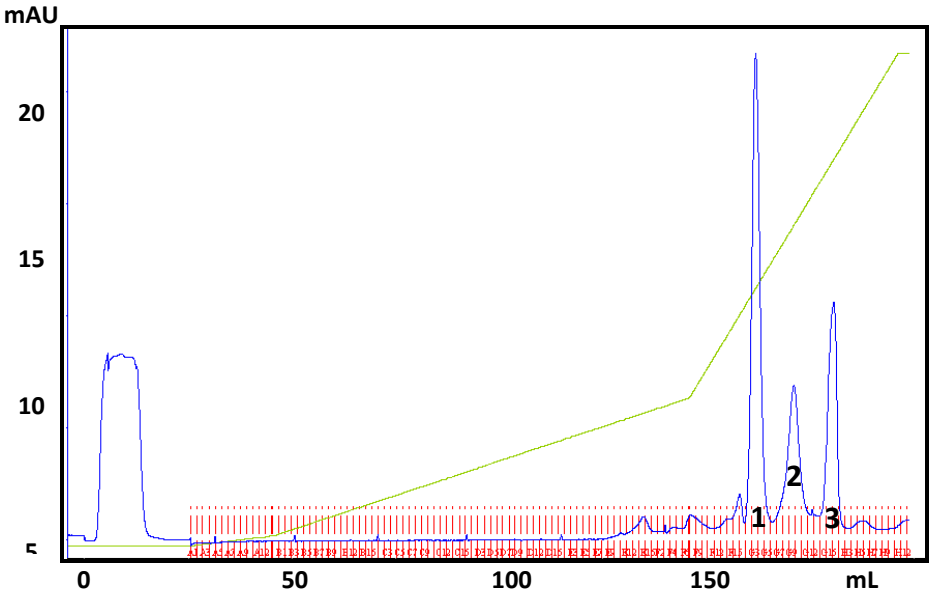
Method 3, the immobilised TCEP resin, was concluded to be the best approach to reduce BCX S22C protein prior to reaction with Permalink Linkers (see chapter 5). However, a clear difference is seen between the extent of PEGylation when the size

of the PEG molecule is increased (figure 6.1.3.1 and 6.1.3.2). With the 20 KDa PEG attached to Permalink 12, FPLC analysis of the reaction shows three distinct peaks, highlighting 3 different components within the reaction mixture (figure 6.1.3.1A). SDS PAGE and coomassie staining demonstrates that the first peak contains PEGylated protein as there is a shift in molecular mass; that is 21KDa of the BCX S22C protein and 20KDa for the PEG. The protein band itself however, falls just under the 72KDa molecular weight marker of the protein ladder; as previously mentioned, due to the migration of the PEG molecule through the polyacrylimide gel, it does not fall at its accurate molecular weight (Kurfust M. M., 1992). Both the second and third peak contain unreacted protein (figure 6.1.3.1B), with the third peak being the dimer of BCX S22C, which had been reduced prior to running on the SDS PAGE.

When this result is compared to the same reaction with PermaLink12-P2K, a stark contrast is seen (figure 6.1.3.2) with the extent of reaction. The FPLC trace (figure 6.1.3.2A) shows a peak eluting off the column, and when analysed by SDS PAGE (figure 6.1.3.2B), this contains the 2KDa PEGylated protein when compared to the BCX S22C control. These results indicate that the reaction has gone to completion, with unreacted protein being undetectable.

Figure 6.1.3.1BCX S22C and PermaLink 12-P20K

(A) FPLC trace of overnight reactions mixture of BCX S22C and PermaLink 12-P20K



(B) SDS PAGE of fractions from FPLC of the reaction

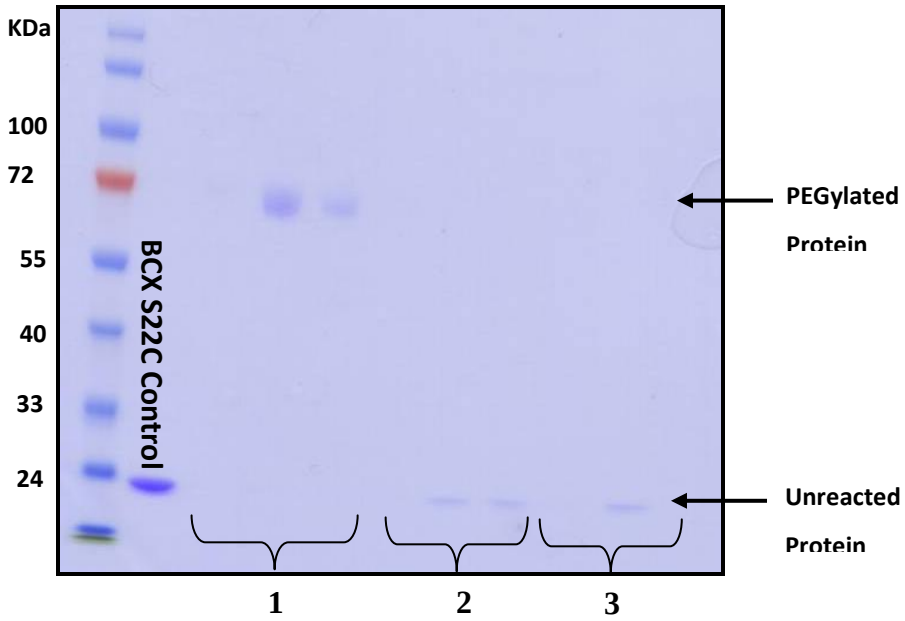


Figure 6.1.3.1 – BCX S22C and PermaLink12-P20K. 1mg of BCX S22C was reduced for 2 hours using an immobilised TCEP resin (method 3) and then reacted with 2 molar equivalents of PermaLink 12-P20K PEG and left to react overnight. **(A)** shows the FPLC trace for the reaction, with three distinct peaks detected. Analysis of the fractions within these peaks by SDS PAGE **(B)** shows they equate to a PEGylated product, the monomer and the dimer.

Figure 6.1.3.2 - BCX S22C and PermaLink 12-P2K

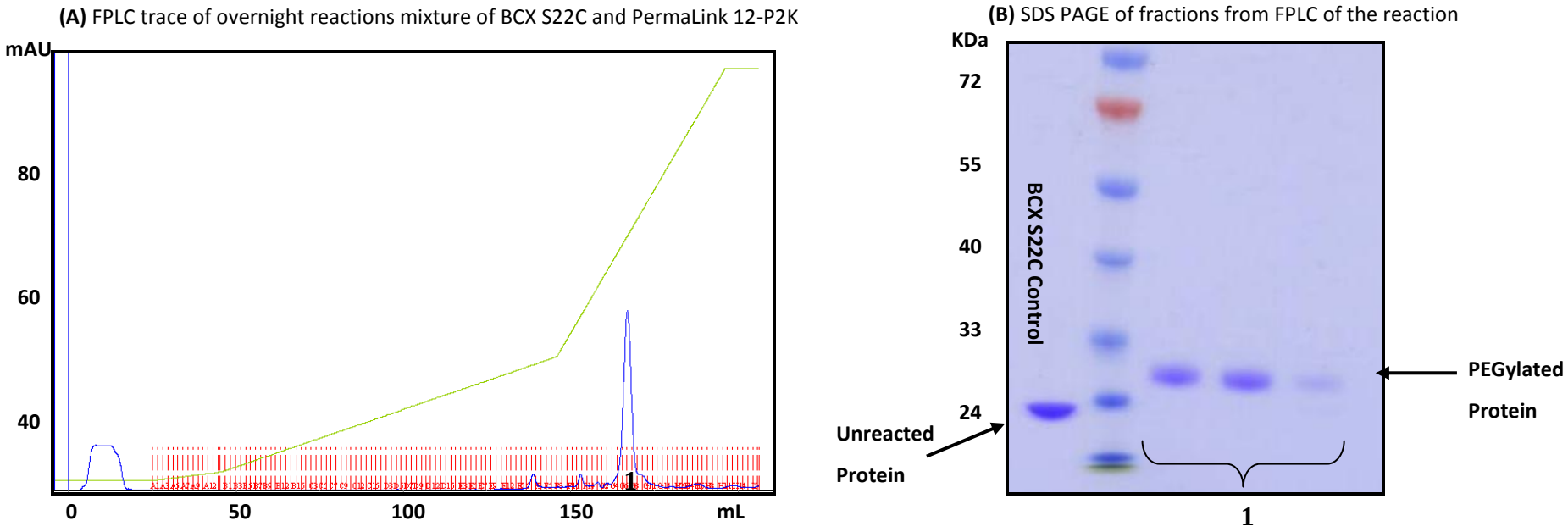


Figure 6.1.3.2 - BCX S22C and PermaLink 12-P2K. 1mg of BCX S22C was reduced for 2 hours using an immobilised TCEP resin (method 3) and reacted with 2 equivalents of PermaLink 12-P2K and left overnight. (A) shows the FPLC trace at the end of the reaction, with only one peak detected. The SDS PAGE in (B) confirms that this one peak is the PEGylated form of BCX S22C, when compared to the control.

6.2 Therapeutic Peptide – sCT

6.2.1 Introduction

Salmon calcitonin (sCT) is the most widely used source of calcitonin for clinical use because of its increased potency and improved analgesic properties (Chesnut *et al.*, 2008). It is commercially available in an injectable form or as a nasal spray; formulations of sCT which would be orally available are also being developed. Low molecular weight peptides such as sCT can show more improved pharmacokinetic properties when PEGylated than proteins. This is because their half life is substantially increased after PEGylation, because usually, they are subject to rapid elimination by renal clearance (Youn *et al.*, 2007). sCT does not require the disulphide bridge to be bioactive (Orlowski *et al.*, 1987), which means that the two cysteine residues 1 and 7 can be targeted for PEGylation for thiol specific conjugation. sCT has previously been PEGylated by maleimide PEG conjugates at both cysteine 1 and 7 (Cheng *et al.*, 2006).

In these experiments, 20 KDa PermaLink12 PEGs are attached to sCT using method 3. Due to the size of the peptide, coomassie staining is not detected on an SDS PAGE very well and therefore PEG dye has been used. It is worth noting here that coomassie staining which was detected did correspond to where the PEG dye stained the SDS PAGE.

6.2.2 Summary of Results

- sCT reacts with PermaLink12-P20K to produce two different PEGylated species of sCT
- PermaLink structures are more specific to cysteine residues than maleimide linkers.
- MonoPEGylated product of sCT and PermaLink12-P20K is formed within 30 minutes, however, the diPEGylated form increases over time.

6.2.3 Results

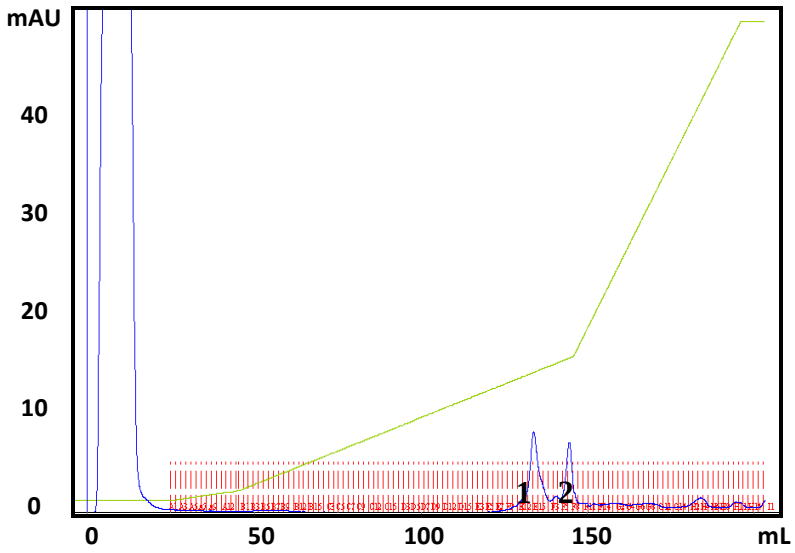
6.2.3.1 sCT and PermaLink12-P20K and P2K reactions

sCT reacts with PermaLink12-P20K to produce two different PEGylated species of sCT. This correlates to the two cysteine residues present available for conjugation, and therefore the mono- and di- PEGylated form of sCT. The FPLC trace of this reaction can be seen in figure 6.2.3.1A where both the mono- and di-PEGylated forms are present in the same UV peak in the FPLC trace. The resultant molecular weights of the products of the reaction would be 3.2 KDa + 20 KDa = 23.2 KDa for mono-PEGylated; and 3.2 KDa and 40 KDa = 43.2 KDa for di-PEGylated. However, analysing the FPLC fractions by SDS PAGE and staining with a PEG dye (figure 6.2.3.1B) shows the bands falling at about 55 KDa and 100 KDa. This is to be expected however, due to how PEGs migrate through an SDS PAGE.

sCT and PermaLink12-P2K react well as can be seen when analysed by FPLC (figure 6.2.3.2A). The content of the FPLC fractions from this reaction were very hard to detect on SDS PAGE due to the size of the conjugates. However, a faint PEGylated band at approximately 15KDa can be seen (figure 6.2.3.2B). In terms of the extent of PEGylation when comparing conjugation of P20K and P2K reactions, quantifying it by SDS PAGE is not practical. The absorbance value can be looked at as an alternative, the reaction with the P2K reaches 30 mAU and the P20K only reaches 10 mAU. Similarly, the observed ratio between PEGylated and non-PEGylated species appears equal with the P20K reaction (figure 6.2.3.1A).

Figure 6.2.3.1 sCT and Permalink12-P20K

(A) FPLC trace of overnight reactions mixture of sCT and PermaLink12-P20K



(B) PEG stained SDS PAGE of fractions from FPLC of the reaction

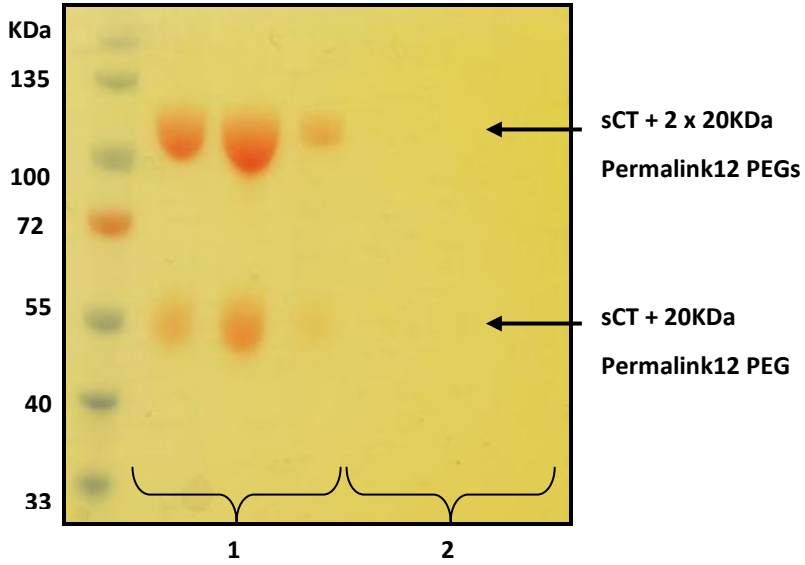


Figure 6.2.3.1 sCT and Permalink12-P20K. 1mg of sCT was reduced for 30 minutes and then reacted with 2 molar equivalents of PermaLink12-P20K and left overnight. There are 2 peaks detected through FPLC analysis **(A)** and through analysis on SDS PAGE **(B)** and staining with PEG dye, they equate to PEGylated and non-PEGylated sCT. Within the PEGylated fractions, two PEGylated species are detected **(B).**

Figure 6.2.3.2 – sCT and Permalink12-P2K

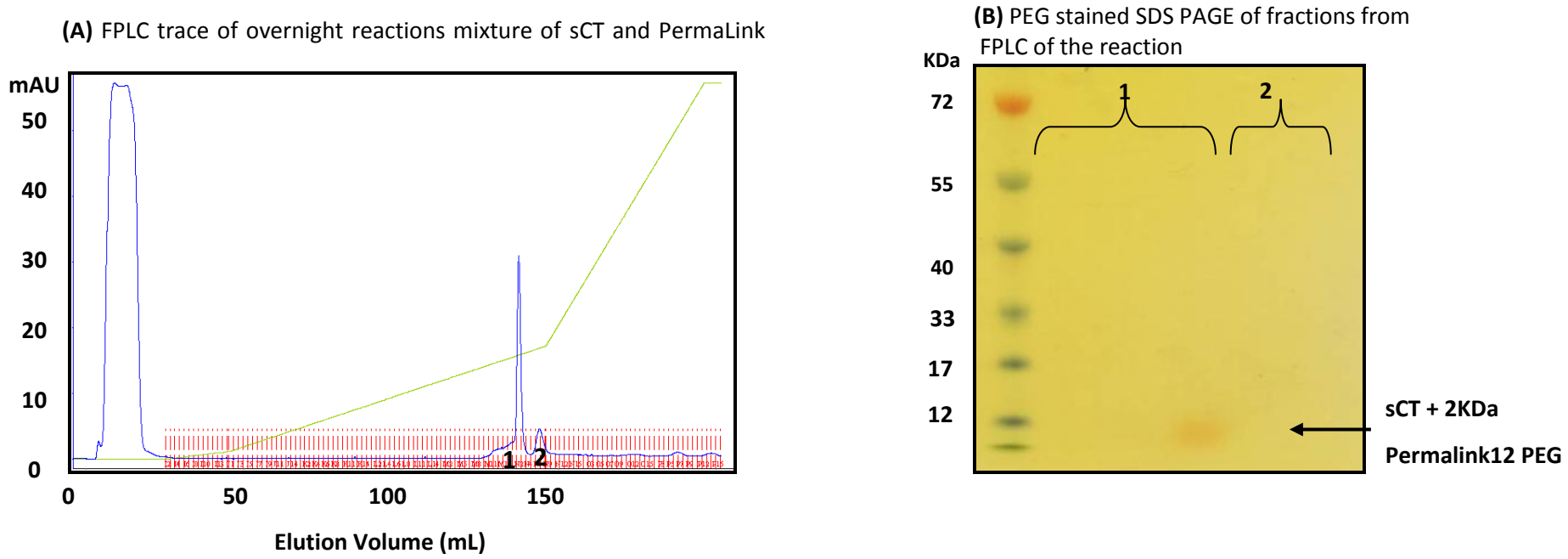


Figure 6.2.3.2 – sCT and Permalink12-P2K. 1mg of sCT was reduced for 30 minutes and reacted with 2 molar equivalents of Permalink 12-P20K. FPLC analysis of the reaction can be seen in **(A)**, where two peaks are detected. Analysis by SDS PAGE and staining with a PEG dye shows a faint band at approximately 12 KDa **(B)** , indicating the larger peak is the PEGylated species.

6.2.3.3.1 - sCT and PermaLink 12-P20K and Maleimide 20K Crude reactions and sCT and PermaLink 12-P20K Time Course

To analyse the complete reaction mixture without it being diluted by FPLC, the crude reaction was run on an SDS PAGE (figure 6.2.3.3A). Here the two bands, which are seen in the reactions separated by FPLC, are detected by the PEG dye at the same molecular weights as seen previously in these reactions (figure 6.2.3.1B) for PermaLink12-P20K. This reaction is directly compared to Maleimide-P20K and there is clear evidence for non-specific and multi-PEGylation with Maleimide chemistry. Several bands of very high molecular weight are detected in the lane with the Maleimide-P20K reaction, none of which are seen with PermaLink12-P20K.

Figure 6.2.3.3 - sCT and PermaLink 12-P20K and Maleimide 20K Crude reactions and sCT and PermaLink 12-P20K Time Course

(A) sCT + PermaLink 12-P20K and sCT + Maleimide-P20K crude reactions

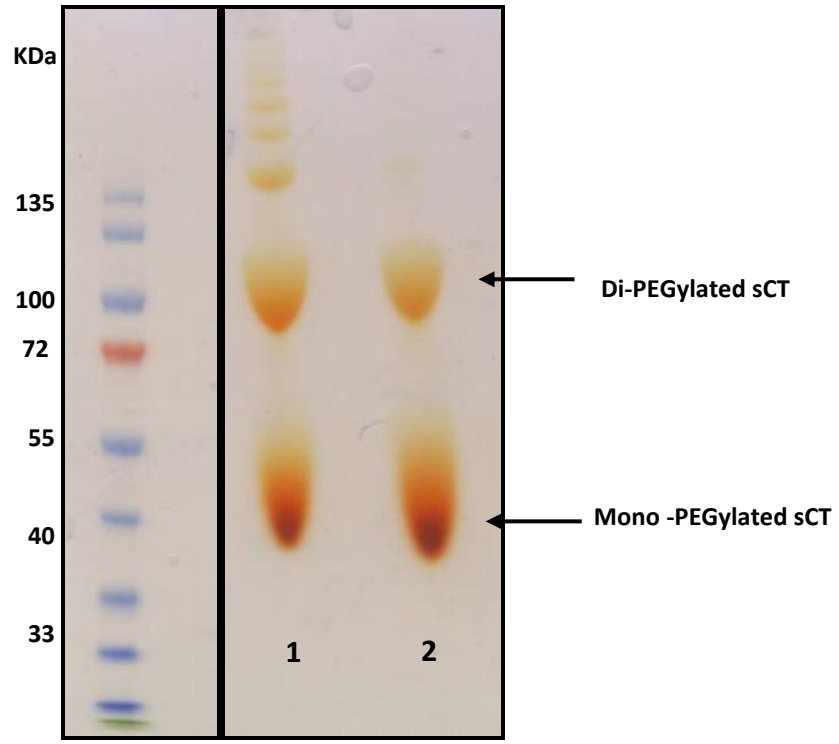


Figure 6.2.3.3 Crude reactions (not separated by FPLC) were run on an SDS PAGE and stained with PEG dye **(A)**. Maleimide-P20K reaction (lane 1) clearly shows non-specific PEGylation, whereas PermaLink 12-P20K and sCT (lane 2) does not.

The speed of the reaction between sCT and PermaLink12-P20K was briefly investigated (figure 6.2.3.3B). It can be seen that after 30 minutes the mono-PEGylated species appears to have reached its maximum levels, with the intensity of the band remaining the same even at the longer time points. However, the intensity of the di-PEGylated species increases over time.

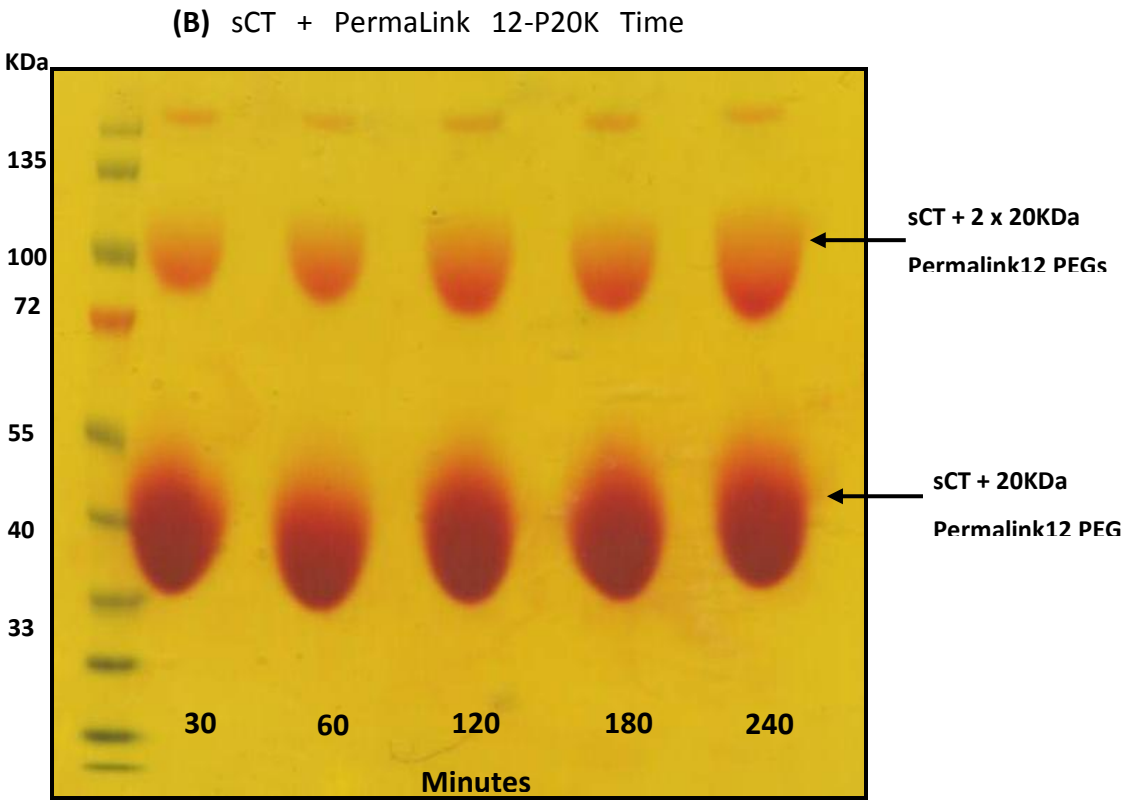


Figure 6.2.3.3(B) A time course looking at PermaLink 12-P20K speed of reaction was conducted and samples were run on an SDS PAGE **(B)**, it can be seen the reaction occurs within 30 minutes, but more of the di-PEGylated species is formed over time.

6.3 Discussion

6.3.1 PermaLink12-P20K reactions with model protein

By analysing the reaction of PermaLink12-P20K with BCX S22C, an idea of the conjugation efficacy can be seen when reacting with therapeutic proteins. When comparing the reactions to those with the P2K PEG attached, it can be inferred that the larger PEG effects the reactivity of the linker as stated in Roberts *et al.*, (2002). These experiments with PermaLink12 had been repeated at least 3 times, however, accurate quantification was not achieved using FPLC analysis. This can be improved upon, potentially by looking at the area under the curve. The structure of this product could also be looked at using mass spectrometry to ensure that the protein-PEG ratio was 1:1.

6.3.2 sCT and PermaLink12 Reactions

sCT acted as a good model peptide to determine PEGylation efficacy with the PermaLink structures. It reacted very quickly with PermaLink12-P20K to form two different PEGylated species, the mono- and di- PEGylated sCT. This was seen with both the 2K and 20K PermaLink structures, however, analysis of the P20K reaction was more relevant to its potential commercial application.

As seen in the figure 6.2.3.1 (A) there is still unreacted peptide within the reaction mix. Although the reaction was faster with the peptide than with the model protein BCX S22C, it perhaps is still not fast enough to react with the peptide before re-oxidation. The two free cysteines reform the disulphide bridge and therefore do not allow further PEGylation. Although accurate quantification needs to be established, the amount of non-PEGylated to PEGylated sCT appears to be greater with the P20K reactions. This could indicate that access to the free cysteine residues is being hindered by the large PEG molecule.

6.3.3 PermaLink P20K and Maleimide P20K Crude Reactions

A direct comparison of Maleimide P20K and PermaLink12 P20K was difficult using FPLC analysis which diluted samples and allowed experimental variation. A crude

reaction mix of PermaLink12-P20K and Maleimide-P20K and sCT was run directly on an SDS PAGE (figure 6.2.2.3(A)). A pure reaction mix of both mono- and di- PEGylated product can be seen with PermaLink12-P20K when compared to multiple PEGylated species detected in the Maleimide reaction. This is in keeping with Sharpless & Flavin (1966) who determined Maleimide can also react with lysine residues on a protein surface, like sCT (Niall *et al.*, 1969). Maleimide has previously been seen to react with these lysine residues (Youn *et al.*, 2006); in this case, non-specific PEGylation highlights PermaLink benefits in a commercial setting.

7.0 Conclusions

Overall conclusions drawn from these data are as follows –

1. Permalink Technologies target cysteines more specifically than maleimide linkers in conjugation reactions

As seen in figure 4.2.2.3.3 the crude total reaction mixture of therapeutic peptide sCT and maleimide 20KDa PEG results in multiple PEGylated species being created in the reaction. This demonstrates maleimide's lack of specificity for cysteine residues when compared to the same reaction but with Permalink12-P20K reaction seen in figure 6.2.3.3, where only the mono- and di- PEGylated species are detected. This is a desirable property of Permalink technology in regards to commercial conjugation chemistry.

2. Increasing the size of the PEG molecule affects the rate and extent of reaction

Chapter 6 highlights the difference in reactivity of the Permalink linkers when the size of the PEG molecule is increased. Extent and speed of reaction is reduced overall, the latter of which can be inferred by the amount of dimer which has reformed by the end of the reaction. Commercially viable PEGd therapeutics need to be at least 20KDa in size to improve the pharmacokinetic properties of the therapeutic. If the reactivity of PermaLink structures are affected by the larger sized PEG molecules then the benefits of specificity are redundant.

3. Further method development is required to improve the conjugation with Permalink Technologies to make them more viable for use in a commercial setting.

a) Protein purification

There are various stages in these experiments where potential for loss of protein or linker-PEG needs to be reduced, as well as more accurate ways of quantifying results. Firstly, in protein production and purification, a substantial loss of protein is very probable due to extensive concentration which is required after FPLC purification. Perhaps protein precipitation as outlined in Gräslund S., *et al.* (2008) would be a faster and more effective way of purifying proteins. Running the precipitated protein

through the FPLC would only have to happen once, reducing the number of times the protein would have to be filtered and concentrated.

b) Reduction of the protein

As in method 2, the ability to have accurately measured the amount of protein in the reaction and not have to move it around or change holding vessels would have been beneficial to reduce protein loss. Unfortunately as it was not plausible to leave TCEP in the solution when attempting conjugation because of TCEP reacting with PermLink structures, a TCEP resin was employed so the protein can be separated from the reducing agent. This was an inaccurate procedure as real care had to be taken to ensure none of the TCEP resin was taken up when removing the protein. I would suggest that more precision could be achieved if this was done on a larger scale, as both visually and practically this would have been easier.

c) Quantification of results

i) With more accurate knowledge of the amount of protein in a reaction, more precise methods for determining the extent of PEGylation can be calculated. ELISAs designed for PEGylated protein could be a very useful tool.

ii) Similarly, characterisation of the product from the conjugation reaction could help find more ways to quantify the results. Tandem Mass Spectometry (MS/MS) could be used to determine the site at which the PEG has attached; a method used to determine the site of glycosylation in carbohydrate chemistry (Domon & Costello, 1998).

iii) Activity assays would also be helpful in establishing whether the protein was still active, even with large PEGs attached.

Overall, PermaLink technologies have benefits of specificity for thiol groups on proteins when compared to maleimide. The efficiency and speed of reaction will undoubtedly be improved with further linker development.

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