Development of Mixed-Charge EK Polypeptides for Protein Protection

Erik Jerry Liu

A dissertation

submitted in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Washington

2019

Reading Committee:

Shaoyi Jiang, Chair

Elizabeth A. Nance

Qiuming Yu

Program Authorized to Offer Degree:

Chemical Engineering

University of Washington

Abstract

Development of Mixed-Charge EK Polypeptides for Protein Protection

Erik Jerry Liu

Chair of Supervisory Committee:

Shaoyi Jiang

Chemical Engineering

The presented work focuses on the development of mixed charge polypeptides and their fusion proteins and protein conjugates for the protection of proteins. Compared to many polymers used to modify proteins, polypeptides are an ideal candidate for protein modification due to their biocompatibility, biodegradability, and uniformity. Polypeptides composed of alternating glutamic acid and lysine residues (EK) function as rationally-designed polypeptide analogues of low-fouling zwitterionic polymers due to their ability to resist nonspecific protein adsorption. Zwitterionic polymers have demonstrated superior properties related to protein protection, pharmacokinetics, and immunogenicity compared to other polymers, including poly(ethylene glycol) (PEG), which has been widely used to modify therapeutic proteins in the clinic. As EK has demonstrated similar zwitterionic properties it can be used in similar capacities when used to modify therapeutic proteins. The use of EK allows for the use of recombinant protein expression techniques, thereby allowing

for the synthesis of well-defined products of uniform composition without any inherent batch to batch variation. Additionally, recombinant techniques allow for EK to be a component of a fusion protein to modify target therapeutic proteins, allowing for one-step generation of any number of EK-modified products.

This work develops the capabilities of EK through various systems from proof of concept through *in vivo* capabilities and demonstrates that EK behaves similarly to zwitterionic polymers when used to modify proteins. A proof of concept system utilized a model β-lactamase system to demonstrate the generation of fusion proteins containing EK. These fusion proteins were able to be expressed at a uniform molecular weight and purified. It was observed that EK demonstrated the conferring of the same protecting properties *in vitro* against environmental stressors and improvements to enzyme kinetics that zwitterionic polymers had previously demonstrated. These *in vitro* properties of EK were again demonstrated when EK was used as a part of a fusion protein with organophosphate hydrolase. Additionally EK exhibited its zwitterionic low-fouling characteristics by preventing the formation of the dimeric structure of wild-type organophosphate hydrolase and leaving stable monomeric structures, demonstrating the ability of EK to protect the surface of the protein from foreign interactions. This effort also represents the first known expression of a stable monomeric unit of the protein. Furthermore, other EK fusion protein systems and standalone EK peptides have also been expressed and studied.

Table of Contents

List of Tables

List of Figures

Acknowledgements

This work would not have been possible without the financial support of the Army Research Office (63926-LS-249 II), National Science Foundation (CBET 1264477), Office of Naval Research (N000141010600), and Defense Threat Reduction Agency (HDTRA1-13-1-0044). The author would like to thank Drs. Ann Kasia Nowinski, Andrew Sinclair, Douglas M. Cerasoli, and Steven P. Harvey and Patrick McMullen and Caroline Tsao for helpful discussions, Dr. Priyesh Jain for synthetic chemistry support, and Drs. François Baneyx and Brandon Coyle of the Baneyx research group for discussions and facility usage. The author would like to thank Dr. Shaoyi Jiang for discussion, advisement, and funding all works. Additionally the author would like to thank Drs. Elizabeth A. Nance, Mark Warner, and Qiuming Yu for serving on the supervisory committee and Drs. Baneyx, Cole A DeForest, and Deok-Ho Kim for previously serving on the supervisory committee.

Chapter 1

Introduction

The conjugation of polymers to proteins has been used to great effect in improving the therapeutic properties of proteins over the last several decades. Most notably PEGylation, the conjugation of (PEG) to proteins, has led to significant advances in improving therapeutic protein properties, beginning with the first major protein modification in the 1970s and later marked by the landmark FDA approval of Adagen® (PEGylated adenosine deaminase) as the first PEGylated protein therapeutic in 1990[1-5]. Currently then there have been fourteen PEGylated protein and peptide therapeutics approved by the FDA that are currently available on the market, as highlighted in **Table 1**[3-13].

The PEGylation of therapeutic proteins has demonstrated multiple beneficial properties of PEG. PEGylation has been shown to significantly increase blood circulation *in vivo* in the clinic while maintaining sufficient bioactivity for therapeutic usage[14,15]. Increased blood circulation is significant, as it then leads to reduced dosage frequency, and thus increased patient compliance, particularly for treatment of chronic conditions that require frequent dosage regimens[16,17]. Additionally PEG has been shown to increase protein stability by maintaining target protein integrity and reduce immunogenicity through protecting the protein surface from foreign interactions[18,19].

This ability of PEG in protecting the protein surface is key in its widespread therapeutic usage over the years. PEGylation covers the surface of the protein, creating a masking effect. This masking effect is able to prevent immunogenicity against the parent protein, thereby significantly reducing severe adverse events and receptor-mediated clearance[20-22]. PEG is also able to protect proteins from proteolytic degradation, further increasing blood circulation[23,24]. Additionally since

PEG is regarded as low-fouling the PEG surfaces of PEGylated proteins is able to avoid non-specific protein adsorption that may detrimentally affect protein function[25,26]. Thus this protecting effect of PEG allows for a wider range of viable target proteins and significantly increases the blood circulation of these PEGylated proteins compared to their unmodified counterpart.

Additionally PEGylation leads to the increase of the apparent size of protein drugs. Increasing the size of a protein through PEGylation is able to reduce renal clearance, thereby slowing protein drug clearance from the body and increasing blood circulation[6]. The association of water molecules to PEG via hydrogen bonding results in the PEG polymer behaving like it has a significantly larger hydrodynamic size compared to a protein of similar molecular weight[27]. This has been particularly useful when targeting proteins and peptides of low molecular weight that exhibit extremely low blood circulation times to significantly increase blood circulation, including those that are non-immunogenic[19,28,29].

PEG is conjugated to target proteins through a variety of methods. First generation PEGylation techniques involved the random targeting of exposed surface lysine residues by linear PEG polymers due to the ease of modification[30,31]. However this approach results in the formation of multiple isomers, greatly reducing product reproducibility. Amine-modifying PEGylation generally falls into two types of chemistry: alkylating PEGylation which maintains the positive charge of the lysine residue and acylating PEGylation which results in the loss of the lysine residue's positive charge[24]. Second generation PEGylation techniques include the use of new polymers such as branched PEG to increase molecular weight and apparent size and more chemistries to improve conjugation uniformity[32,33]. These new chemistries, such as the targeting of cysteine residues, targeting of the N-terminal α-amine, and targeting specific isoforms of ε-amine lysine conjugation, allows for better control of protein modification, resulting in improved

repeatability and bioactivity retention[32-35]. While these methods often have to balance reduced potency with increased circulation, third generation PEGylation techniques primarily seek to reduce steric hindrance near the active site such that there is no need to balance circulation versus potency[30].

While PEG had previously been generally regarded as biologically inert, recently there have been increasing reports indicating adverse immunological reactions to PEG in the clinic. The presence of native anti-PEG antibodies in healthy blood donors has significantly increased from approximately .2 percent of the population in 1986 to approximately 25 percent of the population in the early 2000s[36-38]. Several previous studies quantifying anti-PEG antibody prevalence have had their methods criticized, and as such a large scale study using fully validated assays published in 2016 assessed that the occurrence of anti-PEG antibodies in healthy individuals was about 23 to 24 percent of the population[39,40]. As early as the 1950s it was suggested that PEG may exhibit non-specific interactions with blood, and anaphylactic reactions were observed in the 1980s in patients undergoing Oncaspar® treatment[6,41]. Omontys®, a PEGylated erythropoiesis-stimulating agent, was voluntarily recalled in 2013 by its manufacturer after numerous severe anaphylactic events and several deaths, though the cause of these severe adverse events was never publically confirmed[8,42].

The presence of anti-PEG antibodies has led to the accelerated blood clearance (ABC) phenomenon and increased sensitivity. It has been demonstrated in PEGylated proteins and liposomes that the presence of anti-PEG antibodies leads to drastically decreased blood circulation, especially when compared to patients that do not exhibit native anti-PEG antibodies[41,43-46]. Additionally anti-PEG antibodies can also be induced by the injection of PEGylated therapeutics, thereby rendering subsequent injections ineffective through the ABC phenomenon when the first

injection demonstrates good blood circulation[21,47,48]. These ABC effects were noted in a phase I trial of a PEGylated uricase[21]. While no patients experienced severe adverse events, five out of the thirteen patients exhibited induced anti-PEG antibodies, leading to increased clearance and thus reduced overall efficacy as shown in **Figure 1.1**. Continued ABC effects were also observed in multiple other studies of PEGylated uricase, including a larger study where 67 out of 169 patients developed anti-PEG antibodies[49,50]. This phenomenon is not unique to PEGylated uricase and has also been observed in several other systems as well[43,44,51-53]. Anaphylactic reactions have also occurred, including incidences when sensitivity events against the PEGylated protein did not occur during the first injection but occurred during subsequent injections in conjunction with the ABC phenomenon[41]. Further, Omontys®, a PEGylated erythropoiesis-stimulating agent approved by the FDA in 2012, was voluntarily recalled by Takeda in 2013 and formally withdrawn in 2014 due to hypersensitivity reactions resulting at least seven known patient deaths[54,55]. While there has been academic speculation, a root cause for any of the patient deaths has not been announced[56-58]. Thus going forward there are increasing concerns about the use of PEG and the future it holds in the clinic.

While PEG has been the most clinically relevant polymer used for protein modification, there have been a wide number of polymers used to modify therapeutic proteins. However most of these polymers are chemically synthesized and, while some may be regarded as biocompatible, are generally not biodegradable[31,59,60]. Many that claim to be degradable disperse cleavable groups throughout the polymer backbone, resulting in the creation of smaller polymeric or oligomeric units that are not biodegradable, or using labile linkers to cleave the polymer from the target protein[61-64]. There has also been significant work done involving biological polymers, primarily polysaccharides to polypeptides, to improve pharmacological properties of protein therapeutics, as

highlighted in **Table 1.2**[31,65-67]. However few of these have been demonstrated at the clinical level, and are primarily limited to polypeptides and proteins modifying target proteins through chemical conjugation or generation of fusion proteins[31,65,68]. Early works utilizing polypeptides and proteins, such as Fc fusions and albumin, are able to extend the half-life of target proteins due to increased size, but do not otherwise protect target proteins[31,66,69]. Recently XTEN and PAS technologies were developed as fusion proteins that also serve to protect target proteins.

PAS is a polypeptide sequence designed to mimic PEG[70]. The PAS sequence was designed to contain uncharged polar residues with restrictions to prevent aggregation, structure formation, and solubility issues, resulting in PAS being composed of proline, alanine, and serine. Multiple constructs of PAS sequences were screened, however only desirable proline- and alanine-heavy sequences ranging from 100 to 600 amino acids are described and there is no discussion on design of any specific sequences[70,71]. PAS exhibits many desirable features, such as it being hydrophilic and exhibiting an ideal random chain structure. Additionally PAS exhibits increased hydrodynamic volume compared to PEG polymers of the same molecular weight and reduced concentration-dependent viscosity. PASylation, the generation of fusion proteins comprising PAS and the target protein, has been used to modify a number of receptor-binding protein with promising results and a number of products are in preclinical studies[72-75]. However no study involving PAS has used PAS to modify an inherently immunogenic protein.

XTEN, first reported in 2009, used similar design principles in order to attempt to achieve similar properties to achieve a polypeptide comprised of alanine, glutamic acid, glycine, proline, serine, and threonine[76]. A library of sequences 36 amino acids in length was screened to determine which sequences were best expressing, and these sequences were appended together to generate sequences ranging from 144 to 1728 amino acids in length, though XTEN sequences used to

modify proteins are generally capped at 912 residues[76,77]. Similar to PAS, XTEN exhibits a generally lacks secondary structure and a relatively large hydrodynamic volume. XTEN has been used both as a part of a fusion protein to modify receptor-binding proteins and also has been chemically conjugated to receptor-binding peptides[78-80]. While XTEN has been shown to reduce protein immunogenicity, XTEN has not been used to modify any inherently immunogenic proteins in the clinic[76]. XTEN has made significant advances in the clinic, with at least three products currently in the clinical phase for at least five indications, with VRS-317, a recombinant human growth hormone modified with XTEN, in phase III trials for pediatric growth hormone deficiency (GHD) indications and in phase II trials for adult GHD indications[31,78,81,82]. While VRS-317 has struggled through phase III trials, XTEN has demonstrated promise in improving pharmacological properties of proteins[83].

Superhydrophilic zwitterionic polymers, including poly(carboxybetaine) (pCB), have been demonstrated to confer ultra-low fouling properties when used to modify surfaces[84-87]. Compared to amphiphilic PEG, modifications of surfaces using these zwitterionic polymers demonstrates superior non-fouling performance[85,88]. This increased performance is due to the ability of zwitterionic pCB in attracting water molecules in an electrically induced manner and forming a stronger hydration layer on the modified surface compared to the weaker hydrogen bonding-associated hydration exhibited by PEG[89,90]. The unique non-fouling properties of zwitterionic pCB are strong enough for surfaces to resist nonspecific biofouling when challenged using undiluted serum and plasma, when many other types of materials are only able to be challenged by 10% serum and plasma, and to allow for the functioning of medical devices for over 40 days while in the presence of whole blood[85,91].

These superhydrophilic properties of pCB have been exploited for use in protein-polymer conjugates. The conjugation of pCB to proteins has conferred increased stability upon proteins without sacrificing biological activity[47,92]. Additionally these protein conjugates demonstrate increased substrate affinity when compared to both the native protein and PEGylated variants of the native protein. This is due to the amphiphilic properties from PEG giving rise to increased hydrophobicity around the protein surface and near the active site, thereby leading to competition against and weakening of the hydrophobic interactions between the substrate and the active site, whereas superhydrophilic pCB is able to recruit water away from the active site to promote hydrophobic active site-substrate interactions[92,93]. These hydrophobic properties of PEG leads to the generation of another mean force potential minimum, leading to increased energy requirements for the active site-substrate interaction.

pCB modification has also drastically improved the pharmacokinetics of proteins, especially concerning immunogenicity. pCB-modified uricase demonstrated improved blood circulation compared to PEGylated and native uricase, dramatically increasing bioavailability[47,94]. Additionally, both PEGylated and native uricase exhibited the ABC phenomenon upon subsequent injections as shown in **Figure 1.2**, while the pCB-modified uricase exhibited minimal variation in circulation profile between injections. PEGylated uricase and pCB-modified uricase were both able to protect the protein, however the presence of anti-PEG antibodies and the marked lack of anti-pCB antibodies leads to pCB-modified proteins demonstrating superior pharmacokinetics compared to their PEGylated counterparts.

pCB-modified organophosphate hydrolase (OPH), a catalytic bioscavenger of poisonous organophosphate compounds, also demonstrated improved properties[95]. Since OPH is an innately immunogenic protein, it demonstrates reduced circulation upon subsequent injections, whereas

OPH-pCB demonstrates a consistent circulation profile with no OPH or pCB immunogenicity. This increased blood circulation coupled with bioactivity retention allowed for animals to survive longer when challenged with organophosphate compounds. Guinea pigs treated with OPH survived less than two days whereas those treated with OPH-pCB were able to survive over a week when challenged daily with $2xLD_{50}$ of sarin, a G-type nerve agent, as shown in **Figure 1.3**. These studies further demonstrate the ultra-low fouling properties of zwitterionic polymers and their applications in protein and drug therapeutics.

Mixed charge peptides containing residues of opposite charge exhibit zwitterionic properties. These peptides, particularly the combination of glutamic acid (E) and lysine (K) residues (EK), are able to exhibit zwitterionic ultra-low fouling characteristics when used to modify surfaces and nanoparticles[96-98]. Bioinformatics studies have shown that the presence of E and K on the protein surface at balanced ratios potentially plays an important role in protein stability, and that the proportion of E and K on the protein surface increases in proteins that exist in more challenging environments[99]. Thus EK presents the ideal peptide analogue to zwitterionic pCB. This naturally-derived polypeptide is biocompatible and biodegradable and ideal for medical applications. And unlike PAS and XTEN, the repeating EK sequence is rationally designed to mimic the zwitterionic properties of pCB and the surface properties of proteins.

The EK polypeptide carries other advantages compared to polymers. EK can be synthesized using recombinant techniques and chemical peptide synthesis methods, allowing for the generation of a product of uniform sequence and length. In addition to being able to use EK for protein-peptide conjugates, the ability to use recombinant techniques allows for the generation of fusion proteins, as diagramed in **Figure 1.4**. Expressing EK as part of fusion proteins demonstrates that these EK-modified fusion protein products can be synthesized uniformly in one

synthesis step without further modification or batch to batch variation. The use of recombinant techniques further allows for the ability to modify any protein, as chemical conjugation techniques can be constrained by the frequency and location of specific functional groups on the protein surface[33].

This body of work focuses on the development of EK systems from demonstrating a proof of concept through *in vivo* capabilities. The primary focus is on developing EK fusion protein systems as a polypeptide analogue to polymer-protein conjugates. Chapter 2 focuses on a proof of concept model in demonstrating the ability to express, purify, and the characterize EK fusion proteins. These EK fusion proteins are generated using a model β-lactamase system, a well-studied antibiotic enzyme, to demonstrate how EK behaves likes zwitterionic pCB *in vitro*. Chapter 3 describes the use of EK fusion proteins in expressing a dimeric organophosphate hydrolase as a stable and increasingly active monomer by using the low-fouling properties of EK to prevent dimer formation. Chapter 4 describes EK fusion protein system comprising granulocyte colony-stimulating factor to characterize the behavior of EK *in vivo*. Chapter 5 describes preliminary work regarding the expression of standalone EK for use in protein chemical conjugation to expand the number of ways EK may be used to modify protein. Finally, Chapter 6 provides concluding remarks for this body of work.

1.1 Tables

Drug	Parent Drug	PEG chains per protein	PEG chain size (kDa)	Year approved
Adagen®	Adenosine deaminase	11-17	5	1990
Oncaspar®	Asparaginase	14-15	5	1994
PegIntron®	Interferon-α-2b	1	12	2000
Pegasys®	Interferon-α-2a	1	40	2001
Neulasta®	G-CSF	1	20	2002
Somavert®	hGH	4-5	5	2003
Macugen®	Anti-VEGF aptamer	1	40	2004
Mircera®	Erythropoietin	1	40	2007
Cizma®	Anti-TNFα Fab'	1	40	2008
Krystexxa®	Uricase	36	10	2010
Omontys®	ESA	1	40	2012[a]
Plegridy®	Interferon-β-1a	1	20	2014
Adynovate®	Factor VIII	2	20	2015
Palynziq™	PAL	9	20	2018
Jivi®	Factor VIII	1	60	2018

G-CSF: granulocyte colony stimulating factor; hGH: human growth hormone; VEGF: vascular endothelial growth factor; TNF: tumor necrosis factor; ESA: erythropoiesis-stimulating agent; PAL: phenylalanine ammonia-lyase

[a]Omontys® was voluntarily recalled in 2013 and formally withdrawn in 2014

Table 1.1. PEGylated protein and peptide drugs approved by the FDA.

Parent Drug	Drug	Modification
CTLA	Orencia®	Fc fusion
	Nulojix®	Fc fusion
EPO	Aranesp®	Glycosylation
Factor VIII	Elocta®	Fc fusion
	Eloctate®	Fc fusion
Factor IX	Alprolix®	Fc Fusion
	Idelvion®	Albumin fusion
	Iprolix®	Fc fusion
GLP-1	Eperzan®/Tanzeum™	Albumin fusion
	Trulicity®	Fc fusion
	Victoza®	Albumin-fatty acid non-covalent binding
Insulin	Levemir®	Albumin-fatty acid non-covalent binding
IL-2	ONTAK®	Fc fusion
LFA-3	Amevive®	Fc fusion
Thrombopoietin	Nplate™	Fc fusion
TNFR	Enbrel®	Fc fusion
VEGFR	Eylea®/Zaltrap®	Fc fusion

CTLA: cytotoxic T-lymphoctye-associate protein 4; EPO: erythropoietin; GLP-1: glucagon-like peptide-1; IL-2: interleukin-2; LFA-3: lymphocyte function-associated antigen 3; TNFR: tumor necrosis factor receptor; VEGFR: vascular endothelial growth factor receptor

Table 1.2. Selection of proteins modified by biomolecules that are FDA-approved.

1.2 Figures

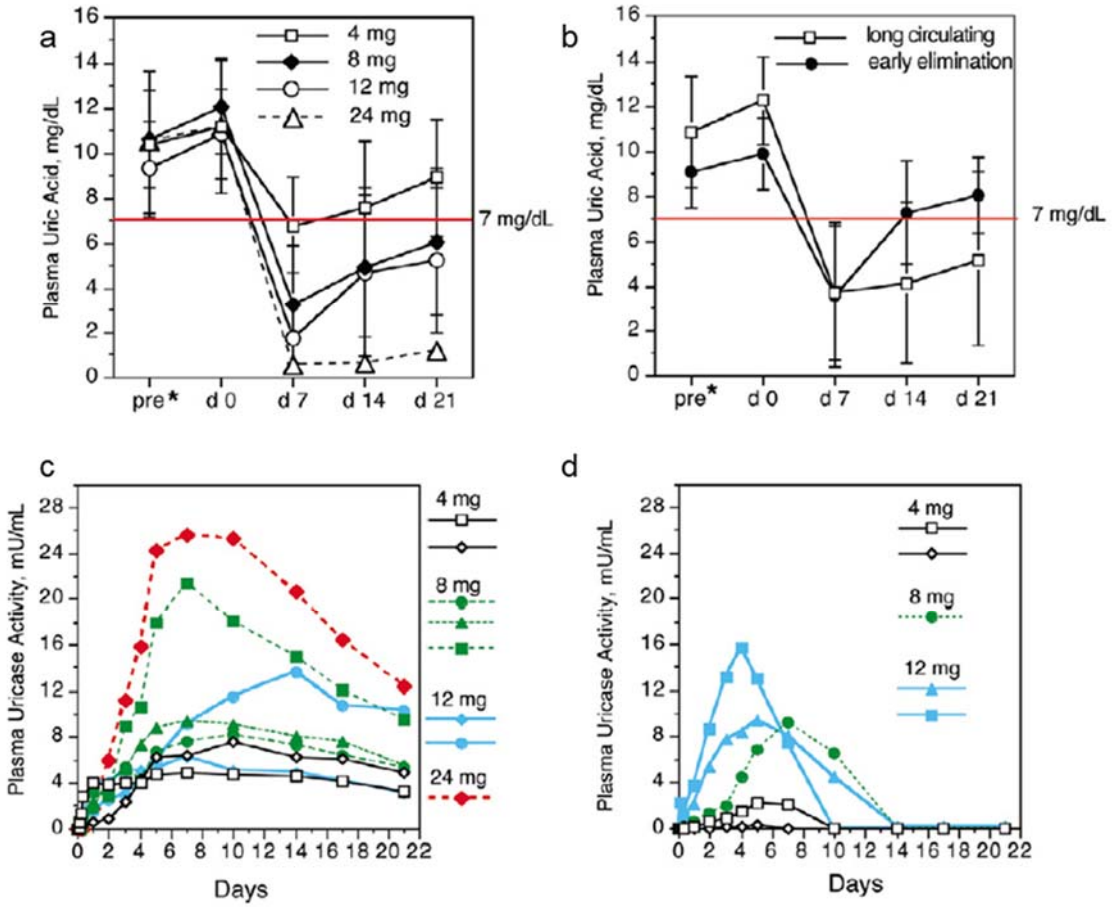

Figure 1.1. Demonstration of the ABC phenomenon in PEG-uricase. (a) Patient response to PEG-uricase is dependent on dosage, though it is noted that (b) patients exhibiting the ABC phenomenon (early elimination) demonstrate a notably reduced drug efficacy. This is also visualized in the plasma uricase activity of (b) patients that do not exhibit the ABC phenomena and (c) patients that do exhibit the ABC phenomena[21].

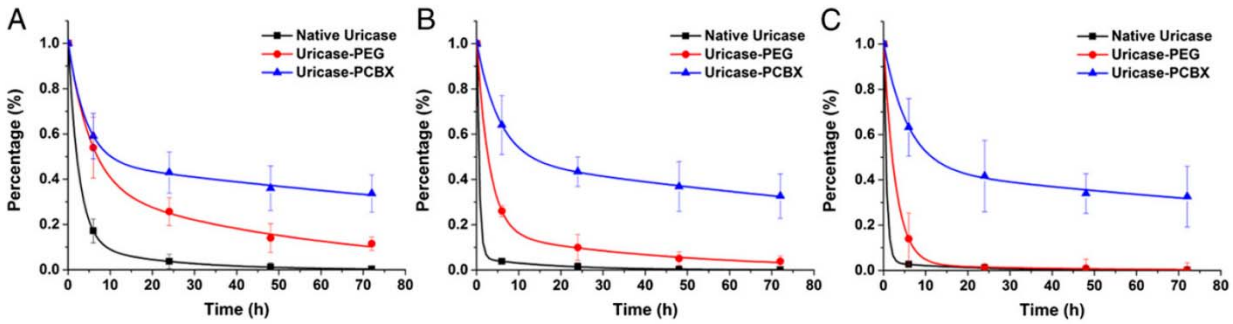

Figure 1.2. Blood circulation of native uricase and uricase modified withi PEG and PCB. Circulation curves are shown for samples after the (a) first, (b) second, and (c) third injections, respectively[47].

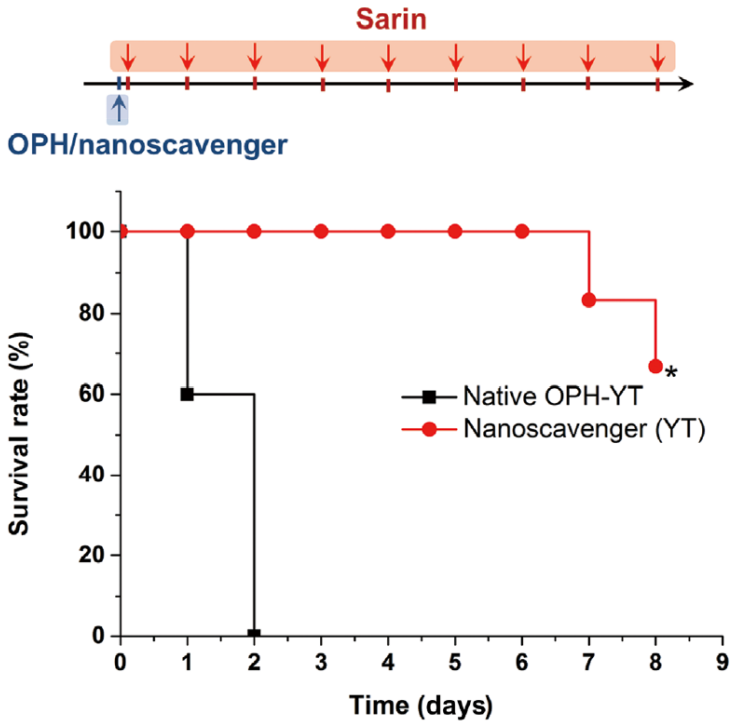

Figure 1.3. Survival rates of guinea pigs after repeated sarin exposure. The bioscavenger was injected, with 2xLD_{50} sarin was administered daily. *Four out of six animals did not show intoxication signs 1 hour after the ninth exposure on day 8[95].

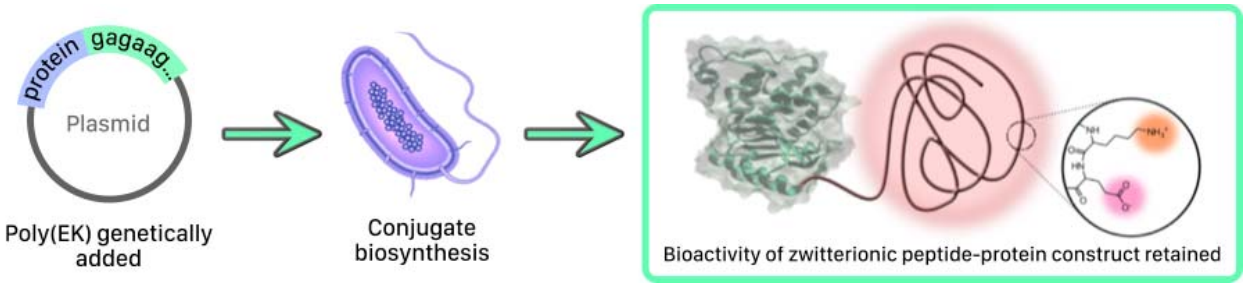

Figure 1.4. Schematic of EK fusion proteins.

Chapter 2

Using a model β-lactamase system to generate and characterize EK fusion proteins

β-lactamase (Bla) is a well-characterized protein that is used to confer antibiotic resistance when secreted from the cellular cytoplasm. Bla is often used in recombinant protein expression applications as an antibiotic resistance marker against ampicillin and carbenicillin. Bla is a relative stable protein that has been well-studied and whose expression in prokaryotic organisms is relatively straightforward and routine. Additionally studies have generated multiple variants of Bla offering various levels of stability that differ by one or two point mutations that were generated through directed evolution and are easily accessible through site-directed mutagenesis. Thus Bla and a destabilized Bla mutant (TEM-19) presented themselves as an appropriate model system to demonstrate expression of EK fusion proteins and how the presence of EK affects differing proteins. EK polypeptides of varying length were appended to the C-terminus of both Bla and TEM-19. All the proteins were successfully expressed into the periplasmic space in *E. coli* and purified using affinity chromatography and size exclusion chromatography. The addition of EK to the Bla proteins lead to increased protein stability when exposed to challenging environmental stressors while maintaining bioactivity. EK of varying lengths had differing effects upon the stability and kinetics of the model proteins. The effect of EK on these proteins is very similar in how pCB has protected proteins, indicating the promise of the EK fusion protein approach for modifying therapeutic proteins.

2.1 Introduction

There has been a significant amount of work done by the Jiang group in utilizing pCB to modify proteins[47,92,94]. These protein-polymer conjugates demonstrate increased stability and kinetics without sacrificing bioactivity. Additionally these pCB-protein conjugates exhibit

superior *in vivo* pharmacokinetics and reduced immunogenicity compared to both their wild-type and PEGylated counterparts. These marked improvements are due to the superhydrophilic nature of zwitterionic pCB and its ability to form a stronger non-fouling hydration layer on the surface of the protein compared to amphiphilic PEG. This is of particular note given the rise of antibodies against PEG[40].

The mixed charge EK polypeptide has demonstrated similar ultra low-fouling techniques compared to zwitterionic pCB when used to modify surfaces, thus presenting it as a natural peptide analogue to pCB. The use of the EK polypeptide allows for the use of recombinant techniques to both express EK by itself and to utilize it as a part of a fusion protein. These techniques allow for a one-step synthesis of a well-defined EK-modified product that is completely uniform without batch to batch variation. Additionally the use of recombinant techniques allows for the expression of polypeptides significantly longer than what can be achieved using peptide synthesis[100]. Thus EK presents itself as a promising candidate for protein modification due to its biocompatibility and biodegradability in addition to its zwitterionic properties and the ability to use recombinant techniques.

However EK polypeptide sequences had not been previously expressed using recombinant techniques and previous EK synthesis had been limited to short sequences achieved through peptide synthesis[96-98,101]. The usage of solid-phase peptide synthesis techniques is generally limited to peptides below 10 kDa in length, and the use of chemical ligation to link multiple peptide fragments together oftentimes introduces extra amino acids or functional groups that may be undesirable or non-natural[100,102]. The limits of peptide synthesis often results in products that exhibit short circulation profiles and variable selectivity[103]. Thus as a result most PEGylated and pCB-modified proteins are either modified with polymers of considerably longer length that which

can be achieved through solid-phase peptide synthesis or modified with a larger number of smaller polymers as shown in **Table 1.1**, thus further necessitating the use of recombinant techniques to synthesize EK.

β-lactamases are a class of enzymes that confers antibiotic resistance by hydrolyzing β-lactam-containing antibiotic agents[104,105]. These antibiotics function by inhibiting transpeptidase enzymes necessary for the formation of the peptidoglycan layer in bacterial cell walls[104,106]. Bla is primarily categorized in a molecular classification system based on characteristic motifs. Class A Bla was the first group of recognized Bla proteins, with class C and D families being recognized once molecular differences between the various types were recognized, and the class B family being characterized as being comprised of zinc-containing metallo-β-lactamases[107]. Bla is commonly used as an antibiotic resistance selection marker for protein expression due to its relative ease of use compared to other antibiotics, particularly in bacterial systems, and is secreted from the host organism to neutralize antibiotics[108,109]. The variant of Bla that is used in the popular pET system is the TEM-1 variant, a class A Bla, which is suggested to be potentially responsible for up to 90% of ampicillin resistance in *E. coli*[110].

Bla is a relatively stable protein that is well-characterized that can be routinely expressed in *E. coli*, particularly as an inclusion body in the cell cytoplasm[105,111,112]. Expression and secretion of Bla into the periplasm or solution leads to the expression of a soluble protein without the need to refold the protein from an insoluble inclusion body[109,113]. Multiple variants of the TEM-1 variant of Bla (herein referred to as Bla) of differing stability levels are easily achieved through site-directed mutagenesis due to their sequences differing by one or two point mutations[114]. This allows for the facile study of proteins of varying stability levels and elucidating how EK can effect similar proteins of differing stability levels. These factors make Bla and its variants an appropriate model

system to demonstrate the expression of EK fusion proteins how EK affects the resulting expressed proteins.

Herein EK fusion protein systems containing Bla variants are described. The wild-type Bla (TEM-1) gene and a destabilized mutant TEM-19 (G238S) were selected to be modified with EK. Genes encoding for repeating EK sequences approximately 10kDa and 30kDa in length are appended to the C-terminal end of both Bla and TEM-19, as that terminus is further from the active site and thus should reduce deleterious effects to the active site structure. These EK fusion proteins demonstrate the ability to further stabilize proteins without drastically affecting bioactivity while improving the enzyme kinetics. This method of modifying therapeutic proteins using a rationally designed mixed-charge polypeptide demonstrates potential in addressing drawbacks related to polymer conjugation methods by providing a homogeneously replicable product that is biocompatible.

2.2 Results and Discussion

The gene encoding for Bla was amplified from the pJG108 plasmid and quick-change mutagenesis was used to generate the TEM-19 sequence. The genes contained an OmpA signaling sequence to direct secretion of the expressed proteins into the periplasmic space, where the OmpA sequence is cleaved from the expressed protein, to express Bla and TEM-19 as soluble proteins[109]. EK sequences encoding for peptides approximately 10kDa ($(EK)_{41}$)and 30kDa ($(EK)_{120}$) in length were appended to the C-termini of both proteins, generating four EK fusion proteins: EK(30k)-Bla, EK(10k)-Bla, EK(30k)-TEM19, and EK(10k)-TEM19. The genes were inserted into a pBLN2000 expression vector, which contains an arabinose-inducible pBAD promoter, and the plasmids were verified to be correct[115,116]. Expression of the target proteins was performed using *E. coli* BL21 (DE3) cells. After undergoing osmotic shock to isolate the periplasmic fraction of

the expression hosts, the target proteins were purified using affinity chromatography and further purified using size exclusion chromatography as needed. The expression of the desired target proteins was validated through mass spectrometry as shown in **Figure 2.1**, and visualized using SDS-PAGE as shown in **Figure 2.2**.

During the expression of the target proteins it was noted that the addition of EK to the protein sequence dramatically reduced protein yield, with increasing EK length further decreasing expression yield. However overall yields for all proteins including unmodified Bla and TEM-19 relatively low, in the .5mg/L to 5mL range, due to the relatively low yields achievable through periplasmic expression compared to cytosolic expression[117,118]. This is due to a variety of factors, but one cause of reduced expression of wild-type Bla is low secretion efficiency as observed in **Figure 2.2**. Once the low secretion efficiency is considered, it becomes evident that EK fusion proteins express at a considerably lower level than their wild-type counterparts. While the expression of EK fusion proteins in usable quantities was demonstrated, this indicates that the expression of EK fusion proteins is creating a significant burden on the host. Additionally experiments where the media was supplemented with extra glutamic acid and lysine did not appreciably increase yields, indicating that the culture is not completely depleting the available supply of amino acids and that issues with expression yields lie elsewhere in the protein expression process. One possible hypothesis is that the expression of EK is leading to rapid depletion of the charged tRNAs for lysine and glutamic acid, leading to the reduced expression of necessary host proteins and thus resulting in reduced cell function. The use of the periplasmic expression tag, which originally led to the expression of soluble Bla and TEM-19, could also have been the reason for improved host viability by diverting energy from overexpressing the EK fusion proteins and

thus rapidly depleting charted tRNAs to instead secreting the synthesized EK fusion proteins into the periplasm.

Once expressed, the effect of EK on the stability and kinetics on Bla and TEM-19 were studied. Kinetic parameters were studied using benzylpenicillin as the substrate. Overall the addition of EK did not strongly affect the kinetic properties of the proteins, as summarized in **Table 2.1**. The addition of the shorter 10kDa EK did not change the catalytic activity (k_{cat}) of both Bla and TEM-19, while the addition of the longer 30kDa EK led to retention of approximately 70% and 60% of activity of Bla and TEM-19, respectively. Appending 30kDa and 10kDa EK to Bla reduced the Michaelis-Menten constant (K_m) from 70μM to 25μM and 45μM, respectively, and appending them to TEM-19 reduced the K_m from 50μM to 5μM and 30μM, respectively. The reduction of K_m, and thus the increase in substrate affinity, was previously demonstrated in pCB-protein conjugates, and this can be explained by the superhydrophilic pCB locally promoting hydrophobic interactions required for the binding of the substrate to the active site of the enzyme[92]. Overall the addition of EK led to marked increases in the overall catalytic efficiencies of both Bla and TEM-19.

Similar to conjugation of pCB to proteins, the addition of EK confers increased thermal stability when used to modify Bla and TEM-19, as shown in **Figure 2.3**. The responses to thermal stress highlighted the influence of the EK length on the ability of EK in protecting proteins. The addition of EK(10k) increased the transition midpoint temperature by approximately 15°C without affecting the shape of the unfolding curve. The addition of EK(30k) stabilized the enzymes at extremely high temperatures and increased the midpoint transition temperature by approximately 20-30°C. This indicates that there is a demonstrable trade-off between maintaining enzymatic activity at lower temperatures and retaining stability at extreme temperatures, suggesting that the

length of EK can be optimized to suit specific applications (i.e. catalytic or storage conditions). Additionally it may suggest that differing lengths of EK may affect unfolding mechanisms differently, with the longer EK(30k) potentially interacting with the protein during the unfolding process. Remarkably, proteins modified with EK(30k) were able to retain 30-40% of their initial activities after being incubated at 95°C for an hour, up to a 300% improvement over wild-type enzyme in terms of activity retention when exposed to the same conditions.

In addition to thermally challenging these fusion proteins, EK demonstrated the ability to protect proteins in the presence of high salt, as shown in **Figure 2.4**. Both EK(30k)-Bla and EK(30k)-TEM19 retained approximately 80 percent activity after 90 minutes of incubation at 80°C in the presence of 2M NaCl while wild-type Bla and TEM-19 were completely inactivated. The more pronounced effect stabilizing effect of EK in high salt conditions indicates that E and K may interact with counterions in solution to buffer deleterious ionic interactions experienced by proteins at higher temperatures and at high salt concentrations and prevent its destabilization[119]. Similar studies were conducted without the use of a blocking agent in the assay buffer. This resulted in a much more rapid loss of enzymatic activity of unmodified proteins as shown in **Figure 2.5**, suggesting that EK reduces interactions with other proteins and surfaces that may lead to denaturation, particularly through deleterious hydrophobic interactions. Thus EK may behave as a general protein stabilizer by preventing physical and chemical stressors from interfering with protein structure and activity.

2.3 Conclusions

In summary this work demonstrates the ability to clone, express, and purify EK fusion proteins using recombinant protein expression techniques and the ability of EK in stabilizing proteins. EK was rationally developed as a peptide analogue of ultra low-fouling zwitterionic pCB

that exhibits similar ultra low-fouling properties. EK polypeptide sequences of varying lengths were appended to the C-termini of a model β-lactamase and destabilized TEM-19 variant. These fusion proteins retained biological activity but exhibited increased proteins stability, consistent with previous results with pCB-protein conjugates. EK of differing lengths led to different protecting effects, with shorter EK sequences conferring additional stability without negatively impacting bioactivity and longer EK sequences imparting a small loss in bioactivity but a significant increase in thermal stability, particularly at extremely high temperatures. EK was also found to reduce K_m, thereby demonstrating increased substrate affinity by promoting hydrophobic protein-substrate interactions. Such marked stability improvements conferred through the use of a biologically designed and well-defined method should find applications in fields ranging from drug delivery to protein therapeutics.

2.4 Materials and methods

All materials were purchased commercially unless otherwise noted and were used without further purification. All buffers were made from commercially available products and Milli-Q® water (EMD Millipore). All DNA primers were synthesized commercially (Invitrogen). All error bars represent one standard deviation from the mean.

2.4.1 Cloning

E. coli DH10B (Life Technologies) was used for cloning. PCR was used to extract the Bla gene containing the OmpA periplasmic secretion tag from the pJG108 vector (New England Biolabs) using forward primer 5'-TAATAACATATGAAAAAGACAGCTATCGCGATTG-3' and reverse primer 5'-TAATAAAGCTTACCAATGCTTAATCAGTGAGGC-3'. The resulting fragment was inserted into the pBLN2000 expression vector conferring kanamycin resistance at the NdeI

and HindIII restriction sites. The stop codon was removed using QuikChange mutagenesis (Agilent Technologies) using forward primer 5'-GATTAAGCATTGGAAGCTTGCGGCC-3' and reverse primer 5'-GGCCGCAAGCTTCCAATGCTTAATC-3'. Genes encoding for EK(30k) and EK(10k) were commercially synthesized (GenScript) and inserted at the HindIII and XhoI restriction sites to generate the EK-Bla genes with the OmpA periplasmic secretion tag. The TEM-19 derivatives were obtained by introducing the G238S mutation in the Bla sequence through site-directed mutagenesis using forward primer 5'-TCTCAGAATGACTTGGTTAAATACTCACCAGTCACAGAA-3' and reverse primer 5'-TTCTGTGACTGGTGAGTATTTAACCAAGTCATTCTGAGA-3'. All sequences were verified by a third party (Eurofins Scientific) for accuracy.

2.4.2 Protein expression and purification

Protein expression was performed using *E. coli* strain BL21 (DE3) (Life Technologies) cultured in Terrific Broth (TB) supplemented with kanamycin. TB was supplemented with 15% w/v sucrose for the expression of unmodified Bla and TEM-19[120]. Cultures were induced with 0.2% w/v arabinose once the culture reached OD600 0.5, and protein expression was allowed to occur for 4 hours. Cultures were then subjected to osmotic shock. In an osmotic shock, the culture is harvested and washed in 30 mM Tris, pH 7.3. The culture is then resuspended in 50% 30mM Tris, pH 7.3 and 50% 30mM Tris, 1.5mM EDTA, 40% sucrose, pH 7.3 at room temperature for 15 minutes before being centrifuged. The pellet was then resuspended quickly in ice cold 1 mM $MgCl_2$ and put on ice for 10 minutes before being centrifuged at 4°C. The periplasmic fraction is contained in the supernatant, and is dialyzed against the loading buffer used in the maminophenylboronic acid-agarose purification step (subsequently described). Bla and TEM-19 variants were purified from

the periplasmic fractions with maminophenylboronic acid-agarose (Sigma-Aldrich), as previously described with a modified flow rate of approximately 0.2 mL/min[105]. In brief, the loading and wash buffer used was 20 mM triethanolamine, 500 mM NaCl, pH 7 and the eluting buffer used was 500 mM borate, 500 mM NaCl, pH 7. Further purification was performed, as necessary, by Superdex 75 10/300 size exclusion chromatography (Phenomenex) or HiTrap DEAE FF ion exchange chromatography (GE Healthcare). Anion exchange was carried out using a loading buffer of 20 mM Tris-HCl, 10 mM NaCl, pH 8, with an increasing NaCl gradient. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) spectra of purified samples were obtained in linear mode using a Bruker Autoflex II machine.

2.4.3 Enzyme kinetics and activity assays

Bla and TEM-19 enzymes and their respective EK derivatives were diluted to 5 nM in 20 mM sodium phosphate, pH 7.0, with 10 μg/mL bovine serum albumin (BSA, Sigma-Aldrich) added as a blocking agent. Benzylpenicillin (BP) (Life Technologies) solution in the same buffer was added to enzyme samples in UV-transparent 96-well microplates (Corning) at varying final concentrations from 5 to 2000 μM. Enzyme activity (V) was measured using a Cytation3 microplate reader (BioTek) as the initial linear rate of loss in substrate absorbance at 232 nm. Activity (V) versus BP substrate concentration [S] was modeled using standard Michaelis−Menten kinetics using DataGraph 3.2 (Visual Data Tools). After determination of kinetics parameters, all further activity assays were performed at BP concentrations at least 10× K_m, typically 1000 μM. All kinetics and activity experiments were performed in quadruplicate at room temperature.

2.4.4 Thermal stability assays

Enzymes were diluted to 20 nM in 20 mM sodium phosphate, pH 7.0, with 10 μg/mL BSA added as a blocking agent. Enzymes were incubated at temperatures ranging from 25 to 95 °C, and each

solution was incubated at the heightened temperature for 45 min, after which they were placed on ice until being returned to room temperature immediately before activity assay at room temperature, as previously described.

2.4.5 Salt Stability assays

Enzymes and conjugates were diluted to 20 nM in sodium phosphate buffer (20 mM, pH 7.0) or PBS with 2M total NaCl (high-salt PBS). Temperature incubation at 80 °C was performed as previously described, with aliquots removed at specified times and placed on ice until assay was run at room temperature.

2.5 Tables

Enzyme	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} μM^{-1})
Bla	1180	70	17
EK(30k)-Bla	870	25	35
EK(10k)-Bla	1140	45	25
TEM19	29	50	0.6
EK(30k)-TEM19	18	5	3.6
EK(10k)-TEM19	26	30	0.9

Table 2.1. Kinetic parameters measured for Bla and TEM-19 constructs.

2.6 Figures

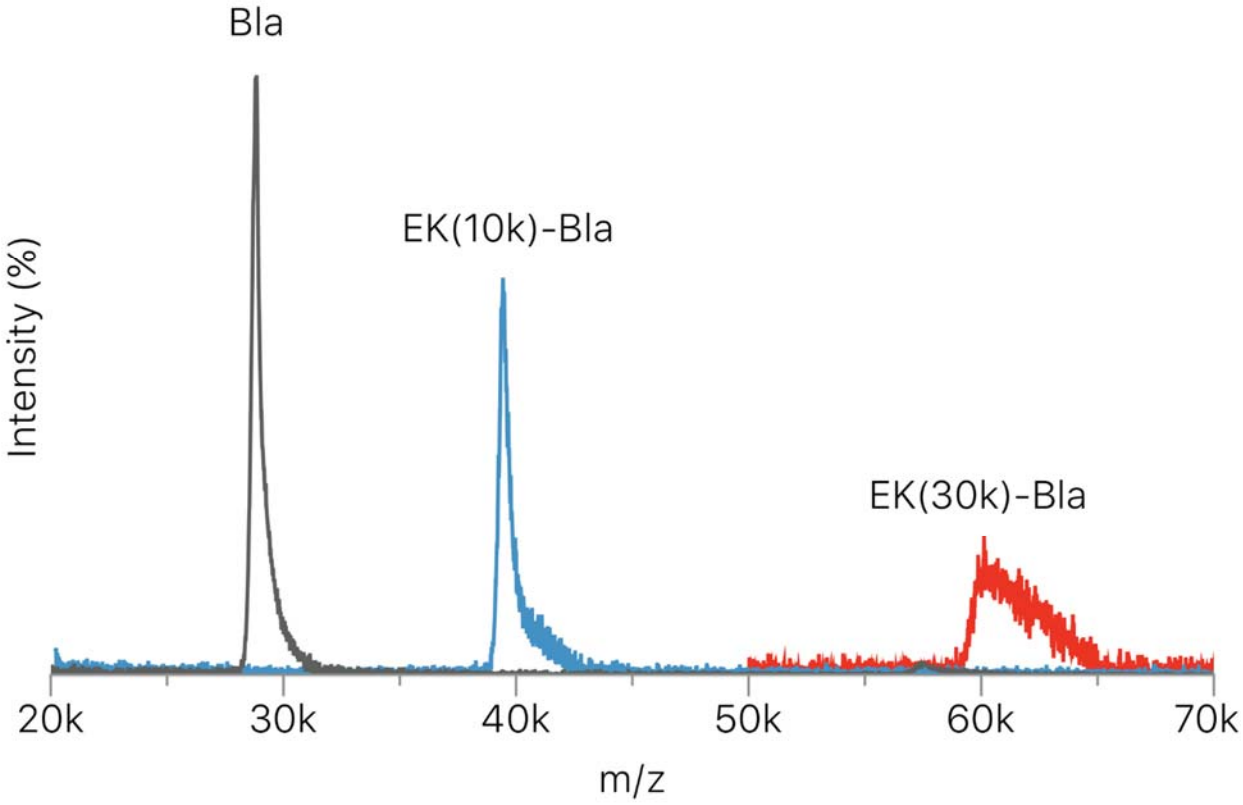

Figure 2.1. MALDI-TOF spectra of Bla, EK(10k)-Bla, and EK(30k)-Bla.

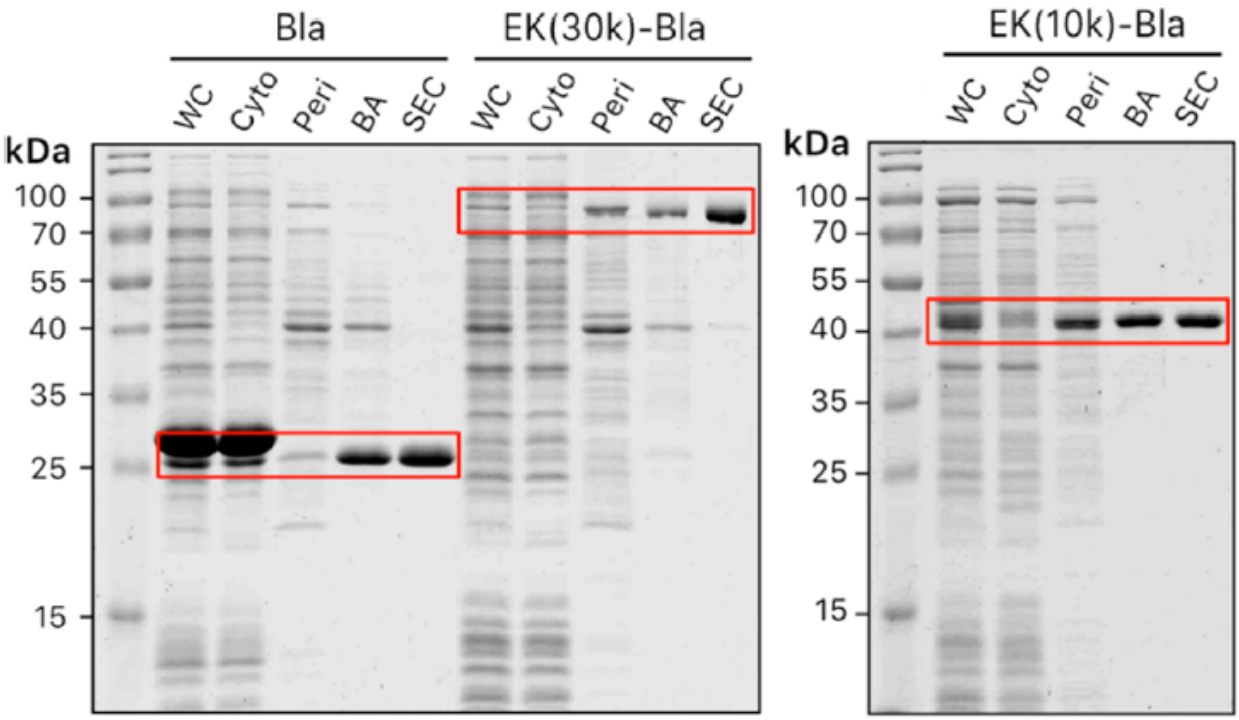

Figure 2.2. Expression and purification of EK-Bla fusion proteins. Representative SDS-PAGE gels demonstrating the expression and purification of Bla, EK(30k)-Bla, and EK(10k)-Bla showing whole cell (WC), cytosolic (Cyto), and periplasmic (Peri) fractions and products after affinity (BA) and size exclusion (SEC) chromatography.

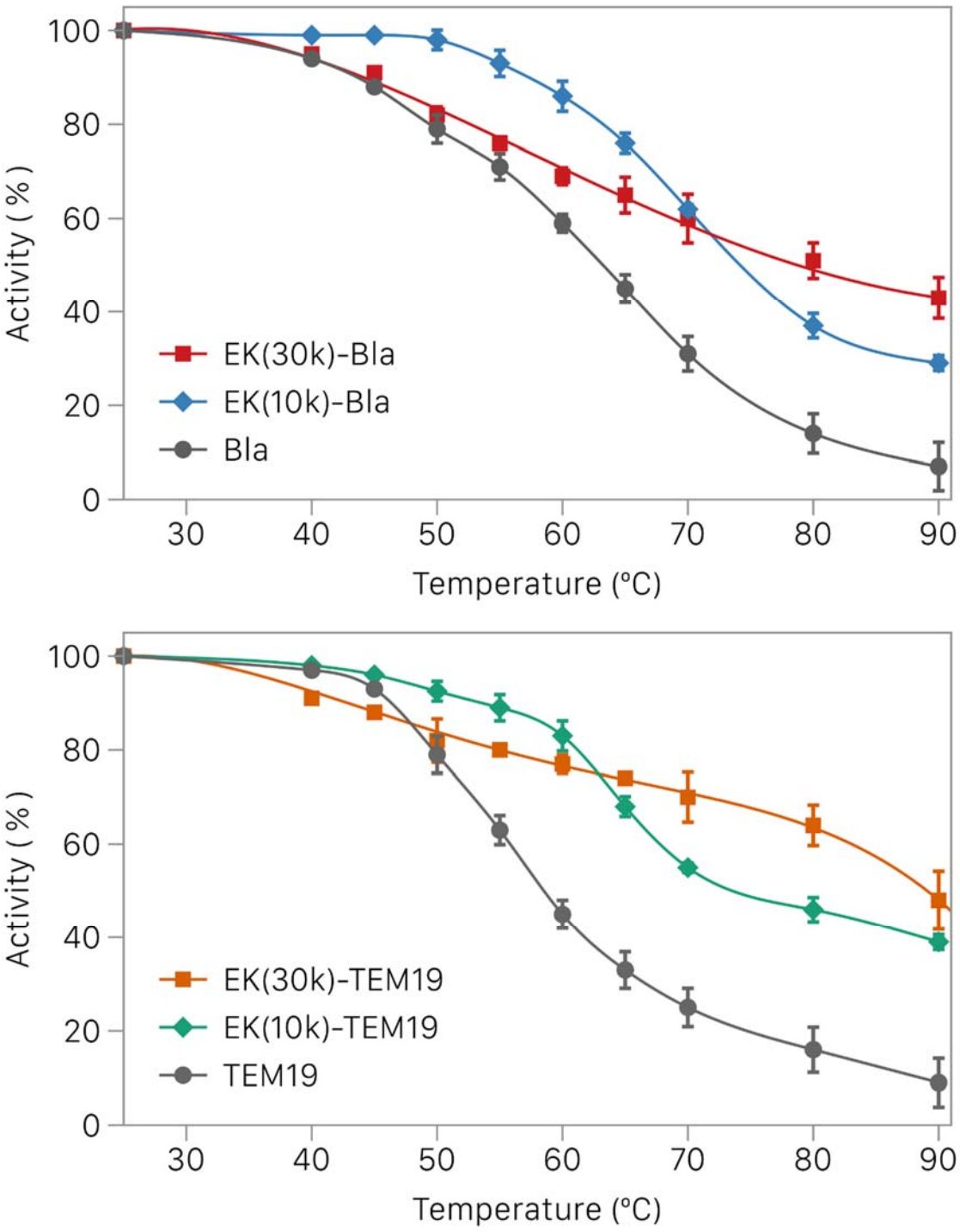

Figure 2.3. Thermal stability of EK fusion proteins. For Bla (top) and TEM-19 (bottom) fusion proteins, the addition of EK(10k) confers a similar stability benefit throughout, while the EK(30k) modification increases stability most under more extreme conditions above T_m.

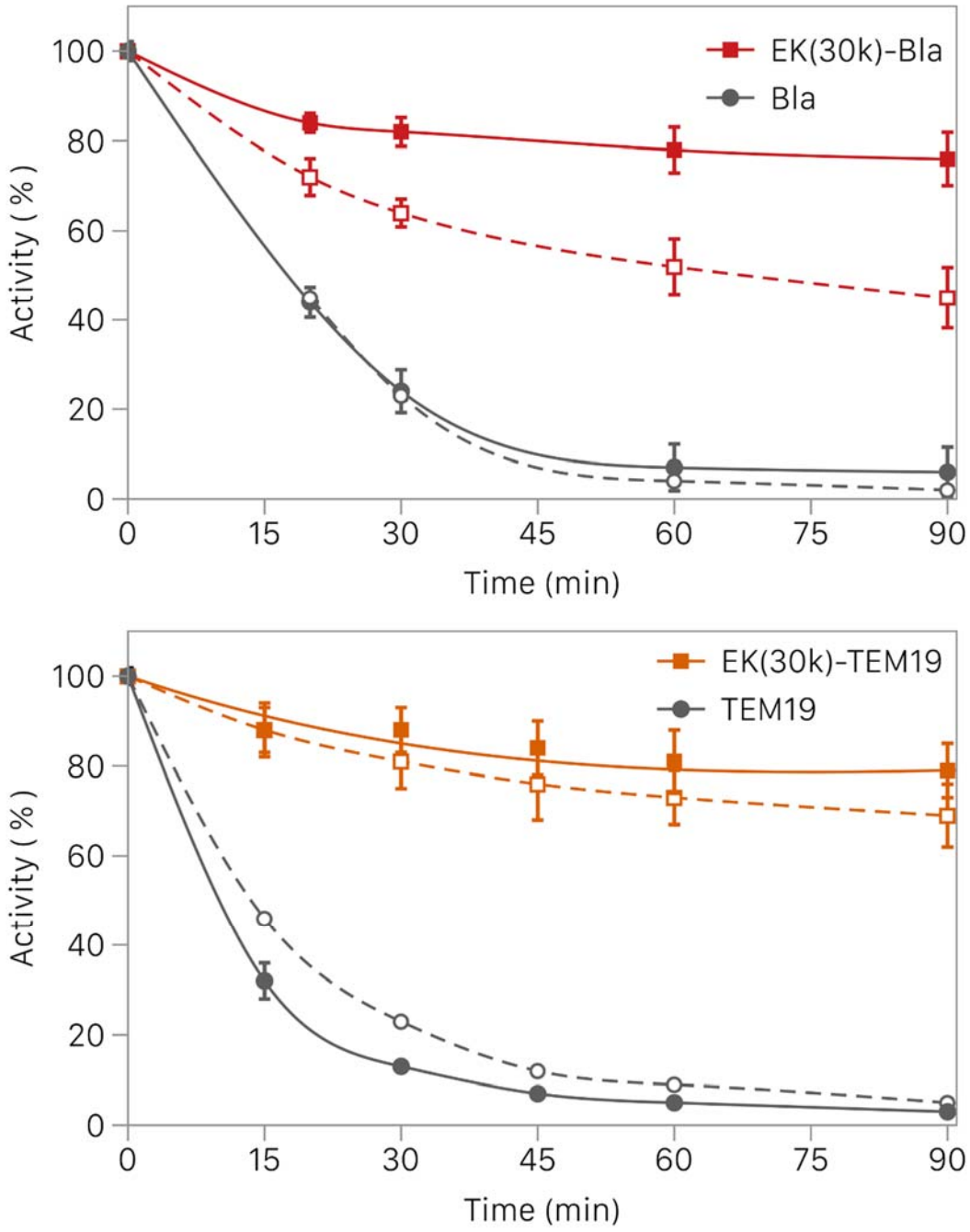

Figure 2.4. Thermal stability of EK fusion proteins under variable salt conditions. EK(30k)-Bla and Bla (top) and EK(30k)-TEM19 and TEM-19 (bottom) were incubated at 80°C under standard 20mM (dashed lines) and high 2M (solid lines) salt conditions. Similar substantial activity loss is observed for unmodified proteins, whereas EK fusion proteins retain high activity, particularly in high salt.

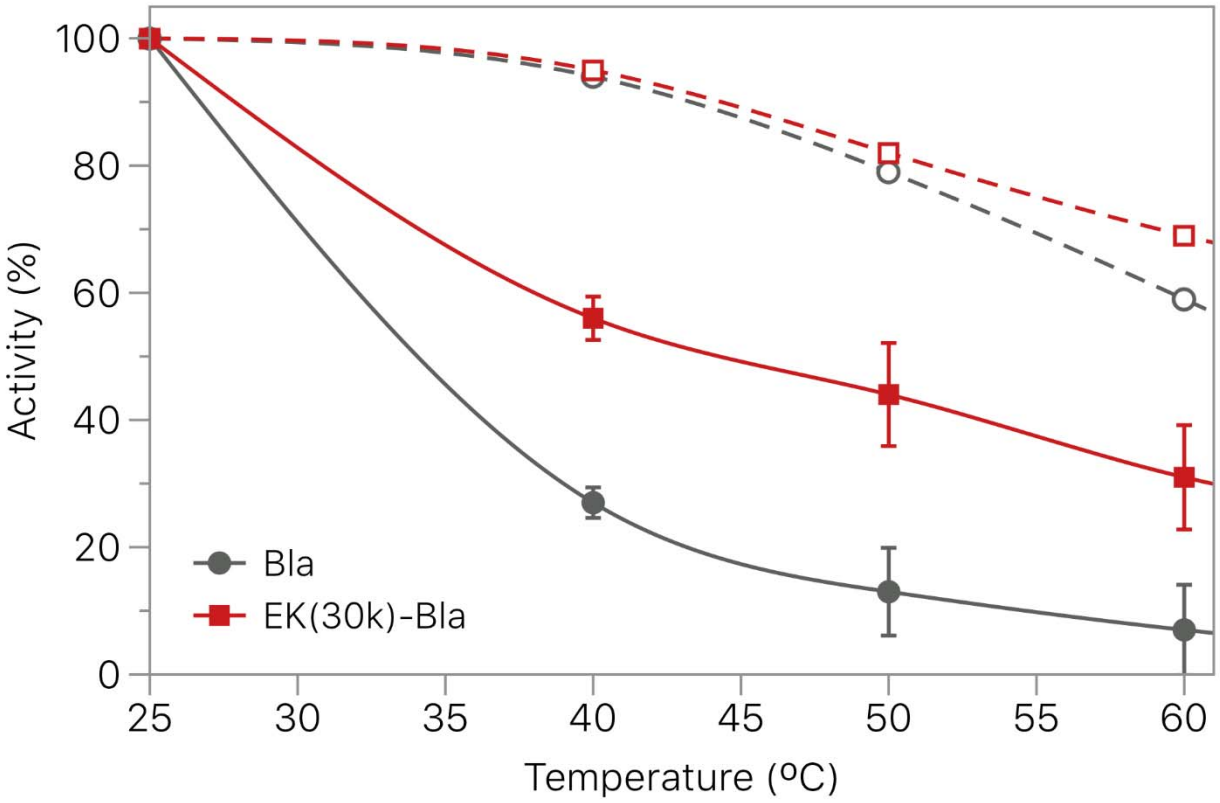

Figure 2.5. Temperature stability of Bla proteins with and without blocking agents. Thermal stability assays Bla and EK(30k)-Bla were run without BSA (solid lines) and with BSA (dashed lines). The higher retained activity by EK(30k)-Bla in BSA-free solutions that EK may prevent activity loss by reducing foreign interactions.

Chapter 3

Expression of a monomeric organophosphate hydrolase as an EK fusion protein

Organophosphate hydrolase (OPH), also known as phosphotriesterase, is a bacterial paraoxonase that demonstrates wide substrate promiscuity against various organophosphate (OP) compounds. These many OP compounds comprise nerve agents and pesticides that present significant risks and dangers worldwide. OPH is expressed as a stable dimeric protein in prokaryotic hosts. We demonstrate, to the best of our knowledge, the first example of a stable OPH monomeric unit by expressing a fusion protein containing a 30kDa EK sequence at the C-terminus end of OPH. This method was able to disrupt formation of the dimer interface found in native OPH due to the presence of EK and its ultra low-fouling properties. This OPH-EK fusion protein demonstrated a 70 percent increase in catalytic activity per active site by potentially reducing steric hindrance near the active site and increased substrate affinity as well by reducing K_m by approximately 70 percent. In addition, stability conferred by the addition of EK was able to overcome the stability loss caused by the elimination of the dimer interface. The structure of EK was probed using circular dichroism (CD). By deconvoluting the CD spectra using a structure predictor with improved β-sheet predictive capabilities, it was shown that EK samples a wide range of conformations, but does not necessarily settle into specific conformations, and is able to increase the solubility of hydrophobic OPH. While it was not able to be demonstrated in the OPH-EK system, the use of EK as a part of EK fusion proteins demonstrates significant promise for use in *in vivo* systems. This strategy can potentially be used to aid the expression of prokaryotic proteins in eukaryotic hosts.

3.1 Introduction

OP compounds exhibit physiological toxicity through inhibition of acetylcholinesterase[121]. OPs center around a pentavalent phosphorus and can be broadly categorized as pesticides (insecticides) and G-type and V-type nerve agents depending on the side groups, as shown in **Figure 3.1**[122,123]. The use of OP pesticides in agriculture and nerve agents in chemical warfare presents significant potential risks worldwide. OP compounds derive their toxicity from their ability to bind to and inactivate acetylcholine esterase, leading to a buildup of acetylcholine and ultimately resulting in improper nervous system function[124]. The use of pesticides has demonstrated increased cancer risks and is responsible for upwards of 200,000 deaths annually in Asia alone due to self-harm alone[125,126]. G-type and V-type chemical warfare agents are exceedingly more potent than pesticides, with dermal LD_{50} values as low as 6mg in healthy 70kg males[127]. The ability to synthesize OP compounds, including G-type and V-type chemical warfare agents, with relative ease coupled with large current stockpiles indicates the great importance and urgency in developing capabilities to neutralize OP compounds both chemically on a large scale and in the bloodstream of individuals affected by OP poisoning[128,129].

Catalytic bioscavengers have demonstrated great efficacy at neutralizing OP compounds, especially compared to stoichiometric bioscavengers[130-132]. Catalytic bioscavengers carry the inherent advantage that they can hydrolyze a large amount of OP compounds with high turnover numbers, whereas stoichiometric bioscavengers can only directly bind to and thus neutralize a set amount of OP compounds. Despite those advantages, stoichiometric bioscavengers such as butyrylcholineesterase (BuChE) have been explored in significantly more detail in clinical applications compared to their catalytic bioscavenger counterparts[133-135]. Additionally, BuChE is a protein that is naturally synthesized in humans and has been recombinantly expressed in

mammalian and transgenic systems, resulting in increased compatibility with regards to safety, whereas many effective catalytic bioscavengers are of bacterial origins and those of human origins lack efficacy[135].

Examples of catalytic bioscavengers include organophosphorus acid anhydrolase (OPAA), which is specific to substrates containing P-F bonds, and OPH, which has a much broader substrate specificity, including activity against various G- and V-type nerve agents[121,136]. OPH is a prokaryotic paraoxonase first isolated from *Pseudomonas diminuta* and *Flavobacterium* sp. (ATCC27551) that demonstrates wide substrate promiscuity against OP compounds[132,137]. The promiscuity of the enzyme has been utilized to great effect in evolving for catalytic activity against specific substrates of interest[123,138,139]. With approximately 11% of each monomer's surface involved as a part of the dimerization interface, OPH exists as a very stable dimer[140]. The stability conferred to OPH by the dimer interface is great enough such that the individual subunits of the dimer will denature and unfold before the primarily hydrophobic interactions that form the dimer interface becomes unstable and dissociate[141]. Each monomeric unit is composed of an $(\beta/\alpha)_8$ barrel folded around two divalent metallic cofactors[142]. The cofactors play a central role in the hydrolysis of OP compounds, coordinating with residues at the active site as well as coordinating OP compounds in the first step of OP hydrolysis mechanism. Currently there are no known examples of a bacterial OPH being expressed as a stable standalone monomeric subunit in any expression host.

As OPH exists natively as a bacterial protein, it has been challenging to express OPH in higher-order systems beyond prokaryotes. OPH has been expressed in eukaryotic organisms such as yeast, fungal, and plant systems, but it remains a challenge to express in a mammalian host[143-146]. Challenges in mammalian expression of OPH include low secretion from the host, poor protein

folding, and low bioactivity[147,148]. Sequence analysis of OPH also reveals that there are multiple potential sites for post-translational modification via N-linked glycosylation when OPH is expressed in mammalian systems, providing another barrier in expressing a functional OPH in mammalian systems.

Previously work appending mixed-charge EK polypeptides to proteins led to the expression of fusion proteins that are more stable and demonstrated increased substrate specificity compared to their wild-type counterparts in a similar manner that zwitterionic pCB protects proteins, as discussed in Chapter 2[47,92,149]. However challenges remain in expressing EK fusion proteins in sufficiently high yields for practical applications. Herein OPH-EK fusion proteins are described. The OPH gene is modified at the C-terminus with a 30kDa EK gene. The addition of EK led to the expression of OPH-EK as a functional monomeric unit, which had not been previously demonstrated. This new monomeric OPH-EK demonstrated similar stability compared to dimeric OPH but greatly improved kinetics. This work further demonstrates the properties of EK previously observed and further exhibits the non-fouling properties of EK on the surface of the protein.

3.2 Results and discussion

This work examined the effect of EK on the properties of OPH, a dimeric bacterial paraoxonase. The 30 kDa OPH gene was used as previously described[132]. A sequence encoding for a 30 kDa EK polypeptide was appended to the C-terminus of OPH gene to generate an OPH-EK fusion protein gene. Immediately the challenges observed in expressing the Bla-EK fusion proteins manifested again when expressing OPH-EK in *E. coli*. Expression of OPH-EK in the cell cytosol resulted in significant cell death and extremely no yield (<10μg/L). Thus, similar to the Bla systems, the OPH-EK gene was placed under the control of a pelB leader sequence for

expression and secretion of the fusion protein into the periplasmic space. This approach increased cell viability by reducing cell death, allowing for increased yields of OPH-EK, though albeit not dramatically (1mg/L) considering that native OPH variant itself expresses at yields of up to 100mg/L. Additionally OPH-EK demonstrated better expression at 30°C instead of 37°C, further demonstrating the benefits of slowing down the expression of these difficult to express proteins. Thus the expression of EK fusion proteins without further modifications to the expression system or significant optimization remains extremely challenging for future systems. Both OPH and OPH-EK were expressed in *E. coli*, purified, and characterized. Enzyme kinetics was measured using paraoxon as the substrate while circular dichroism was used to probe protein structure; particularly that of EK. Initially it was planned to be able to perform *in vivo* studies in order to demonstrate the effects of EK on target therapeutic proteins, with particular interest in seeing how EK would be able to protect immunogenic OPH.

During the purification process, it became apparent that 30kDa EK was not sufficient to completely protect OPH sufficiently for *in vivo* applications. If the EK was able to completely protect the protein, its hydrophilic properties would be sufficient for OPH-EK to pass in the flow-through during hydrophobic interaction chromatography (HIC). However during HIC OPH-EK sticks to the column strongly and only elutes under low salt conditions, demonstrating that OPH-EK maintains some surface hydrophobic characteristics characteristic of OPH. OPH-EK does, however, elute at a higher salt concentration than OPH does, indicating that EK does demonstrate noticeable protecting effects through reducing surface hydrophobicity, but not in a sufficient amount to move forwards with *in vivo* studies. Thus, while it would have been desirable, *in vivo* studies were not pursued for this system due to the immunogenicity of OPH and the inability for 30kDa EK to completely protect the protein[150]. This is not entirely unsurprising, given that EK

comprises just under 50 percent of the total protein, while proteins modified with other related polymers and peptides oftentimes are comprised primarily of the modifying agent, particularly when it comes to immunogenic therapeutic proteins[70,76,77]. Further, while a 10kDa EK fusion protein was initially pursued, its study was discontinued early on for similar reasons for not being able to sufficiently protect the surface of OPH and for not appreciably increasing expression yields of the fusion protein.

Another phenomena observed during the HIC purification process was how the EK seems to affect the elution of the protein. Normally proteins are eluted from the column as part of a standard bell curve-shaped peak. However in this instance the protein gradually eluted from the column, forming more of a step than a nice peak, as shown in **Figure 3.2**. This is further evidence that EK is able to partially protect the protein, since any given EK could be covering a random amount of surface hydrophobicity, resulting in a distribution of surface hydrophobicity throughout the OPH-EK population. This phenomenon had not been initially observed when working with the Bla and TEM-19 systems previously described since they were run as gravity columns by hand so there was no real-time monitoring of the purification and the elution was performed as an isocratic elution instead of a gradient elution.

It is well known that OPH is expressed as a dimer in native bacterial hosts. In this instance, OPH-EK is expressed primarily as a monomer. Over 90% of OPH-EK is expressed as a monomer, however the more limited dimer quantity is isolated during the purification process due to its relative ease of purification. The recovered purified OPH-EK dimer immediately once again demonstrates a similar equilibrium between the monomeric and dimeric forms and reverts to its primarily monomeric form, while the initially recovered dimer form is almost undetectable, as shown in **Figure 3.3**. This is verified by showing that the OPH dimer, at 72 kDa, is larger than the

OPH-EK monomer, at 66 kDa, and smaller than the OPH-EK dimer when both proteins are run through size exclusion chromatography. This further demonstrates the zwitterionic non-fouling properties of EK, as its addition to OPH is able to confer demonstrable protection to the surface of the protein and prevent a sufficiently large amount of the strong hydrophobic interactions that cause the dimerization of OPH. This indicates that the addition of EK may also be able to prevent other nonspecific and undesirable protein interactions. For example, this may potentially allow for the expression of prokaryotic proteins in eukaryotic hosts without post-translational modifications. This represents, to the best of our knowledge, the first known expression of a stable monomeric unit of OPH in any expression system.

The loss of the dimer interface due to the monomer state does alter the kinetic properties of OPH, as summarized in **Table 3.1**. The k_{cat} values of OPH and OPH-EK are $4.4 \cdot 10^3$ and $3.8 \cdot 10^3$ s^{-1}, respectively. However, this compares the catalytic rate constant between the OPH dimer and the OPH-EK monomer. Once the k_{cat} values are adjusted for each active site, each active site of OPH-EK has a catalytic rate constant that is approximately 70% higher than that of each active site of OPH. It can be suggested that this increased catalysis per active site is due to multiple residues of the active site being relatively near the dimer interface and that its loss reduces some of the rigidity and thus steric influence inherent in the OPH dimer, which is shown in **Figure 3.4**[151,152]. While these residues may not be part of the catalytic mechanism, they do play strong roles in the stereochemistry of substrate hydrolysis[136]. This is particularly noted in regards to the leaving group pocket, which is directly adjacent to the dimer interface, and allowing for the products to leave the active site could allow for increased overall catalytic efficiency.

The K_m values for OPH and OPH-EK are 714 and 277 μM, respectively. This is consistent with previous results when the addition of EK and pCB to proteins increases substrate

affinity[47,92,149]. This has been explained by demonstrating that the superhydrophilic characteristics of these zwitterionic polymers promote specific hydrophobic interactions required for the substrate binding to the active site. Overall the catalytic efficiencies of OPH and OPH-EK are $6.2 \cdot 10^8$ and $1.4 \cdot 10^9$ $M^{-1}s^{-1}$, respectively with OPH-EK demonstrated improved kinetics compared to OPH overall.

The thermal stability of OPH and OPH-EK kinetics was compared. Both OPH and OPH-EK exhibit similar losses of catalytic activity with increasing temperature, indicating that the stability conferred by the addition of EK is able to not only account for the loss in stability caused by the loss of the dimer interface, but also EK confers a very slight increase in stability compared to native OPH, as shown in **Figure 3.5**. With the profiles being very similar, it suggests that the monomeric units, and thus the active sites, of both OPH and OPH-EK unfold and are inactivated in very similar manners. This further demonstrates the influence of the dimer interface upon stability of OPH and its influence on the active site and the importance of lysine and glutamic acid and their roles in promoting protein stability.

Circular dichroism (CD) was performed on OPH and OPH-EK to study the structure of EK and how it affected protein stability and unfolding, as shown in **Figure 3.6**. The 30kDa EK tail demonstrates formation of some parallel β-sheets and α-helices, as shown in **Table 3.2**, but there is also a significant amount of unstructured protein exhibited by the EK tail. While this does not mean that EK exhibits completely unstructured properties, it does suggested that EK is able to sample a wide variety of structures without settling into a specific conformation and can thus be assumed as non-rigid. For this analysis BeStSel (β-structure selection) was used to deconvolute the CD spectra taken, as it demonstrates improved efficacy in differentiating between different types of β sheets that demonstrate differing CD spectra and thus improves overall prediction

accuracy[153,154]. By using a wider database to populate the software and by taking a wider range of structures into consideration, BeStSel is better able to predict all the structural elements of OPH and OPH-EK compared to other methods.

Analysis of OPH by CD reveals two unfolding events. The first event is the gradual loss of antiparallel β-sheets and the gradual increase in parallel β-sheets. Above 70°C OPH loses essentially all of its helices, resulting in increased parallel β-sheets and unstructured protein, before becoming insoluble between 80°C and 90°C. This is consistent with previously reported results, indicating that OPH unfolds in two steps, with the first step involving the denaturing of the two individual subunits while the dimer interface is maintained and with the second step involving the loss of the dimer interface[141]. This is also shown in the thermal kinetics data as mentioned previously, where activity is lost with the denaturing of the individual subunits at temperatures below 70°C.

OPH-EK denatures in a different manner than OPH due to the presence of EK and the lack of the dimer interface affecting its structure. OPH-EK still observes two unfolding events like OPH does, but the events are slightly different and less extreme. Below 60°C OPH-EK slowly loses all of its structures. This first unfolding event is very similar to that of OPH, where the monomeric unit is denaturing, in both the thermal transition temperature and the overall kinetic activity profile. This slower loss in structure is also visualized in the slight increase in thermal kinetic stability, demonstrating the ability of EK in conferring protein stability. Above 70°C OPH-EK appears to partially collapse into an antiparallel β-sheet structure. This second unfolding event, which differs from the prevalence of parallel β-sheets seen with OPH, is particularly interesting as OPH-EK is not observed to be insoluble at 90°C despite β-sheets demonstrating strong potential for aggregation, suggesting that EK is able to increase the solubility of proteins as well. This is as

expected, given that EK is highly charged and hydrophilic and thus should confer increased solubility properties to target proteins.

3.3 Conclusions

In summary, this work demonstrates the first known expression of a stable monomeric unit of OPH by using EK to generate recombinant OPH-EK fusion proteins. EK demonstrated zwitterionic ultra-low fouling properties by preventing dimer formation caused by strong hydrophobic interactions that are exhibited by unmodified OPH. The stability conferred by EK was able to overcome that which was lost through the elimination of the dimer interface, and this was demonstrated through thermal catalytic activity and CD studies. CD studies further demonstrated that while EK is not entirely unstructured, it generally exhibits non-rigid properties. EK fusions were able to demonstrate increased catalytic activity per active site due to potentially reducing steric hindrance around the active site, particularly in the leaving group pocket, and increasing substrate affinity. OPH-EK further demonstrated improved solubility compared to OPH, particularly at elevated temperatures. Such characteristics of EK make it an ideal candidate for expression of prokaryotic proteins in higher-level hosts. This is especially relevant in the case of OPH, as its sequence contains multiple sites that can potentially be modified through N-linked glycosylation if expressed in advanced eukaryotic systems. While the amount of EK was not sufficient to protect the entire protein surface in this particular case, the ability of EK in protecting the surface of the protein demonstrates the promise of EK for use in *in vivo* applications. Future target systems can be protected for *in vivo* applications by increasing the proportion of EK in the fusion protein for proteins that need protection or selecting a protein system that solely requires modification to increase its hydrodynamic size to increase blood circulation.

3.4 Materials and methods

All materials were purchased commercially unless otherwise noted and were used without further purification. All buffers were made from commercially available products and Milli-Q® water (EMD Millipore). All primers were synthesized commercially (Integrated DNA Technologies). All error bars represent one standard deviation from the mean.

3.4.1 Cloning

E. coli strain DH10B (Life Technologies) was used for cloning. The OPH gene was provided by Drs. Andrew Bigley and Frank Rauschel as previously described[132]. Briefly, the VRN-VQFL variant of OPH was inserted between NdeI and EcoRI restriction sites of a pET-20b(+) expression vector. A gene encoding for a 30kDa EK sequence ($(EK)_{120}$) with HindIII and XhoI restriction sites was synthesized as previously described (GenScript). PCR was performed on the OPH gene in order to change the restriction sites to PciI and HindIII while removing the stop codons using 5'-CGAGCTACATGTCCATCGGGACCGG-3' and 5'-TGGAAGCTTGGAAGCACGCAGGGTC-3' as the forward and reverse primers, respectively. The EK gene was inserted into a pET-20b(+) expression vector at the HindIII and XhoI restriction sites following by the insertion of the modified OPH gene digested with PciI and HindIII at the NcoI and HindIII restriction sites to generate the OPH-EK gene. All sequences were verified by a third party (Genewiz).

3.4.2 Expression and purification of OPH

E. coli strain BL21 (DE3) (Life Technologies) was used for protein expression. All cultures were grown at 37°C unless otherwise noted. 5mL of TB supplemented with ampicillin were inoculated with a single colony for 8 hours. 1mL of the inoculate was added to 1L of TB supplemented with ampicillin and grown for 16 hours at 30°C. The culture was supplemented with 1mM $CoCl_2$ and

induced with 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and allowed to express for 24 hours at 30°C. The culture was harvested and resuspended in 100mL purification buffer (50mM HEPES 100μM $CoCl_2$, pH 8.5). The cells underwent three freeze-thaw cycles and were lysed using a probe sonicator (Fisher Scientific) and underwent centrifugation at 4°C. .5g protamine sulfate in 20mL purification buffer was added to the supernatant and allowed to incubate for 20 minutes on ice before centrifugation at 4°C. The resulting supernatant was brought to 60 percent ammonium sulfate saturation and mixed for 30 minutes at 4°C before centrifugation at 4°C. The resulting pellet was solubilized in purification buffer, filtered, and purified using an NGC Quest 10 FPLC system (Bio-Rad). The target protein was purified using a HiLoad 16/600 Superdex 200pg size exclusion chromatography column (GE Healthcare) equilibrated in purification buffer and a Capto Q anion exchange column (Bio-Rad) equilibrated in purification buffer with a linearly increasing NaCl gradient.

3.4.3 Expression and purification of OPH-EK

E. coli strain BL21 (DE3) (Life Technologies) was used for protein expression. All cultures were grown at 37°C unless otherwise noted. 4 cultures each containing 5mL of LB supplemented with ampicillin were inoculated with a colony each for 16 hours. The overnight cultures were added to 1L of TB supplemented with ampicillin and grown at 30°C. Once the culture reached OD_{600} .5, the culture was supplemented with 1mM CoCl2 and 1mM IPTG and allowed to express for 24 hours at 30°C. The culture was harvested and resuspended in 10mL purification buffer per gram of wet cell pellet. The cells underwent three freeze-thaw cycles and were lysed using a probe sonicator (Fisher Scientific) and underwent centrifugation at 4°C. The supernatant was brought to 2M NaCl and mixed for 30 minutes at 4°C before centrifugation at 4°C. The target protein was purified on an NGC Quest 10 FPLC system (Bio-Rad) using a Capto Butyl hydrophobic interaction

chromatography column (GE Healthcare) equilibrated with purification buffer containing 2M NaCl with a linearly decreasing NaCl gradient and an ENrich SEC 650 column (Bio-Rad) equilibrated with purification buffer.

3.4.4 Enzyme kinetics and activity assays

The concentration of both OPH and OPH-EK used in activity assays was 2nM in purification buffer. Paraoxon was used as the substrate with concentrations ranging from 20 to 2000μM. 100uL each of both enzyme and substrate were added to wells of 96-well UV-transparent microplates (Corning) such that the final concentration of protein in the assay was 1nM and the final concentrations of substrate in the assay ranged from 10 to 1000μM. Substrate hydrolysis was monitored through the increase in absorbance at 400nm using a Cytation 3 microplate reader (BioTek). Enzyme activity was calculated using the initial linear hydrolysis rate. Michaelis-Menten kinetics were modeled using Mathematica (Wolfram Research). All data points were measured in triplicate.

3.4.5 Enzyme stability assays

The concentration of OPH and OPH-EK used was 2nM in purification buffer. Paraoxon was used as the substrate at a concentration of 2mM. The enzymes were heated to temperatures ranging from 37°C to 80°C for 10 minutes. The activity assay was then performed as previously described, with the final protein concentration in the assay at 1nM and the final substrate concentration in the assay at 1mM.

3.4.6 Circular dichroism

The concentration of OPH and OPH-EK used was 1mM in 500μM HEPES 1μM CoCl2 pH 8.5. Samples were loaded into 1mM quartz cuvettes and run on a Jasco J-720 circular dichroism spectrophotometer (Jasco Inc.). Samples were run at temperatures ranging from 30 °C to 90°C.

Samples were incubated at the desired temperature for 10 minutes before being run. Spectra were taken from 260-190nm with .02nm pitch, scan speed 100nm/min, and 2nm bandwidth. Spectra were averaged over 6 individual scans and analyzed using BeStSel after the background was subtracted[153,154].

3.5 Tables

Protein	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} M^{-1})
OPH	$4.4 \cdot 10^3$	714	$6.2 \cdot 10^8$
OPH-EK	$3.8 \cdot 10^3$	227	$1.9 \cdot 10^9$

Table 3.1. Kinetic properties of OPH and OPH-EK

Temp (°C)	OPH						OPH-EK					
	α_R	α_D	β_A	β_P	T	U	α_R	α_D	β_A	β_P	T	U
30	18.5	10.0	20.9	4.4	11.9	34.3	14.2	15.7	7.5	8.8	12.1	41.7
40	10.3	8.5	25.9	4.6	10.8	39.8	19.3	9.6	4.8	9.5	10.6	47.3
50	12.6	6.9	22.1	9.0	11.7	37.7	10.7	12.4	7.8	9.0	13.3	46.9
60	10.5	10.7	15.8	11.5	10.6	40.8	5.3	6.7	1.7	0.0	17.9	68.4
70	15.8	8.5	11.2	13.1	10.3	41.1	9.8	9.0	2.8	6.4	15.3	56.9
80	0.0	1.3	1.8	20.6	12.6	63.8	3.5	6.8	14.5	7.9	15.2	52.1
90	-	-	-	-	-	-	0	5.7	12.5	5.4	14.5	61.9

Table 3.2. Estimated secondary structure of OPH and OPH. Values were calculated from circular dichroism spectra and are a percentage of total protein structure. OPH was insoluble at 90°C. α_R: regular α-helix, α_D: distorted α-helix, β_A: antiparallel β-sheet, β_P: parallel β-sheet, T: turns, U: unordered.

3.6 Figures

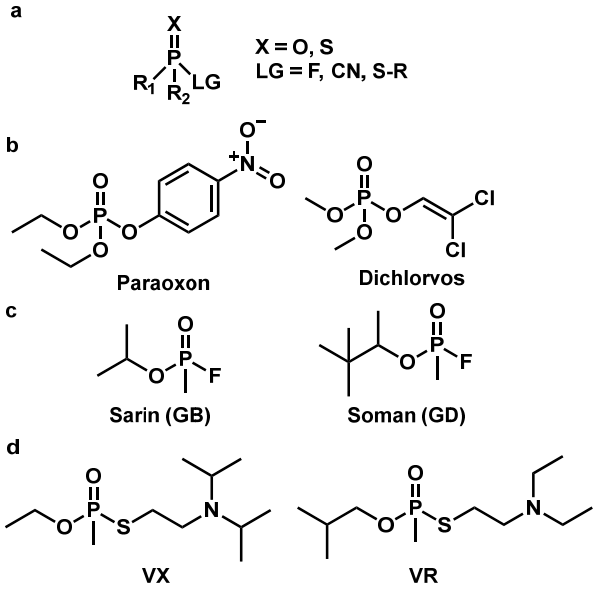

Figure 3.1. Representative organophosphate compounds. (a) The general structure of organophosphates comprises (b) pesticides, (c), G-type nerve agents, and (d) V-type nerve agents[122,123].

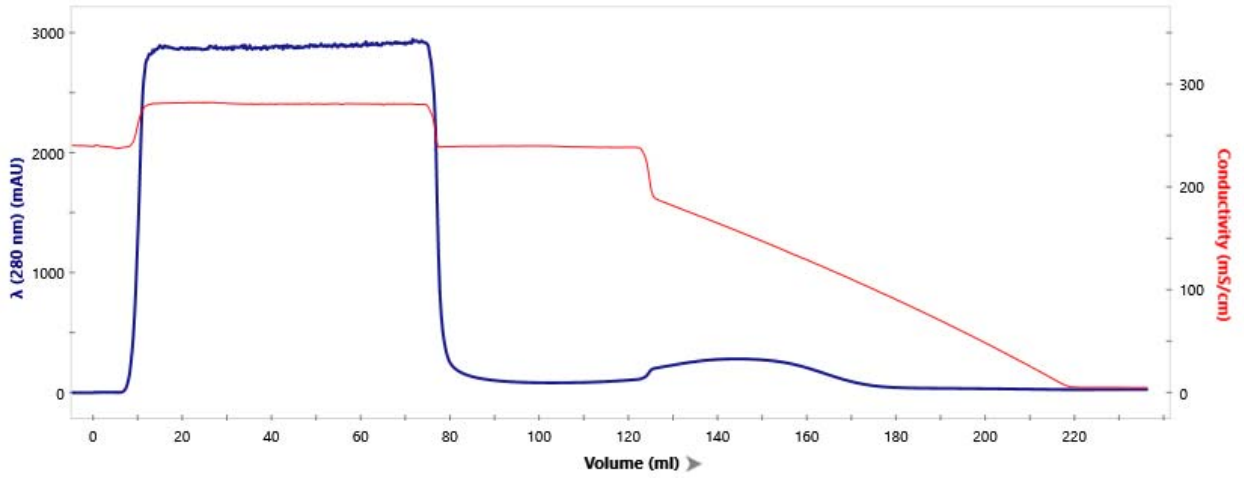

Figure 3.2. Hydrophobic interaction chromatography purification step of OPH-EK. The chromatogram shows absorbance (purple) and conductivity (red), which serves as a proxy for sodium chloride concentration. The two peaks in absorbance are the flow-through (left) and the elution of OPH-EK and impurities (right.).

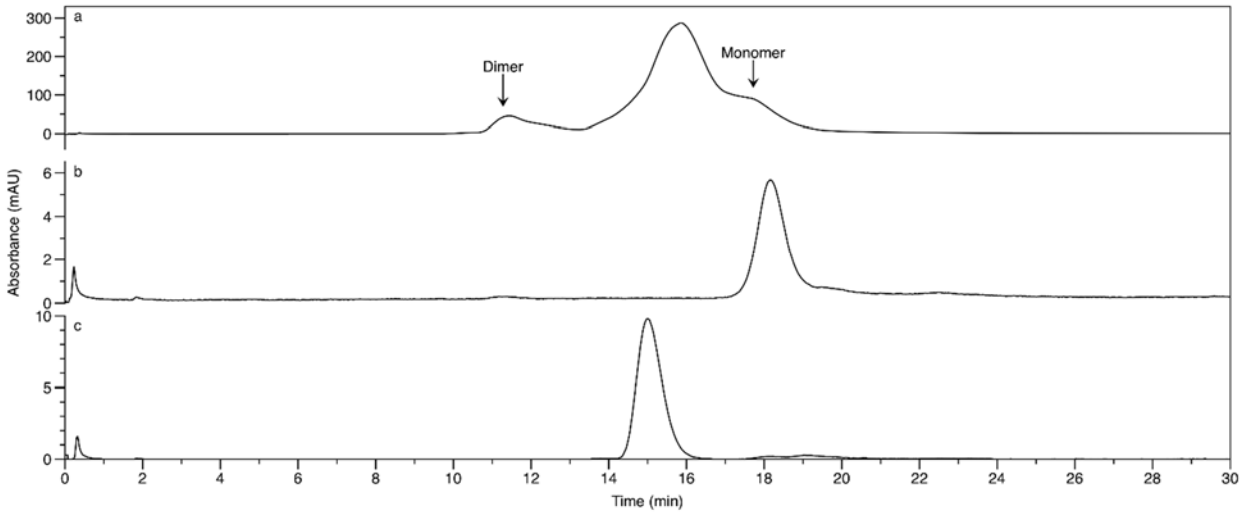

Figure 3.3. Size exclusion chromatography of OPH and OPH-EK. The purification of OPH-EK (a) isolates the dimer, due to its relative ease of isolation. OPH-EK (b) exists primarily as a monomer. Dimeric OPH (c) has a larger molecular weight (72 kDa) compared to monomeric OPH-EK (66 kDa) and thus elutes earlier during size exclusion.

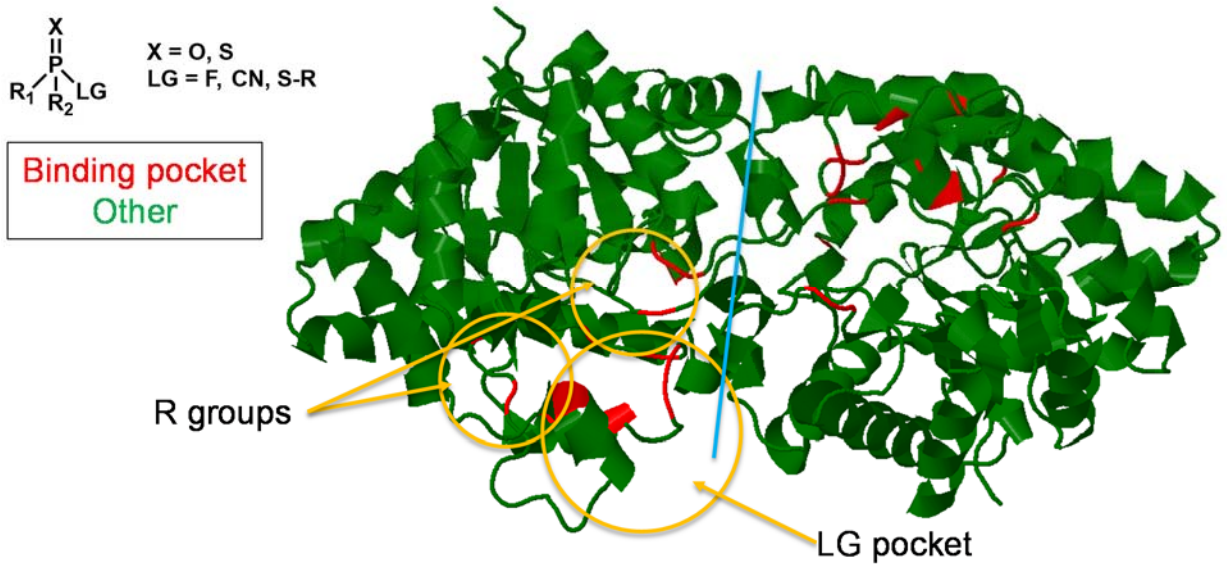

Figure 3.4. Active site of OPH. Residues that comprise the binding pocket are labeled in red, with various areas of the binding pocket corresponding to regions within OP compounds labeled. The dimer interface region is approximated by a blue line.

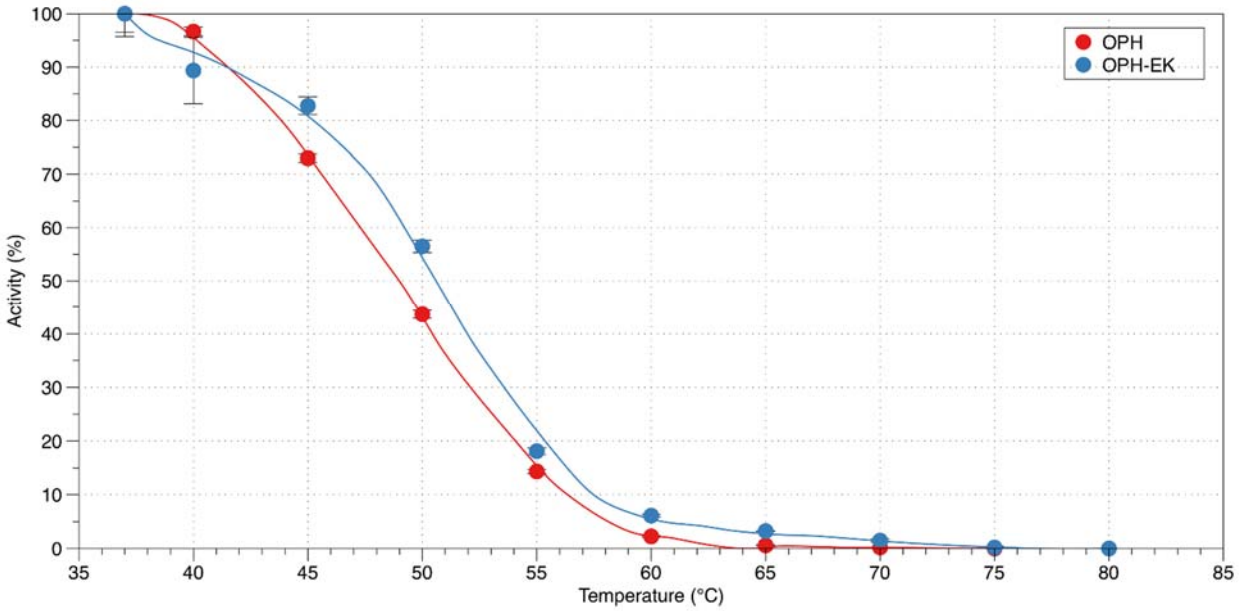

Figure 3.5. Temperature stability of OPH and OPH-EK. OPH and OPH-EK demonstrate similar temperature kinetic stability despite OPH-EK losing the dimer interface present in OPH, demonstrating the stabilizing effects of OPH.

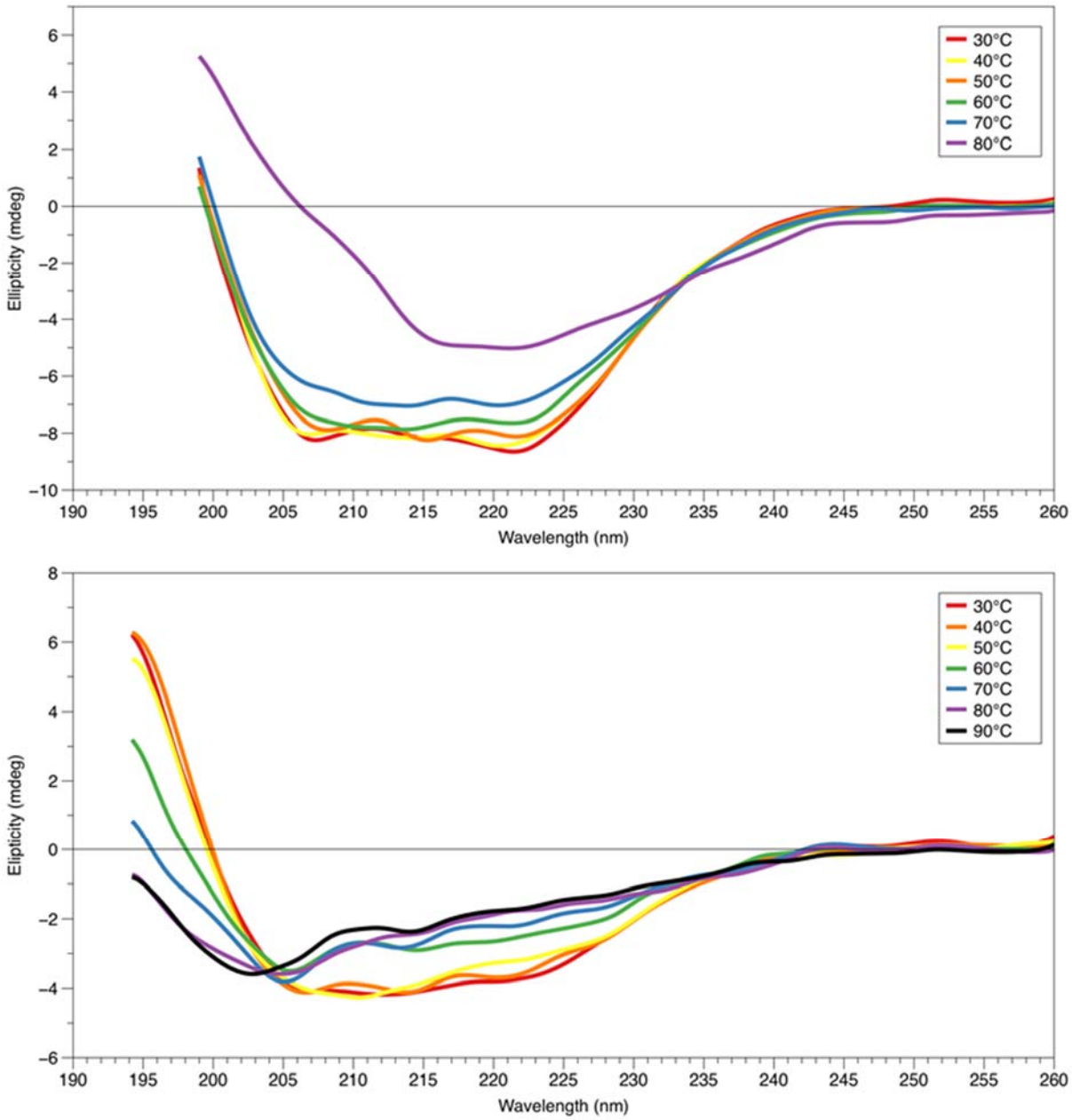

Figure 3.6. Circular dichroism spectra as a function of temperature. OPH (top) and OPH-EK (bottom) unfolding were studied using circular dichroism. There is no spectra for OPH at 90°C as it is insoluble at that temperature.

Chapter 4

Expression and *in vivo* properties of a granulocyte colony-stimulating factor-EK fusion protein

Granulocyte colony-stimulating factor (G-CSF) is a cytokine that plays a strong role in the production of neutrophils. Recombinant G-CSF is used to treat both isolated and chronic neutrophina and is used in bone marrow transplants. However G-CSF is plagued by short blood circulation, and treatment oftentimes requires consistent daily injections. An EK-G-CSF system is described. G-CSF is modified with a 30kDa EK at its N-terminus and is expressed as a fusion with maltose binding protein to improve yield and solubility and purified. The use of a maltose binding protein fusion system dramatically increases expression yields of EK-G-CSF. Standalone EK-G-CSF has been achieved, though optimization remains in the cleavage of the fusion to generate EK-G-CSF. Current works are in place to prepare EK-G-CSF for *in vivo* studies to determine the effects of EK on the blood circulation and efficacy of G-CSF.

4.1 Introduction

Neutrophils are the most abundant form of white blood cells in the body and play a critical role in the innate immune system. G-CSF plays a strong role in the production of neutrophil granulocytes in response to infection or impairment of bone marrow activity[155]. Additionally G-CSF prolongs the survival of mature neutrophils and enhances many of their primary functions[156]. G-CSF levels in the blood generally low until produced as necessary as part of a response; after which blood cell levels decline during recovery[157].

G-CSF was one of the early cytokines identified for therapeutic usage[157]. It is used to treat a variety of conditions related to low white blood cell count including neutropenia, chemotherapy-induced myelosuppression, aplasia recovery after bone marrow transplantation, and conditions

related to HIV/AIDS[158,159]. For example treatment with G-CSF to mitigate induced neutropenia in cancer patients increases the likelihood that chemotherapy treatments could proceed at the intended dosage and on schedule[160]. G-CSF treatment also reduces the use of antibiotics in cancer patients, thus further reducing patient hospitalization time[161].

Commercially available recombinant G-CSF is available in two forms: filgrastim and lenograstim. The amino acid sequence of both filgrastim and lenograstim are identical to the more active 174 amino acid (19kDa) form, with the difference being that filgrastim is expressed in *E. coli* without further modification and lenograstim is expressed in Chinese hamster ovary cells and is glycosylated[162,163]. G-CSF has a relatively short half-life of approximately 4 hours and the half-life of G-CSF displays an inverse relationship with respect to the absolute neutrophil count[164,165]. While these forms of G-CSF have been successful as neutropina-related treatments, daily injections, and in some instances multiple daily injections, are required for uninterrupted G-CSF treatment, particularly for individuals with chronic neutropenia[166]. While G-CSF is generally viewed as non-immunogenic and is available on the market, anti-G-CSF antibodies have been discovered in healthy individuals[167].

Pegfilgrastim is a PEGylated version of filgrastim that was developed to increased blood circulation and was approved by the FDA in 2002 and had a biosimilar approved in 2018[165,166,168]. Pegfilgrastim is a monopegylated filgrastim with a 20kDa PEG conjugated to the N-terminal amine of filgrastim. PEGylation of the N-terminal amine is favorable compared to surface lysine residues or the unbound cysteine residue due to increased retention of bioactivity and the repeatability of performing a site-specific conjugation[169,170]. The PEGylation of filgrastim greatly reduced renal clearance, making neutrophil-mediated receptor clearance the primary clearance mechanism and increasing circulation half-life from 4 hours to 42 hours[171]. These improvements to blood

circulation have resulted in the reduction of administration of pegfilgrastim to once per myelo-suppressive chemotherapy cycle that is well-tolerated by patients[172,173]. While patients treated with Pegfilgrastim have been found to have anti-PEG antibodies, currently there is currently no indication that they adversely affect Pegfilgrastim treatment[174]. However this is something that needs to be tracked in future treatments.

Generally the overexpression of G-CSF in prokaryotic system is performed through the generation of insoluble inclusion bodies[155,159]. The formation of inclusion bodies in recombinant protein expression is not unique to G-CSF, as up to 70% of recombinant proteins expressed in *E. coli* result in inclusion body formation[175]. These inclusion bodies form due to the inability of the target to correctly fold, resulting in the formation of insoluble aggregates. This requires that the inclusion body is harvested, resolublized, and refolded into its functional form. Generally soluble expression of G-CSF has been limited to eukaryotic systems, where native post-translational glycosylation prevents the protein from aggregating, or expression in prokaryotic system utilizing certain fusion proteins that either demonstrate improved protein solubility or promote protein folding[176-178].

Fusion protein tags have demonstrated great efficacy in promoting protein expression in *E. coli*, improving protein purification, and enabling characterization. Maltose binding protein (MBP) is a well-expressing *E. coli* protein that functions to improve protein expression yield and solubility and able to prevent the formation of inclusion bodies[179-181]. This has been exploited to great effect for a wide number of targets, including proteins of eukaryotic origins such as antibodies[182]. In addition to improvements in protein expression and solubility, the use of MBP allows for a facile purification of the fusion protein through the use of an amylose resin, with capture and elution occurring under non-denaturing conditions. Oftentimes MBP fusion protein systems contain

cleavage sites after the MBP sequence, allowing of the cleavage of the target protein from the MBP once the fusion protein has been sufficiently purified. Currently MBP fusion protein systems are commercially available for expressing MBP fusion proteins.

Herein the expression and characterization of an EK-G-CSF is described. G-CSF was chosen since it has been shown that N-terminal modification of G-CSF does not strongly reduce bioactivity. Additionally, G-CSF, at 19kDa, is relatively small and the conjugation of 20kDa PEG has been shown to significantly increase circulation half-life. 30 kDa EK ($(EK)_{120}$) appended appended to the N-terminus of G-CSF to generate the EK-G-CSF fusion protein. Previous EK fusion proteins have been expressed into the periplasm primarily to increase cell viability and have not greatly focused on optimizing expression yields. The EK-G-CSF sequence will contain a cleavable MBP sequence at the N-terminus in order to increase protein yield, increase protein solubility, and simplify the purification protocol. The primary goal will be to characterize the effects of EK on EK-G-CSF *in vivo*, necessitating the need for a system that enhances protein expression of EK fusion proteins. This system will the first to demonstrate *in vivo* properties of EK fusion proteins. While the size of the EK is not significantly larger compared to that of G-CSF, the immunogenicity of the unmodified G-CSF is not of great concern, considering that G-CSF is currently available on the marketplace and widely used in the clinic.

4.2 Results and discussion

Currently this work is in progress, with expression and purification of G-CSF and EK-G-CSF achieved and plans for an upcoming *in vivo* study. The wild-type G-CSF (filgrastim) had demonstrated expression in *E. coli* as an insoluble inclusion body without the use of an inducing agent and instead relies on the leaky expression inherent in pET systems[155,183]. However expression as an inclusion body can lead to reduced yield, as recovering active protein from the

inclusion body can be extremely challenging and inefficient[184]. While recovered yields of approximately 2-3 mg/L are acceptable for *in vivo* studies for unmodified EK-G-CSF, it would be extremely undesirable if EK-G-CSF would express as an inclusion body given how thus far it has been demonstrated that using the exact same expression strategy for both wild-type and EK-modified protein has resulted in the EK-modified variants demonstrating significantly reduced expression.

For EK-G-CSF expression, the initial approach was to express it as a standalone protein in an attempt to see if that it could be expressed as a soluble protein with sufficient yield without exhibiting inefficiencies caused by inclusion body recovery. However, while the expressed protein was soluble, once again expression yields of any useful quantity were not achieved. Thus EK-G-CSF was placed in a pMAL-c5e expression vector, generating an MBP-EK-G-CSF fusion protein, as shown in **Figure 4.1**. This MBP fusion protein demonstrated much improved expression of a soluble protein, with yields of up to 8 mg/L. Thus the use of MBP demonstrates improved expression of EK fusion proteins and strongly mitigates expression loss when compared to the expression of wild-type protein. As noted, this expression yield is significantly more compared to the expression of a non-useful amount of unmodified EK-G-CSF. Additionally, given that the pMAL-c5e expression vector builds in an enterokinase cleavage site after the MBP sequence, isolating the EK-G-CSF post-cleavage can be readily accessible. Standard cleavage protocols are not viable, as EK-G-CSF does exhibit a non-trivial amount of aggregation. That, coupled with the potential ultra low-fouling properties of EK, prevents the enterokinase from cleaving MBP from the EK-G-CSF. This can be resolved by supplementing the buffer conditions with 3M urea to reduce protein interactions, which allows for accessibility to the cleavage site while not denaturing enterokinase[185]. The cleavage of MBP using these modified conditions is achievable as shown in

Figure 4.2, though for this particular system the cleavage efficacy of enterokinase is demonstrably reduced when compared to the cleavage efficacy against the substrate where the activity of enterokinase is defined. The cleavage has only been demonstrated at small scales, and if complete cleavage of all of the MBP from EK-G-CSF expressed in a 1L liquid culture is achievable, expression yields for EK-G-CSF under current expression conditions of 5mg/L are accessible.

Following the generation of EK-G-CSF it is planned to begin *in vivo* experiments demonstrating the effects of EK on the efficacy and blood circulation of G-CSF. Standard Sprague Dawley rats will be used, with efficacy being tested by measuring white blood cell count and blood circulation being calculated using ELISA. Due to the increase in size, it is expected that blood circulation will increase due to reduced renal filtration. It will be intriguing to see how EK affects the biological activity of G-CSF, as it has been previously demonstrated that polymer conjugates of receptor-binding proteins exhibit reduced biological activity, and if it exhibits any similar trade-off between reduced activity and increased circulation.

This work is being done in parallel with other studies concerning the expression and properties of EK. These other EK sequences contain low quantities of glycine and proline spaced in a non-repeated manner. Initially these other peptide sequences were explored to improve the expression yield of EK fusion proteins by "diluting" the sequence such that E and K occurred less frequently in the sequence, as that has been an obstacle throughout the exploration of these EK fusion proteins[186]. Glycine and proline were considered for these EK modifications since glycine and proline are generally known to have a low propensity for forming alpha helices[187]. Especially given how proline exhibits an unusual shape due to its cyclic side chain, it is also interesting to explore how these EKG/EKP/EKGP sequences exhibit different behavior compared to EK. A

variety of these modified EK sequences are currently being explored to determine which demonstrates the most promise.

4.3 Conclusions

This work is in the process of demonstrating the *in vivo* properties of EK fusion proteins through the generation of EK-G-CSF. G-CSF was selected due to its poor circulation and a known pathway to improve its *in vivo* properties. A 30kDa EK was appended to the N-terminus of G-CSF and the protein was expressed as a MBP fusion, with EK-G-CSF being isolated after cleaving the MBP portion. While some optimization of the EK-G-CSF cleavage and purification remains, *in vivo* studies will be able to be carried out using EK-G-CSF. These *in vivo* studies will be able to demonstrate the effects of EK on blood circulation of G-CSF and whether or not it affects the bioactivity of the drug. These studies will be able to shed light onto how promising EK can be for *in vivo* applications.

4.4 Methods and materials

All materials were purchased commercially unless otherwise noted and were used without further purification. All buffers were made from commercially available products and Milli-Q® water (EMD Millipore).

4.4.1 Cloning

E. coli strain DH10B was used for cloning. The gene encoding for G-CSF was synthesized (GenScript) with NdeI and XhoI restriction sites and was inserted into a pET-20b(+) expression vector. An additional gene encoding for G-CSF for use in the EK fusion protein system was synthesized (GenScript) with HindIII and XhoI restriction sites. A gene encoding EK-G-CSF sequence with the EK sequence comprising $(EK)_{120}$ was synthesized (GenScript) with NdeI and

EcoRI restriction sites and was inserted into a pMAL-c5e expression vector to generate an MBP-EK-GCSF gene. All sequences were verified by a third party (Genewiz).

4.4.2 Expression, resolublizing, and purification of G-CSF

E. coli strain BL21 (DE3) (Life Technologies) was used for protein expression. All cultures were grown at 37°C unless otherwise noted. 4 cultures of 5mL of TB supplemented with ampicillin were each inoculated with a colony for 16 hours. The overnight cultures were added to 1L TB supplemented with ampicillin and allowed to express without an inducing agent for 24 hours. The culture was harvested and resuspended in 10mL 100mM Tris 20mM EDTA, pH 7.9 per gram of wet cell mass. The cells underwent three freeze-thaw cycles and were lysed using a probe sonicator (Fisher Scientific) and underwent centrifugation. The pellet was washed twice with 30mL 20mM Tris 5mM EDTA .5% TritonX-100, pH 8 before being solubilized in 60mL 8M urea 50m glycine 80μM β-mercaptoethanol, pH 8 for one hour. After centrifugation, the supernatant was collected and dialyzed against 800mM urea 50mM glycine 8μM β-mercaptoethanol pH 8 and refolded for 16 hours at room temperature. The pH was then reduced to 5.5 using phosphoric acid to precipitate contaminants, with the target protein product purified on an NGC Quest 10 FPLC system (Bio-Rad) using a Capto S cation exchange column (GE Healthcare) equilibrated with 800mM urea 50mM glycine 8μM β-mercaptoethanol pH 5.5 with a linearly increasing NaCl gradient from 0-1M.

4.4.3 G-CSF ELISA detection assay

All wash steps were performed in triplicate. 100μL of desired sample was added to wells of a 96-well high binding plate for 1 hour at 37°C and washed with 1x PBS. Wells were blocked with 1mg/mL BSA in 1x PBS .02% Tween® 20 at 37°C for 45 minutes and washed with 1x PBS .02% Tween® 20. 5μg/mL mouse IgG anti-G-CSF was added to wells and incubated at 37°C for 30

minutes and washed with 1x PBS .02% Tween® 20. Goat anti-mouse IgG conjugated to horseradish peroxidase diluted 2,500-fold was added to wells and incubated at 37°C for 25 minutes and washed with 1x PBS. 50µL 3,3',5,5'-tettramethylbenzidine (TMB) was added and incubated at 37°C for 10 minutes. Absorbance of the wells was read at 652nm using a Cytation3 microplate reader (BioTek).

4.4.4 Expression and purification of EK-G-CSF

E. coli strain BL21 (DE3) (Life Technologies) was used for protein expression. All cultures were grown at 37°C unless otherwise noted. 4 cultures of 5mL of TB supplemented with ampicillin were each inoculated with a colony for 16 hours. The overnight cultures were added to 1L TB supplemented with ampicillin and .3% glucose and grown at 30 °C. At OD600 .5 100µM was added and allowed to express for 24 hours. Alternatively the overnight cultures can be added to 1L TB supplemented with ampicillin and an Overnight Express™ Autoinduction System 1 (Novagen) and allowed to express for 24 hours. The culture was harvested and resuspended in 10mL 20mM Tris 200mM NaCl pH 7.4 per gram of wet cell weight. The cells underwent three freeze-thaw cycles and were lysed using a probe sonicator (Fisher Scientific) and underwent centrifugation. The supernatant was purified on an NGC Quest 10 FPLC system (Bio-Rad) using an MBP Trap HP column (GE Healthcare) equilibrated with 20mM Tris 200mM NaCl pH 7.4 with the elution buffer being supplemented with 10mM maltose. The eluted product was buffer exchanged with 20mM Tris 50mM NaCl 2mM $CaCl_2$ 3M urea pH 7.4 and concentrated to 4mL, after which 900U enterokinase was added and the mixture was placed in the dark at room temperature for 2 days. The mixture is then purified on an NGC Quest 10 FPLC system (Bio-Rad) using a HiLoad 16/600 Superdex 200pg size exclusion chromatography column (GE Healtcase) equilibrated with 20mM Tris 200mM NaCl pH 7.4 and an MBP Trap HP column (GE Healthcare)

equilibrated with 20mM Tris 200mM NaCl pH 7.4 with the elution bugger being supplemented with 10mM maltose. The resulting EK-G-CSF product was equilibrated with 8M urea 50mM glycine 80μM β-mercaptoethanol pH 8 for an hour and dialyzed against 800mM urea 50mM glycine 8μM β-mercaptoethanol pH 8and refolded for 16 hours at room temperature.

4.5 Current and Future work

4.5.1 *in vivo* studies

Sprague Dawley rats weighing between 75 and 100 grams will be used as the animal model for *in vivo* pharmacokinetic studies. Studies for both G-CSF and EK-G-CSF will be performed in triplicate. G-CSF and EK-G-CSF will be injected intravenously through the tail vein at a dosage of .5mg/kg and .1mL blood samples will be collected 0 minutes, 5 minutes, 15 minutes, 30 minutes, 2, hours, 4 hours, 8 hours, 12 hours, 24 hours, 48 hours, and 72 hours post-injection. Blood samples will be analyzed for blood neutrophil count and remaining administered protein in the bloodstream. The blood neutrophil count will demonstrate the efficacy of G-CSF and EK-G-CSF. ELISA will be used to quantify the amount of target protein remaining in the blood to indicate blood circulation and how EK affects the circulation profile. Target proteins will be administered weekly over the course of three weeks with one week interval time between injections to demonstrate whether there is any change in the response to the administered compounds. Pharmacokinetic properties will be calculated using PKSolver[188]. Five weeks after the first injection 5mL of blood will be drawn and prepared for antibody detection. ELISA will also be used to determine if there are any anti-protein present against G-CSF and EK-G-CSF and if there are any antibodies present against EK.

4.6 Figures

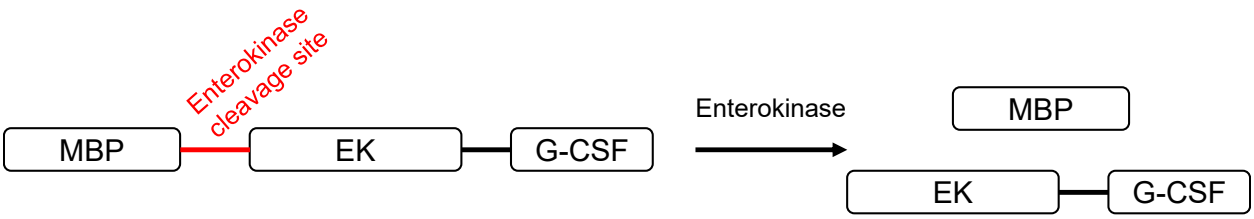

Figure 4.1. Schematic of MBP-EK-G-CSF fusion protein expression system.

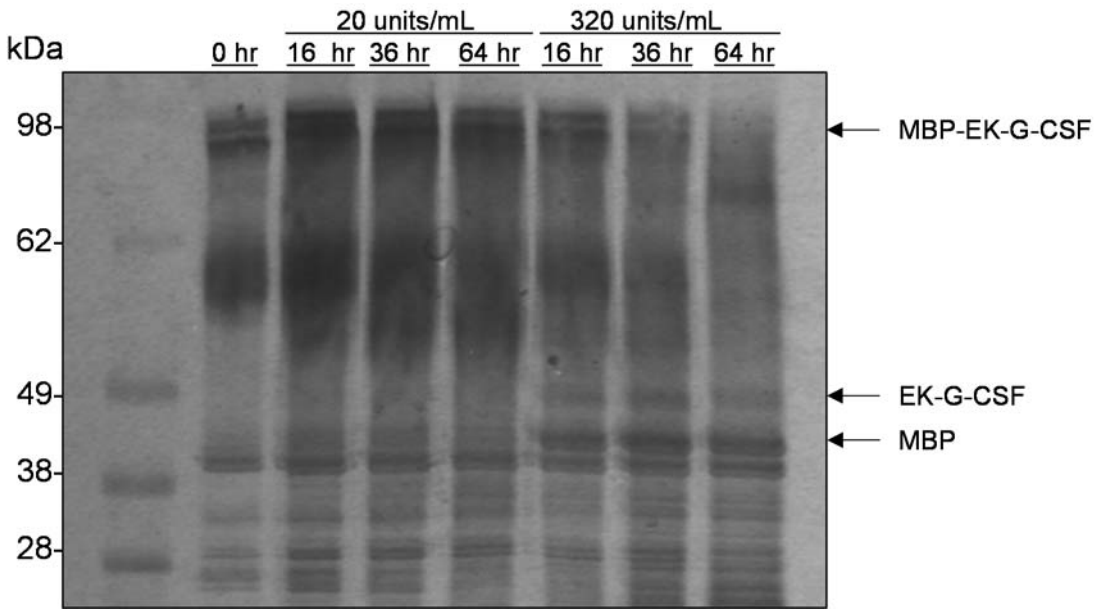

Figure 4.2. Demonstration of cleavage of MBP-EK-G-CSF using enterokinase. Increased enterokinase results in increased cleavage of MBP.

Chapter 5

Expression of standalone EK for protein conjugation

EK has been primarily used as part of a fusion protein to protect and stabilize proteins. However, it is still worthwhile to pursue a chemical conjugation techniques, which would open up additional ways to modify proteins with EK. Protein modification by EK conjugation is studied using both chemical peptide synthesis and recombinant protein expression to generate standalone EK polypeptides. Given the restrictions in chemical peptide synthesis, peptides synthesized using peptide synthesis were demonstrated to not sufficiently improve properties of the target protein, thus necessitating the use of recombinant techniques to generate sufficiently large EK to afford protein protection. Herein systems describing chemical peptide synthesis and recombinant protein expression are described for applications relating to protein conjugation.

5.1 Introduction

While the EK system, like many polypeptide systems, has acted primarily as part of a fusion protein, the conjugation of polymers to proteins has proven extremely useful over a long period of time[76,149,189,190]. This has allowed for the modification of proteins with materials that impart different types of properties on proteins. While PEGylation has been the gold standard, various other polymers such as polyglycerol and polysaccharides such as hyaluronic acid have been conjugated to the surface of proteins[191,192].

The use of polymer conjugation allows for a wide range of chemistries for targeting protein surfaces. The targeting of surface primary amines is relatively facile, however specificity is difficult due to the abundance of surface primary amines[7,20,193]. However the N-terminal amine can be selected due to differing pK_a values between the N-terminal amine and the primary amine on the lysine side chain[18,194]. The terminus of polymers can be modified to contain one of a variety

of reactive groups such as an N-hydroxysuccinimide (NHS) ester, isocyanate, or carbonyl group to target primary amines[189].

There are several other reactive groups on the protein surface that may be targeted, which often results in more specific conjugation location. Thiol chemistry has been used to great effect especially with antibody drug conjugates, particularly since the presence of thiols on the protein surface is rare[195-197]. Oftentimes mutagenesis is performed on the target protein to generate an exposed cysteine residue, though this approach does carry the risk of misfolding and thus inactivating the target protein[198,199]. Additionally there are many other chemistries available that rely on either enzymatic or chemical modification of the protein surface or the expression of non-canonical amino acids to generate unique functional groups[200-205].

For therapeutic usage polymers are used in relatively large quantities compared to the target protein that is being modified. PEGylated proteins demonstrate this in **Table 1.1**, with proteins being modified with many smaller PEG chains or fewer PEG chains of significantly larger molecular weight. Proteins that have been modified by other polymers that have reached clinical trials also demonstrate the use of significant quantity of polymers compared to the target protein as well[77,81,206]. Many of these recent efforts include usage of longer and/or branched polymers, thereby reducing the conjugation sites necessary to either increase the hydrodynamic size or to protect proteins from deleterious interactions. This allows for more selective targeting to better retain bioactivity and increased accuracy in describing protein-polymer conjugate formulations for FDA approval[35,207,208].

Chemical peptide synthesis involves the repetition of reactions to append amino acids to lengthen the polypeptide. Use of solid-phase peptide synthesis greatly simplifies the synthesis and purification when compared to solution phase peptide synthesis[209]. Peptide synthesis relies on the

use of excess reagents to generate accurate sequences. Peptides are synthesized from a residue anchored to the solid support, with the sequence being synthesized from the C-terminus to N-terminus. The α-amine and side chain of amino acids are protected in order to control coupling and prevent undesired side reactions. At each step, the N-terminal amine is deprotected from the peptide and the next amino acid is coupled to the existing peptide chain. However, given that the process of peptide synthesis is essentially a series of chemical reactions, the nonquantitative coupling efficiency generally limits the length of routinely chemically synthesized peptides to about 50 residues, though chemical ligation of peptide fragments is able to achieve polypeptides and proteins over 200 residues in length[100,102].

Like previously-described MBP, small ubiquitin-related modifier (SUMO) is one of many peptides and proteins used to enhance protein expression and solubility through the generation of fusion proteins[210-213]. At approximately 11.5kDa, it is significantly smaller in size than most other systems that are used for similar applications[214]. It is one a number of fusion protein tags that is cleavable to leave a relatively unmodified target protein. SUMO protease is used to cleave the SUMO sequence without leaving any extra residues or non-canonical amino acids, and has been used in combination with a large variety of protein types[210,214]. In addition to recognizing a specific cleavage sequence, SUMO protease also recognizes the structure of SUMO itself, thereby reducing potential incidences where the target protein itself is cleaved by the protease[210]. Additionally, most SUMO fusion protein systems are designed such that both the SUMO sequence and the SUMO protease contain a 6xHis sequence at the N-terminus, further simplifying the purification of the target protein by only requiring immobilized metal ion affinity chromatography (IMAC), since the cleaved target protein is the only component that does not demonstrate IMAC affinity[215].

Herein systems for the generation of standalone EK for peptide synthesis are described. EK has demonstrated zwitterionic protecting properties when modifying protein *in vitro* and an *in vivo* system has been proposed as previously described[149]. All of these systems thus far have utilized EK as part of a fusion protein, and the many benefits of using a fusion protein system have been previously described. Expanding the capabilities of EK to include chemical conjugation would greatly increase the ways in which EK can be used to modify proteins. This will allow for the modification of larger proteins than previously used and immunogenic proteins due to the ability to modify proteins with multiple EK chains. It is desirable to synthesize EK polypeptides for this application, thus allowing for the use of chemical peptide synthesis for shorter length EK and use recombinant techniques to express longer EK. For shorter EK a 2.5kDa peptide was targeted. As 30kDa EK has been expressed as part of fusion proteins, it is the target size for longer EK. The prevalence of primary amines on the lysine residues will necessitate that the conjugation of EK to proteins will requiring targeting other functional groups. Thus the sequence will contain a solitary cysteine residue in order to utilize thiol chemistry for protein conjugation.

5.2 Results and discussion

A short 2.5kDa $(EK)_{10}C$ polypeptide was synthesized using solid phase peptide synthesis and used for conjugation to chymotrypsin, as using peptide synthesis is a relatively straightforward method to achieve short peptides. A bifunctional crosslinker was used to conjugate EK to chymotrypsin. The NHS ester of the crosslinker was first conjugated to the surface lysine residues of chymotrypsin, with the maleimide-functionalized end of the cross-linker subsequently reacted to the cysteine residue of the EK peptide. Of the 12 lysine residues on chymotrypsin, an average of 9 residues were modified by EK per protein. While the resulting protein conjugates demonstrated the retention of bioactivity, the addition of EK had an almost negligible zwitterionic

protecting effect upon conjugation even though on average 9 peptides were conjugated to each chymotrypsin, as shown in **Figure 5.1**. The minimal protecting effects demonstrates that longer EK sequences are necessary in order to confer good protein protection properties, and thus recombinant strategies would be necessary to express sufficiently longer EK. In the instance of CB it was also demonstrated that longer CB, when conjugated to the same protein, was able to demonstrate improved protection properties[92].

This recombinant EK system is utilizing multiple approaches for achieving a 30kDa EK. The $(EK)_{120}C$ sequence is appended to the C-termini of both MBP and SUMO proteins to generate MBP-EK and SUMO-EK. The SUMO-EK variant was studied first as it allowed for the study of both SUMO and MBP systems concurrently with the MBP system described in Chapter 4. The cysteine residue exists to aid in protein conjugation, as the significant portion of lysine prevents the use of amine-target chemistry involving EK. Currently the SUMO-EK construct has demonstrated expression at the test expression level, with current efforts studying large-scale expression of the construct. Expressed at a larger scale, the SUMO-EK is isolated using IMAC; the EK will then be cleaved using a SUMO protease and the EK will be isolated through IMAC again, as shown in **Figure 5.2**. However the cleavage of SUMO from EK has thus far proved challenging even in the presence of urea, demonstrating that the SUMO system to be less ideal than the MBP system[210]. The relative small size of SUMO (11.5kDa) could indicate that 30kDa EK is better able to protect SUMO from cleavage, particularly since the SUMO protease requires both recognition of the cleavage sequence and the structure of the SUMO protein. The MBP-EK system is in the exploratory phase, as it has recently been demonstrated that EK can be cleaved from MBP as described in Chapter 4. The purification of EK from the other products will most likely have to be achieved through reverse-phase chromatography, particularly since standalone

EK does not absorb at 280nm. Once the longer EK sequences has been generated it will be used for protein conjugation applications.

As previously discussed in Chapter 4, other EK sequences will also be explored. Once other studies identify the most promising EK sequences that contain one or both of glycine and proline, those sequences will also be expressed as a standalone peptide in whichever of SUMO or MBP demonstrates to be the better-expressing system for standalone EK. Once these modified EK and base EK sequences are achieved, they can all be used for protein conjugation applications to determine the *in vivo* properties of each of their respective protein conjugates.

5.3 Conclusions

The generation of EK peptides for protein conjugation applications is described in order to increase the number of systems where EK can be used. The EK peptide systems contain a single cysteine residue allows for the use of thiol chemistry as the presence of lysine does not allow for amine-targeting chemistry. Peptide synthesis was used to generate short 2.5kDa EK and conjugated to chymotrypsin. However the limited size of EK achievable wasn't able to sufficiently protect the protein and thus recombinant techniques were required to be able to express longer EK sequences. Initial experiments the expression of a SUMO-EK protein at the test expression level, with current efforts aiming to achieve large-scale expression of SUMO-EK. An MBP-EK system is also under study as an alternate approach to generate long EK. Once expressed, purified, and isolated, the EK can be used in protein conjugation applications to modify proteins that otherwise could not be feasibly modified using an EK fusion protein system. In addition to unmodified EK, other related EK sequences will also be explored.

5.4 Materials and methods

All materials were purchased commercially unless otherwise noted and were used without further purification. All buffers were made from commercially available products and Milli-Q® water (EMD Millipore). All error bars represent one standard deviation from the mean.

5.4.1 Peptide synthesis

Peptide synthesis of $(EK)_{10}C$ was performed using a Titan 357 Peptide Synthesis Machine (AAPPTec). Rink-Amide AM and Rink-Amide MBHA resins (.51mmol/g, 100-200 mesh) were used as the solid phase supports for Fmoc synthesis. All reagents were dissolved in dimethylforamide. Protected residues used were Fmoc-Cys(Trt), Fmoc-Lys(Boc), and Fmoc-Glu(OtBu). 130mg of resin was swelled in 4.5mL 50% dichloromethane for 60 minutes. The addition of each amino acid consisted of a deprotection step, a wash 1 step, two coupling steps, and a wash 2 step. A deprotection step consisted of 2 cycles of resin agitation in 4mL 20% piperadine for 15 minutes. A wash 1 step consisted of 5 cycles of resin agitation in 4.5mL 50% dichloromethane for 3 minutes. A coupling step consisted of resin agitation in 3.6mL 4x excess amino acid with the ratio of amino acid:hexafluorphosphate benzotriazole tetramethyl uranium: hydroxybenzotriazole:N,N-diisopropylethylamine being 1.1:1:1:.5. A wash 2 step consisted of 3 cycles of resin agitation in 4.5mL 50% dichloromethane. After the final amino acid was added, an additional deprotection step was performed to remove the N-terminal Fmoc protecting group before a final wash 1 step.

5.4.2 Peptide purification

The side chain protection groups were cleaved from the peptide and the peptide was cleaved from the solid phase support using a deprotection cocktail of 77.5% trifluoroacetic acid, 15% dichloromethane, 2.5% water, 2.5% triisopropylsilane, 2.5% ethane dithiol and reacted for two

hours at room temperature. The reaction was filtered and the liquid was evaporated using a SpeedVac vacuum concentrator (Fisher Scientific) and dissolved in trifluoroacetic acid. Diethyl ether chilled to -20°C was added dropwise until the EK peptide crashes out of solution. The solid is then washed three times with -20°C ether and dried under high vacuum. The dried pellet was dissolved in 99% water 1% acetonitrile and purified using a 2695 Separations Module (Waters) using a 250x10mm 4μM Jupiter column (Phenomenex) with an increasing acetonitrile gradient. Peptide mass and purity were evaluated using an Autoflex-II (Bruker) and the 2695 Separations Module.

5.4.3 Protein conjugation

The linker shown in **Figure 5.3**, prepared by Priyesh Jain, was used as the heterobifunctional crosslinker. The crosslinker was reacted to chymotrypsin at a 100-fold molar excess in 1xPBS pH 7.4. Unreacted linker was removed and a buffer exchange was performed using a 10kDa desalting column equilibrated with water. The protein-linker complexes were dialyzed against 1.5mM EDTA and water before being lyophilized. 10-fold excess of $(EK)_{10}C$ were reacted with the protein-linker complexes for 24 hours at room temperature in 1x PBS pH 7.4. The unreacted peptide was removed using a 10kDa desalting column equilibrated in water and lyophilized. Conjugation rates were determined using a bicinchoninic acid protein assay coupled with a 2,4,6-trinitrobenzene sulfonic acid amine detection assay.

5.4.4 Conjugate activity and stability assay

Activity assays were performed in .1mg/mL BSA 100mM Tris pH 8. Assays reacted 10nM of chymotrypsin or chymotrypsin conjugates with 1mg/mL N-succinyl Ala-Ala-Pro-Phe p-nitroanilide at varying temperatures. Initial linear changes in absorbance at 412nm were used as a proxy for substrate hydrolysis. All samples were run in triplicate.

5.4.5 Cloning of SUMO-EK

E. coli strain DH10B (Life Technologies) was used for cloning. A 6xHis-SUMO-$(EK)_{120}$C gene was synthesized with the NcoI and HindIII restriction sites (Genscript). The target gene was inserted into a pET-20b(+) expression vector at the NcoI and HindIII restriction sites. All sequences were verified by a third party (Genewiz).

5.4.5 Expression and purification of SUMO-EK

E. coli strain BL21 (DE3) (Life Technologies) was used for protein expression. All cultures were grown at 37°C unless otherwise noted. 4 cultures each containing 5mL of LB supplemented with ampicillin were inoculated with a colony each for 16 hours. The overnight cultures were added to 1L of TB supplemented with ampicillin and grown. Once the culture reached OD_{600} .5, the culture was supplemented with 1mM IPTG and allowed to express for 24 hours. The culture was harvested and resuspended in 10mL 20mM sodium phosphate 500mM NaCl 20mM imidazole pH 7.4 per gram of wet cell pellet. The cells underwent three freeze-thaw cycles and were lysed using a probe sonicator (Fisher Scientific) and underwent centrifugation at 4°C. The target protein was purified from the supernatant on an NGC Quest 10 FPLC system (Bio-Rad) using a HisTrap HP column (GE Healthcare) equilibrated with 20mM sodium phosphate 500mM CaCl 20mM imidazole pH 7.4 with an increasing imidazole gradient.

5.5 Current and Future work

5.5.1 Isolation of EK from SUMO-EK

The isolated SUMO-EK will be buffer exchanged to 50mM Tris 150mM NaCl 20mM imidazole pH 7.4. 10U 6x-His-SUMO protease will be added in order to cleave the SUMO from the EK. The EK will be purified from the rest of the reaction by using an NGC Quest 10 FPLC system (Bio-

Rad) using a HisTrap HP column (GE Healthcare) equilibrated with 50mM Tris 150mM NaCl 20mM imidazole pH 7.4 with an increasing imidazole gradient.

5.5.2 Cloning of MBP-EK

E. coli strain DH10B (Life Technologies) will be used for cloning. A $SSSN_{10}LGDDDK(EK)_{120}C$ gene is being synthesized (Genscript) with SacI and HindIII restriction sites such that the EK sequence is immediately downstream of the enterokinase cleavage site. The target gene will be inserted into a pMAL-c5e expression vector resulting in an MBP-EK construct.

5.5.3 Expression and purification of MBP-EK

E. coli strain BL21 (DE3) (Life Technologies) will be used for protein expression. All cultures will be grown at 37°C unless otherwise noted. 4 cultures each containing 5mL of LB supplemented with ampicillin will be inoculated with a colony each for 16 hours. The overnight cultures will be added to 1L of TB supplemented with ampicillin and .3% glucose and grown at 30°C. Once the culture reaches OD_{600} .5, the culture will be supplemented with 100μM IPTG and allowed to express for 24 hours at 30°C. The culture will be harvested and resuspended in 10mL 20mM Tris 200mM NaCl pH 7.4 per gram of wet cell weight. The cells will undergo three freeze-thaw cycles and will be lysed using a probe sonicator (Fisher Scientific) and undergo centrifugation. The supernatant will be purified on an NGC Quest 10 FPLC system (Bio-Rad) using an MBP Trap HP column (GE Healthcase) equilibrated with 20mM Tris 200mM NaCl pH 7.4 with the elution buffer being supplemented with 10mM maltose.

5.5.4 Isolation of EK from MBP-EK

The eluted MBP-EK product will be buffer exchanged with 20mM Tris 50mM NaCl 2mM $CaCl_2$ 3M urea pH 7.4, after which 900U enterokinase will be added and the mixture will be placed in the dark at room temperature for 2 days. The mixture will then be buffer exchanged with 1%

acetonitrile in water and purified using a 2695 Separations Module (Waters) using a 250x10mm 4μM Jupiter column (Phenomenex) with an increasing acetonitrile gradient.

5.6 Figures

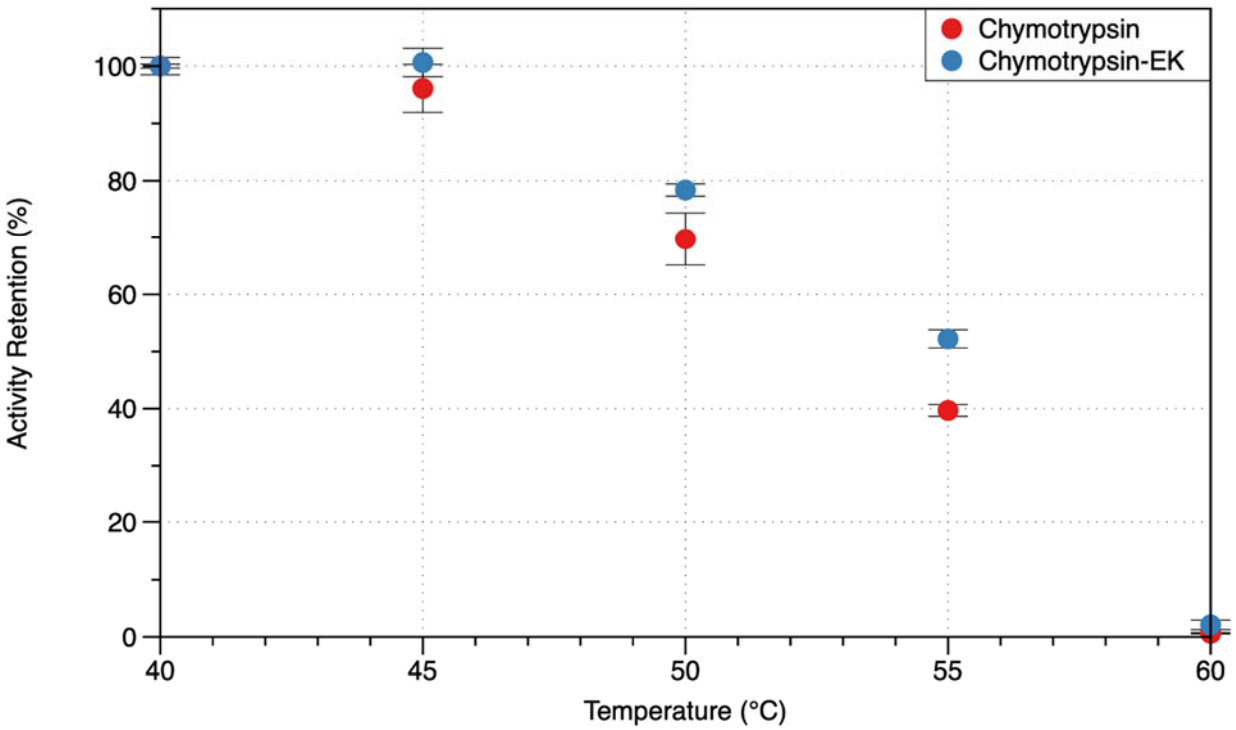

Figure 5.1. Chymotrypsin and chymotrypsin EK stability.

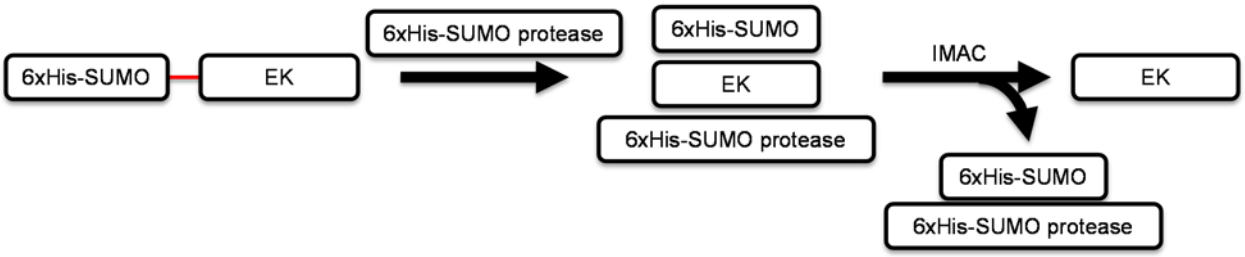

Figure 5.2. Schematic of SUMO-EK purification scheme.

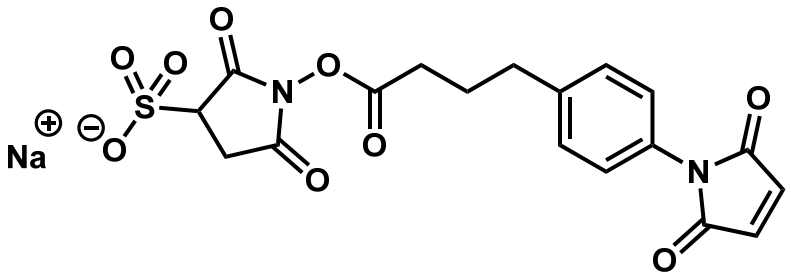

Figure 5.3. A heterobifunctional crosslinker.

Chapter 6

Conclusions

This body of work has demonstrated the ability of EK when used to modify proteins. EK was rationally designed as a polypeptide analogue of low-fouling zwitterionic pCB, which has demonstrated superior properties compared to other polymers when chemically conjugated to proteins. The use of the EK polypeptide further allows for the use of recombinant protein expression techniques to generate products of uniform products in a repeatable fashion, allowing for the creation of EK fusion proteins. EK fusion proteins were successfully expressed and characterized.

The generation of EK fusion proteins was first demonstrated using a model β-lactamase system. The fusion proteins were successfully expressed and purified. These EK fusion proteins demonstrated similar gains in their stability and kinetics compared to proteins that had been modified by pCB, demonstrating that EK is able to mimic pCB. Additionally it was demonstrated that EK sequences of different lengths affected the stability and kinetics of the target proteins differently.

The zwitterionic capabilities of EK were further demonstrated using the OPH system. The generation of OPH-EK fusion proteins was able to successfully disrupt the OPH dimer formation by protecting the monomer from foreign interaction. The resulting monomer was able to demonstrate significantly improved kinetics, possibly due to reduced steric hindrance near the active site.

An EK fusion protein system containing G-CSF is described. G-CSF is a protein plagued by low blood circulation times, requiring frequent injections for treatment. This EK-G-CSF system will be used to elucidate how EK affects the properties of the target protein *in vivo*, with particular

focus on pharmacokinetics and immunogenicity. The goal is to demonstrate improvements in blood circulation through the addition of EK while monitoring the overall efficacy.

Additional work is focusing on the recombinant expression of standalone EK, allowing for the use of long EK polypeptides for other applications including protein conjugation. The continued development of EK for protein modification can lead to significant advancements in protein therapeutics by increasing the number of proteins that can be modified with EK.

References

(1) Abuchowski, A.; Es, T. V. E.; Palczuk, N. C.; Davis, F. F., Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol. *J. Biol. Chem.* **1977**, *252*, 3578-3581.

(2) Abuchowski, A.; McCoy, J. R.; Palczuk, N. C.; Es, T. V. E.; Davis, F. F., Effect of covalent attachment of polyethylene glycol on immunogenicity nad circulating life of bovine liver catalase. *J. Biol. Chem.* **1977**, *252*, 3582-3586.

(3) Fishburn, C. S., The pharmacology of PEGylation: balancing PD with PK to generate novel therapeutics. *J. Pham. Sci.* **2008**, *97*, 4167-4183.

(4) Veronese, F. M.; Mero, A., The impact of PEGylation on biological therapies. *Biodrugs* **2008**, *22*, 315-329.

(5) Li, W.; Zhan, P.; De Clercq, E.; Lou, H.; Liu, X., Current drug research on PEGylation with small molecular agents. *Prog. Polymer Sci.* **2013**, *38*, 421-444.

(6) Knop, K.; Hoogenboom, R.; Fischer, D.; Schubert, U. S., Poly(ethylene glycol) in drug delivery: pros and cons as well as potential alternatives. *Angew. Chem. Int. Ed.* **2010**, *49*, 6288-6309.

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

(8) Kaushik, T.; Yaqoob, M. M., Lessons learned from peginesatide in the treatment of anemia associated with chronic kidney disease in patients on dialysis. *Biologics* **2013**, *7*, 243-246.

(9) Hoy, S. M., Peginterferon beta-1a: a review of its use in patients with relapsing-remitting multiple sclerosis. *CNS Drugs* **2015**, *29*, 171-179.

(10) Turecek, P. L.; Bossard, M. J.; Graninger, M.; Gritsch, H.; Höllriegl, W.; Kaliwoda, M.; Matthiessen, P.; Miterer, A.; Muchitsch, E.-M.; Purtscher, M.; Rottensteiner, H.; Schiviz, A.; Shrenk, G.; Siekmann, J.; Varadi, K.; Riley, T.; Ehrlich, H. J.; Schwarz, H. P., BAX 855, a PEGylated cFVIII product with prolonged half-life. *Hamostaseologie* **2012**, *32 Suppl 1*, S29.

(11) Sarkissian, C. N.; Gámez, A.; Wang, L.; Charbonneau, M.; Pitzpatrick, P.; Lemontt, J. F.; Zhao, B.; Vellard, M.; Bell, S. M.; Henschell, C.; Lambert, A.; Tsuruda, L.; Stevens, R. C.; Scriver, C. R., Preclinical evaluation of multiple species of PEGylated recombinant phenylalanine ammonia lyase for the treatment of phenylketonuria. *Proc. Nat. Acad. Sci.* **2008**, *105*, 20894-20899.

(12) Ekladious, I.; Colson, Y. L.; Grinstaff, M. W., Polymer-drug conjugate therapeutics: advances, insights and prospects. *Nat. Rev. Drug Discov.* **2019**, *18*, 273-294.

(13) Shah, A.; Coyle, T.; Lalezari, S.; Fischer, K.; Kohlstaedde, B.; Delesen, H.; Radke, S.; Michaels, L. A., BAY 94-9027, a PEGylated recombinant factor VIII, exhibits a prolonged half-life and higher area under the curve in patients with severe haemophilia A: comprehensive pharmacokinetic assessment from clinical studies. *Haemophilia* **2018**, *24*, 733-740.

(14) Bailon, P.; APalleroni, A.; Schaffer, C. A.; Spence, C. L.; Fung, W.-J.; Porter, J. E.; Ehrlich, G. K.; Pan, W.; Xu, Z.-X.; Modi, M. W.; Farid, A.; Berthold, W., Rational design of a potent, long-lasting form of interferon: a 40 kDA branched polyethylene glycol-conjugated interferon α-2a for the treatment of hepatitis C. *Bioconjugate Chem.* **2001**, *12*, 195-202.

(15) Pollaro, L.; Heinis, C., Strategies to prolong the plasma residence time of peptide drugs. *Med. Chem. Commun.* **2010**, *1*, 319-324.
(16) Cai, Y.; Xu, M.; Yuan, M.; Liu, Z.; Yuan, W., Developments in human growth hormone preparations: sustained-release, prolonged half-life, novel injection devides, and alternative delivery routes. *Int. J. Nanomedicine* **2014**, *9*, 3527-3538.
(17) Loche, S.; Salerno, M.; Garofalo, P.; Cardinale, G. M.; Licenziati, M. R.; Citro, G.; Nicoletti, M. C.; Cappa, M.; Logobardi, S.; Maghnie, M.; Perrone, R., Adherence in children with grown hormone dificiency treated with r-hGH and the easypod™ device. *J. Endocrinol. Invest.* **2016**, *39*, 1419-1424.
(18) Baker, D. P.; Lin, E. Y.; Lin, K.; Pellegrini, M.; Petter, R. C.; Chen, L. L.; Arduini, R. M.; Brickelmaier, M.; Wen, D.; Hess, D. M.; Chen, L.; Grant, D.; Whitty, A.; Grill, A.; Lindner, D. J.; Pepinsky, R. B., N-terminally PEGylated human interferon-β-1a with improved pharmacokinetic properties and *in vivo* efficacy in a melanoma angiogenesis model. *Bioconjugate Chem.* **2006**, *17*, 179-188.
(19) Hinds, K. D.; Kim, S. W., Effects of PEG conjugation on insulin properties. *Adv. Drug Deliv. Rev.* **2002**, *54*, 505-530.
(20) Bomalaski, J. S.; Holtsberg, F. W.; Ensor, C. M.; Clark, M. A., Uricase formulated with polyethylene glycol (uricase-PEG 20): biochemical rationale and preclinical studies. *J. Rheumatology* **2002**, *29*, 1942-1949.
(21) Ganson, N. J.; Kelly, S. J.; Scarlett, E.; Sundy, J. S.; Hershfield, M. S., Control of hyperuricemia in subjects with refractory gout, and induction of antibody against poly(ethylene glycol) (PEG), in a phase I trial of subcutaneous PEGylated urate oxidase. *Arthritis Res. Ther.* **2006**, *8*, R12.
(22) Kelly, S. J.; Delmondedieu, M.; Oliverio, M. I.; Williams, L. D.; Saifer, M. G. P.; Sherman, M. R.; Coffman, T. M.; Johnson, G. A.; Hershfield, M. S., Diabetes insipidus in uricase-deficient mice: a model for evaluating therapy with poly(ethylene glycol)-modified uricase. *J. Am. Soc. Nephrol.* **2001**, *12*, 1001-1009.
(23) Danial, M.; van Dulmen, T. H. H.; Aleksandrowicz, J.; Pötgens, A. J. G.; Klok, H.-A., Site-specific PEGylation of HR2 peptides: effects of PEG conjugation position and chain length on HIV-1 membrane fusion inhibition and proteolytic degradation. *Bioconjug. Chem.* **2012**, *23*, 1648-1660.
(24) Veronese, F. M., Peptide and protein PRGylation: a review of problems and solutions. *Biomaterials* **2001**, *22*, 405-417.
(25) Diwan, M.; Park, T. G., Pegylation enhances protein stability during encapsulation in PLGA microspheres. *J. Controlled Release* **2011**, *73*, 233-244.
(26) Michel, R.; Pasche, S.; Textor, M.; Castner, D. G., Influence of PEG architecture on protein adsorption and conformation. *Langmuir* **2005**, *21*, 12327-12332.
(27) Kozlowski, A.; Harris, J. M., Improvements in protein PEGylation: PEGylated interferons for treatment of hepatitis C. *J. Control. Release* **2001**, *72*, 217-224.
(28) Foster, G. R., Review acticle: Pegylated interferons: chemical and clinical differences. *Aliment. Pharmacol. Ther.* **2004**, *20*, 825-830.
(29) Hu, X.; Cui, Y.; White, J.; Zhu, Y.; Deykin, A.; Nestorov, I.; Hung, S., Parmacokinetics and pharmacodynamics of peginterferon beta-1a in patiens with relapsing-remitting in the randomized ADVANCE study. *British J. Clinical Pharm.* **2015**, *79*, 514-522.
(30) Swierczewska, M.; Lee, K. C.; Lee, S., What is the future of PEGylated therapies? *Expert Opin. Emerg. Drugs* **2015**, *20*, 531-536.

(31) Zaman, R.; Islam, R. A.; Ibnat, N.; Othman, I.; Zaini, A.; Lee, C. Y.; Chowdhury, E. H., Current strategies in extending half-lives of therapeutic proteins. *J. Control. Release* **2019**, *301*, 176-189.
(32) Harris, J. M.; Chess, R. B., Effect of PEGylation on pharmaceuticals. *Nature Rev. Drug Discov.* **2003**, *2*, 214-221.
(33) Roberts, M. J.; Bentley, M. D.; Harris, J. M., Chemistry for peptide and protein PEGylation. *Adv. Drug Deliv. Rev.* **2002**, *54*, 459-476.
(34) Mero, A.; Grigoletteo, A.; Maso, K.; Yoshioka, H.; Rosato, A.; Pasut, G., Site-selective enzymatic chmistry for polymer conjugation to protein lysine residues: PEGylation of G-CSF at lysine-41. *Polym. Chem.* **2016**, *7*, 6545.
(35) Grace, M. J.; Lee, S.; Bradshaw, S.; Chapman, J.; Spond, J.; Cox, S.; DeLorenzo, M.; Brassard, D.; Wylie, D.; Cannon-Carlson, S.; Cullen, C.; Indelicato, S.; Voloch, M.; Bordens, R., Site of Pegylation and polyethylene glycol molecule size attenuate interferon-α antiviral and antiproliferative activities through the JAK/STAT signaling pathway. *J. Biol. Chem.* **2005**, *280*, 6327-6336.
(36) Richter, A. W.; Akerblom, E., Polyethylene glycol reactive antibodies in man: titer distribution in allergic patients treated with monomethoxy polyethylene glycol modified allergens or placebo, and in healthy blood doners. *Int. Arch. Allergy Appl. Immunol.* **1984**, *74*, 36-39.
(37) Fisher, T. C.; Armstrong, J. K.; Wenby, R. B.; Meiselman, H. J.; Leger, R.; Garratty, G., Isolation and identification of a human antibody to poly(ethylene glycol). *Blood* **2003**, *102*, 559A.
(38) Liu, Y.; Reidler, H.; Pan, J.; Milunic, D.; Qin, D.; Chen, D.; Vallejo, Y. R.; Yin, R., A double antigen bridging immunogenicity ELISA fo rthe detection of antibodies to polyethylene glycon polymers. *J. Pharmacol. Toxicol. Methods* **2011**, *64*, 238-245.
(39) Schellekens, H.; Hennink, W. E.; Brinks, V., The immunogenicity of polyethylene glycol: facts and fiction. *Parm. Res.* **2013**, *30*, 1729-1734.
(40) Lubich, C.; Allacher, P.; de la Rosa, M.; Bauer, A.; Prenninger, T.; Horling, F. M.; Siekmann, J.; Oldenburg, J.; Scheiflinger, F.; Peipert, B. M., The mystery of antibodies against polyethylene glycol (PEG) - what do we know? *Pharm. Res.* **2016**, *33*, 2239-2249.
(41) Ho, D. H.; Brown, N. S.; Yen, A.; Holmes, R.; Keating, M.; Abuchowski, A.; Newman, R. A.; Krakoff, I. H., Clinical pharmacology of polyethylene glycol-L-asparaginase. *Drug Metabolism Disposition* **1986**, *14*, 349-352.
(42) Locatelli, F.; Del Vecchio, L., Peginesatide as anew approach for treating anemia of CKD patient: is it like a falling star? *Expert Opin. Pharmacother.* **2013**, *14*, 1277-1280.
(43) Ishida, T.; Kiwada, H., Accelerated blood clearance (ABC) phenomenon upon repeated injection of PEGylated liposomes. *Int. J. Pharmaceutics* **2008**, *354*, 56-62.
(44) Armstrong, J. K.; Hempel, G.; Koling, S.; Chan, L. S.; Fisher, T.; Meiselman, H. J., Antibody against poly(ethylene glycol) adversely affects PEG-asparaginase therapy in acute lymphoblastic leukemia patients. *Cancer* **2007**, *110*, 103-111.
(45) Zhang, P.; Sun, F.; Liu, S.; Jiang, S., Anti-PEG antibodies in the clinic: current issues and beyond PEGylation. *J. Control. Release* **2016**, *244*, 184-193.
(46) Dams, E. T. M.; Laverman, P.; Oyen, W. J. G.; Storm, G.; Scherphof, G. L.; van der Meer, J. W. M.; Costens, F. H. M.; Boerman, O. C., Accelerated blood clearance and altered biodistribution of repeated injections of sterically stabilized liposomes. *J. Pharmacol. Exp. Ther.* **2000**, *292*, 1071-1079.

(47) Zhang, P.; Sun, F.; Tsao, C.; Liu, S.; Jain, P.; Sinclair, A.; Hung, H.-C.; Bai, T.; Wu, K.; Jiang, S., Zwitterionic gel encapsulation promotes protein stability, enhances pharmacokinetics, and reduces immunogenicity. *Proc. Nat. Acad. Sci.* **2015**, *112*, 12046-12051.
(48) Li, B.; Yuan, Z.; Zhang, P.; Sinclair, A.; Jain, P.; Wu, K.; Tsao, C.; Xie, J.; Hung, H.-C.; Lin, X.; Bai, T.; Jiang, S., Zwitterionic nanocages overcome the efficacy loss of biologic drugs. *Adv. Mater.* **2018**, *30*, 1705728.
(49) Sundy, J. S.; Ganson, N. J.; Kelly, S. J.; Scarlett, E. L.; Rehrig, C. D.; Huang, W.; Hershfield, M. S., Pharmacokinetics and pharmacodynamics of intravenous PEGylated recombinant mammalian urate oxidase in patients with refractory gout. *Arthritis Rheum.* **2007**, *56*, 1021-1028.
(50) Lipsky, P. E.; Calabrese, L., H.; Kavanaugh, A.; Sundy, J. S.; Wright, D.; Wolfson, M.; Becker, M. A., Pegloticase immunogenicity: the relationship between efficacy and antibody development in patients treated for refractory chronic gout. *Arthristis Res. Ther.* **2014**, *16*, R60.
(51) Garratty, G., Progress in Modulating the RBC Membrane To Produce Transfusable Universal/Stealth Doner RBCs. *Transfusion Med. Rev.* **2004**, *19*, 245-256.
(52) Ishihara, T.; Takeda, M.; Sakamoto, H.; Kimoto, A.; Kobayashi, C.; Yuki, K.; Tanaka, K.-i.; Takenaga, M.; Igarashi, R.; Maeda, T.; Yamakawa, N.; Mizushima, Y.; Mizushima, T., Accelerated blood clearance phenomenon upon repeated injection of PEG-modified PLA-nanoparticles. *Pharm. Res.* **2009**, *26*, 2270-2279.
(53) Mima, Y.; Hashimoto, Y.; Shimizu, T.; Kiwada, H.; Ishida, T., Anti-PEG IgM is a major contributor to the accelerated blood clearance of polyethylene glycol-conjugated protein. *Mol. Pharmaceutics* **2015**, *12*, 2429-2435.
(54) DeFrancesco, L., Three deaths sink Affymax. *Nature Biotechol.* **2013**, *31*, 270.
(55) Jelkmann, W., Watch out for a revival of peginesatide in sports. *Drug Test. Anal.* **2016**, *9*, 157-160.
(56) Weaver, J. L.; Boyne, M.; Pang, E.; Chimalakonda, K.; Howard, K. E., Nonclinical evaluation of the potential for mast cell activation by an erythropoietin analog. *Toxicol. Appl. Pharmacol.* **2015**, *287*, 246-252.
(57) Duncan, R., Polymer therapeutics: top 10 selling pharmaceuticals - what next? *J. Control. Release* **2014**, *190*, 371-380.
(58) Acharya, A.; Bingham, K.; Bradner, J.; Burke, W.; Charo, R. A.; Cherry, J.; Choulika, A.; Coles, T.; Cook-Deegan, R.; Crooke, S. T.; Díaz, E.; Erickson, B.; Giddings, L. V.; Giwa, S. E.; Greenwood, J. C.; Gulati, V.; Hall, S.; Harris, J.; Heywood, J.; Hill, C.; Levin, J.; Mangubat, A.; Maraganore, J.; Mariggi, G.; Mazur, B.; McGuire, A. L.; Moll, N.; Moreno, J.; Naughton, G.; Nelsen, L.; Osbourn, J.; Perez, D.; Reed, J.; Schmidt, E.; Seyfert-Margolis, V.; Stoffels, P.; Thorball, J.; O'Toole, T.; Vainu, I.; van Deventer, S.; Zerhouni, E.; Zohar, D., Community crystal gazing. *Nature Biotechol.* **2016**, *34*, 276-283.
(59) Gauthier, M. A.; Klok, H.-A., Polymer-protein conjugates: an enzymatic activity perspective. *Polym. Chem.* **2010**, *1*, 1352-1373.
(60) Pelegri-O'Day, E. M.; Lin, E.-W.; Maynard, H. D., Therapeutic protein-polymer conjugates: advancing beyond PEGylation. *J. Am. Chem. Soc.* **2014**, *136*, 14323-14332.
(61) Filpula, D.; Zhao, H., Releasable PEGylation of proteins with customized linkers. *Adv. Drug Deliv. Rev.* **2007**, *60*, 29-49.

(62) Lee, S.; Greenwald, R. B.; McGuire, J.; Yang, K.; Shi, C., Drug delivery systems employing 1,6-elimination: releasable poly(ethyleen glycol) conjugates of proteins. *Bioconjugate Chem.* **2001**, *12*, 163-169.
(63) Lee, Y.; Koo, H.; Jin, G.-w.; Mo, H.; Cho, M. Y.; Park, J.-Y.; Choi, J. S.; Park, J. S., Poly(ethylene oxide sulfide): new poly(ethylene glycol) derivatives degradable in reductive conditions. *Biomacromolecules* **2005**, *6*, 24-26.
(64) Lundberg, P.; Lee, B. F.; van den Berg, S. A.; Pressly, E. D.; Lee, A.; Hawker, C. J.; Lynd, N. A., Poly[(ethylene oxide)-co-(methylene ethylene oxide)]: a hydrolytically degradable poly(ethylene oxide) platform. *ACS Macro Lett.* **2012**, *1*, 1240-1243.
(65) Kontermann, R. E., Half-life extended biotherapeutics. *Expert Opin. Biol. Ther.* **2016**, *16*, 903-915.
(66) Czajkowsky, D. M.; Hu, J.; Shao, Z.; Pleass, R. J., Fc-fusion proteins: new developments and future perspectives. *EMBO Mol. Med.* **2012**, *4*, 1015-1028.
(67) Lyseng-Williams, K. A., Coagulation factor IX (recombinant), albumin fusion protein (albutrepenonacog alfa; Idelvion®): a review of its use in haemophilia B. *Drugs* **2017**, *77*, 97-106.
(68) Mero, A.; Campisi, M., Hyaluronic acid bioconjugates for the delivery of bioactive molecules. *Polymers* **2014**, *6*, 346-369.
(69) Strohl, W. R., Frusion proteins for half-life extension of biologics as a stragegy to make biobetters. *BioDrugs* **2015**, *29*, 215-239.
(70) Schlapschy, M.; Binder, U.; Börger, C.; Theobald, I.; Wachinger, K.; Kisling, S.; Haller, D.; Skerra, A., PASylation: a biological alternative to PEGylation for extending the plasma half-life of pharmaceutically active proteins. *Protein Eng. Design Selection* **2013**, *26*, 489-501.
(71) Breibeck, J.; Skerra, A., The polypeptide biophysics of proline/alanine-rich sequences (PAS): recombinant biopolymers with PEG-like properties. *Biopolymers* **2018**, *109*, e23069.
(72) Bolze, F.; Morath, V.; Bast, A.; Rink, N.; Schlapschy, M.; Mocek, S.; Skerra, A.; Klingenspor, M., Long-acting PASylated leptin ameliorates obesity by promioting satiety and preventing hypometabolism in leptin-deficient $Lep^{ob/ob}$ mice. *Endocrinology* **2016**, *157*, 233-244.
(73) Kuhn, N.; Schmidt, C. Q.; Schlapschy, M.; Skerra, A., PASylated coversin, a C5-specific complement inhibitor with extended pharmacokinetics, shows enhanced anti-hemolytic activity in vitro. *Bioconjug. Chem.* **2016**, *27*, 2359-2371.
(74) Xia, Y.; Schlapschy, M.; Morath, V.; Roeder, N.; Vogt, E. I.; Stadler, D.; Cheng, X.; Dittmer, U.; Sutter, K.; Heikenwalder, M.; Skerra, A.; Protzer, U., PASylated interferon α efficiently suppresses hepatitis B virus and induces into-HBs seroconversion in HBV-transgenic mice. *Antivir. Res.* **2019**, *161*, 134-143.
(75) Längin, M.; Mayr, T.; Reichart, B.; Michel, S.; Bucholz, S.; Guethoff, S.; Dashkevich, A.; Baehr, A.; Egerer, S.; Bauer, A.; Mihalj, M.; Panelli, A.; Issl, L.; Ying, J.; Fresch, A. K.; Buttgereit, I.; Mokelke, M.; Radan, J.; Werner, F.; Lutzmann, I.; Steen, S.; Sjöberg, T.; Paskevicius, A.; Quiming, L.; Sfriso, R.; Rieben, R.; Dahlhoff, M.; Kessler, B.; Kemter, E.; Klett, K.; Kinkel, R.; Kupatt, C.; Falkenau, A.; Reu, S.; Ellgass, R.; Herzog, R.; Binder, U.; Wich, G.; Skerra, A.; Ayares, D.; Kind, A.; Schönmann, U.; Kaup, F.-J.; Hagl, C.; Wolf, E.; Klymiuk, N.; Brenner, P.; Abicht, J.-M., Consistent success in life-supporting porcine cardiac xenotransplantation. *Nature* **2018**, *564*, 430-433.

(76) Schellenberger, V.; Wang, C.-W.; Geething, N. C.; Spink, B. J.; Campbell, A.; To, W.; Scholle, M. D.; Yin, Y.; Yi, Y.; Bogin, O.; Cleland, J. L.; Silverman, J.; Stemmer, W. P. C., A recombinant polypeptide extends the *in vivo* half-life of peptides and proteins in a tunable manner. *Nature Biotechol.* **2009**, *27*, 1186-1190.

(77) Podust, V. N.; Balan, S.; Sim, B.-C.; Coyle, M. P.; Ernst, U.; Peters, R. T.; Schellenberger, V., Expression of *in vivo* half-life of biologically active molecules by XTEN protein polymers. *J. Control. Release* **2016**, *240*, 52-66.

(78) Ding, S.; Song, M.; Sim, B.-C.; Gu, C.; Podust, V. N.; Wang, C.-W.; McLaughlin, B.; Shah, T. P.; Lax, R.; Gast, R.; Sharan, R.; Vasek, A.; Hartman, M. A.; Deniston, C.; Srinivas, P.; Schellenberger, V., Multivalent antiviral XTEN-peptide conjugates with long in vivo half-life and enhanced solubility. *Bioconjugate Chem.* **2014**, *25*, 1351-1359.

(79) Geething, N. C.; To, W.; Spink, B. J.; Scholle, M. D.; Wang, C.-W.; Yin, Y.; Yao, Y.; Schellenberger, V.; Cleland, J. L.; Stemmer, W. P. C.; Silverman, J., Gcg-XTEN: An Improved Glucagon Capable of Preventing Hypoglycemia without Increasing Baseline Blood Glucose. *PLoS ONE* **2010**, *5*.

(80) Podust, V. N.; Sim, B.-C.; Kothari, D.; Henthorn, L.; Gu, C.; Wang, C.-W.; McLaughlin, B.; Schellenberger, V., Extension of *in vivo* half-life of biologically active peptides via chemical conjugation to XTEN protein polymer. *Protein Eng. Des. Sel.* **2013**, *26*, 743-753.

(81) Moore, W. V.; Nguyen, H. J.; Kletter, G. B.; Millter, B. S.; Rogers, D.; Ng, D.; Moore, J. A.; Humphriss, E.; Cleland, J. L.; Bright, G. M., A randomized safety and efficacy study of Somavaratan (VRS-317), a long-acting rhGH, in pediatric growth hormne definiciency. *J. Clin. Endocrinol. Metlab.* **2016**, *101*, 1091-1097.

(82) Miller, B. S.; Moore, W. V.; Fechner, P. Y.; Van Meter, Q. L.; Fuqua, J. S.; Ng, D.; Humphriss, E.; Charlton, R. W.; Bright, G. M., 3-year safety and efficacy update of the VERTICAL & VISTA trials of somavaratan (VRS-317), a long-acting rhGH, in children with growth hormone deficiency (GHD). *Endocr. Abstracts* **2017**, *49*, GP65.

(83) Versartis announces phase 3 VELOCITY trial of somavaratan in pediatric growth hormone deficiency did not meet primary endpoint, GlobeNewswire, 21 September 2017. https://www.globenewswire.com/news-release/2017/09/21/1126085/0/en/Versartis-Announces-Phase-3-VELOCITY-Trial-of-Somavaratan-in-Pediatric-Growth-Hormone-Deficiency-Did-Not-Meet-Primary-Endpoint.html

(84) Jiang, S.; Cao, Z., Ultralow-fouling, functionalizable, and hydrolyzable zwitterionic materials and their derivatives for biological applications. *Adv. Mater.* **2010**, *22*, 920-932.

(85) Ladd, J.; Zhang, Z.; Chen, S.; Hower, J. C.; Jiang, S., Zwitterionic polymers exhibiting high resistance to nonspecific protein adsorption from human serum and plasma. *Biomacromolecules* **2008**, *9*, 1357-1361.

(86) Zhang, Z.; Chao, T.; Chen, S.; Jiang, S., Superlow fouling sulfobetaine and carboxybetaine polymers on glass slides. *Langmuir* **2006**, *22*, 10072-10077.

(87) Cao, Z.; Yu, Q.; Xue, H.; Cheng, G.; Jiang, S., Nanoparticles for drug delivery prepared with amphiphilic PLGA zwitterionic blocks copolymers with sharp contrast in polarity between two blocks. *Angew. Chem. Int. Ed.* **2010**, *49*, 3771-3776.

(88) Parrott, M. C.; DeSimone, J. M., Relieving PEGylation. *Nature Chem.* **2012**, *4*, 13-14.

(89) He, Y.; Chang, Y.; Hower, J. C.; Zheng, J.; Chen, S.; Jiang, S., Origin of repulsive force and structure/dynamics of interfacial water in OEG-protein interactions: a molecular simulation study. *Phys. Chem. Chem. Phys.* **2008**, *10*, 5539-5544.

(90) Chen, S.; Zheng, J.; Li, L.; Jiang, S., Strong resistance of phosphorylcholine self-assembled monolayers to protein absorption: insights into nonfouling properties of zwitterionic materials. *J. Am. Chem. Soc.* **2005**, *127*, 14473-14478.
(91) Yang, W.; Bai, T.; Carr, L. R.; Keefe, A. J.; Xu, J.; Xue, H.; Irvin, C. A.; Chen, S.; Wang, J.; Jiang, S., The effect of lightly crosslinked poly(carboxybetaine) hydrogel coating on the performance of sensors in whole blood. *Biomaterials* **2012**, *33*, 7945-7951.
(92) Keefe, A. J.; Jiang, S., Poly(zwitterionic)protein conjugates offer increased stability without sacrificing binding affinity or bioactivity. *Nat. Chem.* **2012**, *4*, 59-63.
(93) Shao, Q.; White, A. D.; Jiang, S., Difference of carboxybetaine and oligo(ethylene glycol) moieties in altering hydrophobic interactions: a molecular simulation study. *J. Phys. Chem. B* **2014**, *118*, 189-194.
(94) Liu, S.; Jiang, S., Chemical conjugation of zwitterionic polymers protects immunogenic enzyme and preserves bioactivity without polymer-specific antibody response. *Nano Today* **2016**, *11*, 285-291.
(95) Zhang, P.; Liu, E. J.; Tsao, C.; Kasten, S. A.; Boeri, M. V.; Dao, T. L.; DeBus, S. J.; Cadieux, C. L.; Baker, C. A.; Otto, T. C.; Cerasoli, D. M.; Chen, Y.; Jain, P.; Sun, F.; Li, W.; Hung, H.-C.; Yuan, Z.; Ma, J.; Bigley, A. N.; Rauschel, F. M.; Jiang, S., Nanoscavenger provides long-term prophylactic protection against nerve agents in rodents. *Sci. Trans. Med.* **2019**, *11*, eaau7091.
(96) Chen, S.; Cao, Z.; Jiang, S., Ultra-low fouling peptide surfaces derived from natural amino acids. *Biomaterials* **2009**, *30*, 5892-5896.
(97) Keefe, A. J.; Caldwell, K. B.; Nowinski, A. K.; White, A. D.; Thakkar, A.; Jiang, S., Screening nonspecific interactions of peptides without background interference. *Biomaterials* **2013**, *34*, 1871-1877.
(98) Nowinski, A. K.; White, A. D.; Keefe, A. J.; Jiang, S., Biologically inspired stealth peptide-capped gold nanoparticles. *Langmuir* **2014**, *30*, 1864-1870.
(99) White, A. D.; Nowinski, A. K.; Huang, W.; Keefe, A. J.; Sun, F.; Jiang, S., Decoding nonspecific interactions from nature. *Chem. Sci.* **2012**, *3*, 3488-3494.
(100) Coin, I.; Beyermann, M.; Bienert, M., Solid-phase peptide synthesis: from standard procedures to the synthesis of difficult sequences. *Nature Protocols* **2007**, *2*, 3247-3256.
(101) Nowinski, A. K.; Sun, F.; White, A. D.; Keefe, A. J.; Jiang, S., Sequence, structure, and function of pepetide self-assembled monolayers. *J. Am. Chem. Soc.* **2012**, *134*, 6000-6005.
(102) Kimmerlin, T.; Seebach, D., '100 years of peptide synthesis': ligation methods for peptide and protein synthesis with applications to beta-peptide assemblies. *J. Pept. Res.* **2005**, *65*, 229-260.
(103) Vlieghe, P.; Lisowski, V.; Martinez, J.; Khretchatisky, M., Synthetic therapeutic peptides: science and market. *Drug Discov. Today* **2010**, *15*, 40-56.
(104) Palzkill, T., Metallo-β-lacatamase structure and function. *Ann. N. Y. Acad. Sci.* **2013**, *1277*, 91-104.
(105) Crawford, P. A.; Sharma, N.; Chandrasekar, S.; Signel, T.; Walsh, T. R.; Spencer, J.; Crowder, M. W., Over-expression, purification, and characterization of metallo-β-lactamase Imis from *Aeromonas veronii* bv. *sobria*. *Protein Expression and Purification* **2004**, *36*, 272-279.
(106) Sauvage, E.; Kerff, F.; Terrak, M.; Ayala, J. A.; Charlier, P., The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis. *FEMS Microbiol. Rev.* **2008**, *32*, 234-258.

(107) Bush, K., The ABCD's of β-lactamase nomenclature. *J. Infect. Chemother.* **2013**, *19*, 549-559.
(108) Goh, S.; Good, L., Plasmid selection in *Escherichia coli* using an endogenous essential gene marker. *BMC Biotechnol.* **2008**, *8*, 61.
(109) Rosano, G. L.; Ceccarelli, E. A., Recombinant protein expression in *Escherichia coli*: advances and challenges. *Frontiers Microbiol.* **2014**, *5*, 1-17.
(110) Cooksey, R.; Swenson, J.; Clark, N.; Gay, E.; Thornsberry, C., Patterns and mechanisms of β-lactam resistance among isolates of *Escherichia coli* from hospitals in the United States. *Antimicrob. Agents Chemother.* **1990**, *34*, 739-745.
(111) Georgiou, G.; Telford, J. N.; Shuler, M. L.; Wilson, D. B., Localization of inclusion bodies in *Escherichia coli* overproducing beta-lactamase or alkaline phosphatase. *Appl. Environ. Microbiol.* **1985**, *52*, 1157-1161.
(112) Singh, A.; Upadhyay, V.; Upadhyay, A. K.; Singh, S. M.; Panda, A. K., Protein recovery from inclusion bodies of *Escherichia coli* using mild solubilization process. *Microb. Cell Fact.* **2015**, *14*, 1-10.
(113) Minsky, A.; Summers, R. G.; Knowles, J. R., Secretion of beta-lactamase into the periplasm of *Escherichia coli*: evidence for a disctinct release step associated with a conformational change. *Proc. Nat. Acad. Sci.* **1986**, *83*, 4180-4184.
(114) Wang, X.; Minasov, G.; Shoichet, B. K., Evolution of an antibiotic resistance enzyme contrainted by stability and activity trade-offs. *J. Mol. Biol.* **2002**, *320*, 85-95.
(115) Nannenga, B. L.; Baneyx, F., Reprogramming chaperone pathways to improve membrane protein expression in *Escherichia coli*. *Protein Sci.* **2011**, *20*, 1411-1420.
(116) Ghrayeb, J.; Kimura, H.; Takahara, M.; Hsiung, H.; Masui, Y.; Inouye, M., Secretion cloning vectors in *Escherichia coli*. *EMBO J.* **1984**, *3*, 2437-2442.
(117) Latifi, A. M.; Khajeh, K.; Farnoosh, G.; Hassanpour, K.; Khodi, S., The cytoplasmic and periplasmic expression levels and folding of organophophorus hydrolase in *Escherichia coli*. *Jundishapur J. Microbiol.* **2015**, *8*, e17790.
(118) Klint, J. K.; Senff, S.; Saez, N. J.; Seshadri, R.; Lau, H. Y.; Bende, N. S.; Undheim, E. A. B.; Rash, L. D.; Mobli, M.; King, G. F., Production of recombinant disulfide-rich venom peptides for structural and functional analysis via expression in the periplasm of *E. coli*. *PLoS ONE* **2013**, *8*, e63865.
(119) Tokunaga, H.; Arakawa, T.; Fukada, H.; Tokunaga, M., Opposing effects of NaCl on reversibility and thermal stability of halophilic β-lactamase from a moderate halophile, *Chromohalobacter* sp. 560. *Biophys. Chem.* **2006**, *119*, 316-320.
(120) Bowden, G. A.; Georgiou, G., Folding and aggregation of beta-lactamase in the periplasmic space of *Escherichia coli*. *J. Biol. Chem.* **1990**, *16760-16766*.
(121) Petrikovics, I.; Wales, M. E.; Budai, M.; Yu, J. C. C.; Szilasi, M., Nano-intercalated organophosphorus-hydrolyzing enzymes in organophosphorus antagonism. *AAPS PharmSciTech.* **2012**, *13*, 112-117.
(122) Letort, S.; Balieu, S.; Erb, W.; Gouhier, G.; Estour, F., Interactions of cyclodextrins and their derivatives with toxic organophosphorus compounds. *Beilstein J. Org. Chem.* **2016**, *12*, 204-228.
(123) Cherny, I.; Greisen, P. J.; Ashani, Y.; Khare, S. D.; Oberdorfer, G.; Leader, H.; Baker, D.; Tawfik, D. S., Entineering V-type nerge agents detoxifying enzymes using computationally focused libraries. *ACS Chem. Biol.* **2013**, *8*, 2394-2403.

(124) Munro, N. B.; Ambrose, K. R.; Watson, A. P., Toxicity of the organophosphate chemical warfare agents GA, GB, and VX: implications for public protection. *Environ. Health Perspect.* **1994**, *102*, 18-37.
(125) Lerro, C. C.; Koutros, S.; Andreotti, G.; Friesen, M. C.; Alavanja, M. C.; Blair, A.; Hoppin, J. A.; Sandler, D. P.; Lubin, J. H.; Ma, X.; Zhang, Y.; Freeman, L. E. B., Organophosphate insecticide use and cancer incidence among spouses of pesticide applicators in the Agricultural Health Study. *Occup. Environ. Med.* **2015**, *72*, 736-744.
(126) Eddleston, M.; Buckley, N. A.; Eyer, P.; Dawson, A. H., Management of acute organophosphorus pesticide poisoning. *Lancet* **2008**, *371*, 597-607.
(127) Leikin, J. B.; Thomas, R. G.; Walter, F. G.; Klein, R.; Meislin, H. W., A review of nerve agent exposure for the critical care physician. *Crit. Care Med.* **2002**, *30*, 2346-2354.
(128) Quagliano, J.; Witkiewicz, Z.; Sliwka, E.; Neffe, S., Precursors of nerve chemical warfare agents with industrial relavance: characteristics and significance for chemical security. *Chem. Select* **2018**, *3*, 2703-2715.
(129) Cannard, K., The acute treatment of nerve agent exposure. *J. Neurol. Sci.* **2006**, *249*, 86-94.
(130) Ashani, Y.; Pistinner, S., Estimation of the upper limit of human butyrylcholinesterase dose required for protection against organophosphates toxicity: a mathematically based toxicokinetic model. *Toxicol. Sci.* **2004**, *77*, 358-367.
(131) Trovaslet-Leroy, M.; Musilova, L.; Renault, F.; Brazzolotto, X.; Misik, J.; Novotny, L.; Froment, M.-T.; Gillon, E.; Loiodice, M.; Verdier, L.; Masson, P.; Rochu, D.; Jun, D.; Nachon, F., Organophosphate hydrolases as catalytic bioscavengers of organophosphorus nerve agents. *Toxicol. Lett.* **2011**, *206*, 14-23.
(132) Bigley, A. N.; Xu, C.; Henderson, T. J.; Harvey, S. P.; Raushel, F. M., Enzymatic neutralization of the chemical warfare agent VX: evolution of phosphotriesterase for phosphorothiolate hydrolysis. *J. Am. Chem. Soc.* **2013**, *135*, 10426-10432.
(133) Broomfield, C. A.; Maxwell, D. M.; Solana, R. P.; Castro, A.; Finger, A. V.; Lenz, D. E., Protection of butyrylcholinesterase against organophosphorus poisoning in nonhuman primates. *J. Pharmacol. Exp. Ther.* **1991**, *259*, 633-638.
(134) Lenz, D. E.; Clarkson, E. D.; Schulz, S. M.; Cerasoli, D. M., Butyrylcholinesterase as a therapeutic drug for protection against percutaneous VX. *Chem.-Biol. Interact.* **2010**, *187*, 249-252.
(135) Lenz, D. E.; Yeung, D.; Smith, J. R.; Sweeney, R. E.; Lumley, L. A.; Cerasoli, D. M., Stoichiometric and catalytic scavengers as protection against nerve agent toxicity: a mini review. *Toxicology* **2007**, *233*, 31-39.
(136) Tsai, P.-C.; Bigley, A.; Li, Y.; Ghanem, E.; Cadieux, C. L.; Kasten, S. A.; Reeves, T. E.; Cerasoli, D. M.; Raushel, F. M., Stereoselective hydrolysis of organophosphate nerve agents by bacterial phosphotriesterase. *Biochemistry* **2010**, *49*, 7978-7987.
(137) Harper, L. L.; McDaniel, C. S.; Miller, C. E.; Wild, J. R., Dissimilar plasmids isolated from *Pseudomonas diminuta* MG and a *Flavobacterium* sp. (ATCC 27551) contain identical *opd* genes. *Appl. Environ. Microbiol.* **1988**, *54*, 2586-2589.
(138) Goldsmith, M.; Aggarwal, N.; Ashani, Y.; Jubran, H.; Greisen, P. J.; Ovchinnikov, S.; Leader, H.; Baker, D.; Sussman, J. L.; Goldenzweig, A.; Fleishman, S. J.; Tawfik, D. S., Overcoming an optimization plateau in the direct evolution of highly efficient nerve agent bioscavengers. *Protein Eng. Des. Sel.* **2017**, *30*, 333-345.

(139) Goldsmith, M.; Eckstein, S.; Ashani, Y.; Greisen, P. J.; Leader, H.; Sussman, J. L.; Aggarwal, N.; Ovchinnikov, S.; Tawfik, D. S.; Baker, D.; Thiermann, H.; Worek, F., Catalytic efficiencies of directly evolved phosphotriesterase variants with structurally different oranophosphorus compounds in vitro. *Arch. Toxicol.* **2016**, *90*, 2711-2724.
(140) Benning, M. M.; Kuo, J. M.; Rauschel, F. M.; Holden, H. M., Three-dimensional structure of phosphotriesterase: an enzyme capable of detoxifying organophosphate nerve agents. *Biochemistry* **1994**, *33*, 15001-15007.
(141) Grimsley, J. K.; Scholtz, J. M.; Pace, C. N.; Wild, J. R., Organophophorus hydrolase is a remarkably stable enzyme that unfolds through a homodimeric intermediate. *Biochemistry* **1997**, *36*, 14366-14374.
(142) Aubert, S. D.; Li, Y.; Raushel, F. M., Mechanism for the hydrolysis of organophosphates by the bacterial phosphotriesterase. *Biochemistry* **2004**, *43*, 5707-5715.
(143) Fukuda, T.; Tsuchiyama, K.; Makishima, H.; Takayama, K.; Mulchandani, A.; Kuroda, K.; Ueda, M.; Suye, S.-i., Improvement in organophosphorus hydrolase activity of cell surface-engineered yeast strain using Flo1p anchor system. *Biotechnol. Lett.* **2010**, *32*, 655-659.
(144) Xu, B.; Wild, J. R.; Kenerley, C. M., Enhanced expression of a bacterial gene for pesticide degradation in a common soil fungus. *J. Ferment. Bioeng.* **1996**, *81*, 473-481.
(145) Wang, X.; Wu, N.; Guo, J.; Chu, X.; Tian, J.; Yao, B.; Fan, Y., Phytodegradation of organophosphorus compounds by transgenic plants expressing a bacterial organophosphorus hydrolase. *Biochem. Biophys. Res. Commun.* **2008**, *365*, 453-458.
(146) Shen, W.; Shu, M.; Ma, L.; Ni, H.; Yan, H., High level expression of organophosphorus hydrloase in *Pichia pastoris* by multicopy *ophcM* assembly. *Protein Expr. Purif.* **2016**, *119*, 110-116.
(147) Cerasoli, D. M., **2016** Personal communication.
(148) Kirby, S., **2018** Personal communication.
(149) Liu, E. J.; Sinclair, A.; Keefe, A. J.; Nannenga, B. L.; Coyle, B. L.; Baneyx, F.; Jiang, S., EKylation: addition of an alternating-charge peptide stabilizes proteins. *Biomacromolecules* **2015**, *16*, 3357-3361.
(150) Nivikov, B. N.; Grimsley, J. K.; Kern, R. J.; Wild, J. R.; Wales, M. E., Improved pharmacokinetics and immunogenicity profile of organophosphorus hydrolase by chemical modification with polyethylene glycol. *J. Controlled Release* **2010**, *146*, 318-325.
(151) Koča, J.; Zhan, C.-G.; Rittenhouse, R. C.; Ornstein, R. L., Mobility of the active site bound paraoxon and sarin in zinc-phosphotriesterase by molecular dynamics simulation and quantum chemical calculation. *J. Am. Chem. Soc.* **2001**, *123*, 817-826.
(152) Hong, S.-B.; Rauschel, F. M., Stereochemical constraints on the substrate specificity of phosphotriesterase. *Biochemistry* **1999**, *38*, 1159-1165.
(153) Micsonai, A.; Wien, F.; Bulyáki, É.; Kun, J.; Moussong, É.; Lee, Y.-H.; Goto, Y.; Réfrégiers, M.; Kardos, J., BeStSel: a web serve for accurate protein secondary structure prediction and fold recognition from the circular dichroism spectra. *Nucleic Acids Res.* **2018**, *46*, W315-W322.
(154) Micsonai, A.; Wien, F.; Kernya, L.; Lee, Y.-H.; Goto, Y.; Réfrégiers, M.; Kardos, J., Accurate secondary structure prediction and fold recognition for circular dichroism spectroscopy. *Proc. Nat. Acad. Sci.* **2015**, *112*, E3095-E3103.
(155) Vanz, A. L. S.; Renard, G.; Palma, M. S.; Chies, J. M.; Dalmora, S. L.; Basso, L. A.; Santos, D. S., Human granulocyte colony stimulating factor (hG-CSF): cloning, overexpression, purification, and characterization. *Microb. Cell Fact.* **2008**, *7*, 13.

(156) Roberts, A. W., G-CSF: a key regulator of neutrophil production, but that's not all! *Growth Factors* **2005**, *23*, 33-41.
(157) Bendall, L. J.; Bradstock, K. F., G-CSF: from guanulopoietic stimulant to bone marrow stem cell mobilizing agent. *Cytokine Growth Factor Rev.* **2014**, *25*, 355-367.
(158) Xu, S.; Hoglund, M.; Hâkansson, L.; Venge, P., Granulocyte colony-stimulating factor (G-CSF) induces the production of cytokines *in vivo*. *Br. J. Haematol.* **2000**, *108*, 848-853.
(159) Fallah, M. J.; Akbari, B.; Saeedinia, A. R.; Karimi, M.; Vaez, M.; Zeinoddini, M.; Soleimani, M.; Maghsoudi, N., Overexpression of recombinant human granulycyte colony-stimulating factor in *E. coli*. *Int. J. Mol. Sci.* **2003**, *28*, 131-134.
(160) Trillet-Lenoir, V.; Green, J.; Manegold, C.; Van Pawel, J.; Gatzmeier, U.; Lebeau, B.; Depierre, A.; Johnson, P.; Decoster, G.; Tomita, D.; Ewen, C., Recombinant granulocyte colony stimulating factor reduces the infectious complications of cytotoxin chemotherapy. *Eur. J. Cancer* **1993**, *29*, 319-342.
(161) Crawford, J.; Ozer, H.; Stoller, R.; Johnson, D.; Lyman, G. H.; Tabbara, I.; Kris, M.; Grous, J.; Picozzi, V.; Rausch, G.; Smith, R.; Gradishar, W.; Yahanda, A.; Vincent, M.; Stewart, M.; Glaspy, J., Reduction by granulocyte colony-stimulating factor of fever and neutropenia induced by chemotherapy in patients with small-cell lung cancer. *N. Engl. J. Med.* **1991**, *325*, 164-170.
(162) Ria, R.; Reale, A.; Melaccio, A.; Racanelli, V.; Dammacco, F.; Vaca, A., Filgrastim, lenograstim and pegfilgrastim in the mobilization of peripheral blood protenitor cells in patients with lymphoproligerative malignancies. *Clin. Exp. Med.* **2015**, *15*, 145-150.
(163) Smith, T. J.; Khatcheressian, J.; Lyman, G. H.; Ozer, H.; Armitage, J. O.; Balducci, L.; Bennett, C. L.; Cantor, S. B.; Crawford, J.; Cross, S. J.; Demetri, G.; Desch, C. E.; Pizzo, P. A.; Schiffer, C. A.; Schwartzberg, L.; Somerfield, M. R.; Somlo, G.; Wade, J. C.; Wade, J. L.; Winn, R. J.; Wozniak, A.; Wolff, A. C., 2006 update of recommendations for the use of white blood cell growth factors: and evidence-based clinical practice guideline. *J. Clin. Oncol.* **2006**, *24*, 3187-3205.
(164) Kearns, C. M.; Wang, W. C.; Stute, N.; Ihle, J. N.; Evans, W. E., Disposition of recombinant human granulocyte colony-stimulating factor in children with severe chronic neutropenia. *J. Pediatr.* **1993**, *123*, 471-479.
(165) Almenar, D.; Mayans, J.; Juan, O.; Garcia Bueno, J. M.; Jalon Lopez, J. I.; Frau, A.; Guinot, M.; Cerezuela, P.; Garcia Buscalla, E.; Gasquet, J. A.; Sanchez, J., Pegfilgrastim and daily granulocyte colony-stimulating factor: patterns of use and neutropenia-related outcomes in cancer patiends in Spain-results of the LEARN study. *Eur. J. Cancer Care* **2009**, *18*, 280-186.
(166) Do, B. H.; Kang, H. J.; Song, J.-A.; Nguyen, M. T.; Park, S.; Yoo, J.; Nguyen, A. N.; Kwon, G. G.; Jang, J.; Jang, M.; Lee, S.; So, S.; Sim, S.; Lee, K. J.; Osborn, M. J.; Choe, H., Granulocyte colony-stimulating factor (GCSF) fused with Fc domain produced from *E. coli* less effective than polyethylene glycol-conjugated GCSF. *Sci. Rep.* **2017**, *7*, 6480.
(167) Laricchia-Robbio, L.; Moscato, S.; Genua, A.; Liberati, A. M.; Revoltella, R. P., Naturally occuring and therapy-induced antibodies to human granulocyte colong-stimulating factor (G-CSF) in human serum. *J. Cell. Physiol.* **1997**, *173*, 219-226.
(168) Traynor, K., Pain clinics grapple with naloxone issues. *Am. J. Health-Syst. Pharm.* **2018**, *75*, 1005.

(169) Tamada, T.; Honjo, E.; Maeda, Y.; Okamoto, T.; Ishibashi, M.; Tokunaga, M.; Kuroki, R., Homodimeric cross-over structure of the human granylocyte colony-stimulating factor (GCSF) receptor signaling complex. *Proc. Nat. Acad. Sci.* **2006**, *103*, 3135-3140.
(170) Arvedson, T.; O'Kelly, J.; Yang, B.-B., Design rationale and development approach for pegfilgrastim as a long-acting granulocyte colny-stimulating factor. *BioDrugs* **2015**, *29*, 185-198.
(171) Johnson, E.; Crawford, J.; Blackwell, S.; Bjurstrom, T.; Lockbaum, P.; Roskos, L.; Yang, B.-B.; Gardner, S.; Miller-Messana, M. A.; Shoemaker, D.; Garst, J.; Schwab, G., Randomized, dose-escalation study of SD/01 compared with daily filgrastim in patiends receiving chemotherapy. *J. Clin. Oncol.* **2000**, *18*, 2522-2528.
(172) Holmes, F. A.; O'Shaughnessy, J. A.; Vukelja, S.; Jones, S. E.; Shogan, J.; Savin, M.; Glaspy, J.; Moore, M.; Meza, L.; Wiznitzer, I.; Neumann, T. A.; Hill, L. R.; Liang, B. C., Blinded, randomized, multicenter study to evaluate single administration pegfilgrastim once per cycle versus daily filgrastim as an adjunct to chemotherapy in patiends with high-risk stage II or stage III/IV breast cancer. *J. Clin. Oncol.* **2002**, *20*, 727-731.
(173) Holmes, F. A.; Jones, S. E.; O'Shaughnessy, J. A.; Vukelja, S.; George, T.; Savin, M.; Richards, D.; Glaspy, J.; Meza, L.; Cohen, G.; Dhami, M.; Budman, D. R.; Hackett, J.; Brassard, M.; Yang, B.-B.; Liang, B. C., Comparable efficacy and safety profiles of once-per-cycle pegfilgrastim and daily injection filgrastim in chemotherapy-induced neutropenia: a multicenter dose-finding study in women with breast cancer. *Ann. Oncol.* **2002**, *13*, 903-909.
(174) Harbeck, N.; Lipatov, O.; Frolova, M.; Udovitsa, D.; Topuzov, E.; Ganea-Motan, D. E.; Nakov, R.; Singh, P.; Rudy, A.; Blackwell, K., Randomized, double-blind study comparing proposed biosimilar LA-EP2006 with reference pegfilgrastim in breast cancer. *Future Oncol.* **2016**, *12*, 1359-1367.
(175) Yang, Z.; Zhang, L.; Zhang, Y.; Zhang, T.; Feng, Y.; Lu, X.; Lan, W.; Wang, J.; Wu, H.; Cao, C.; Wang, X., Highly efficient production of souble proteins from insoluble inclusion bodies by a two-step-denaturing and refolding method. *PLoS ONE* **2011**, *6*, e22981.
(176) Do, B. H.; Ryu, H.-B.; Hoang, P.; Koo, B.-K.; Choe, H., Soluble prokaryotic overexpression and purification of bioactive human granulocyte colony-stimulating factor by maltose binding protein and protein disulfide isomerase. *PLoS ONE* **2014**, *9*, e89906.
(177) Jin, H.; Cantin, G. T.; Maki, S.; Chew, L. C.; Resnick, S. M.; Ngai, J.; Retallack, D. M., Soluble periplasmic production of human granulocyte colony-stimulating factor (G-CSF) in *Pseudomonas fluorescens*. *Protein Expr. Purif.* **2011**, *78*, 69-77.
(178) Oh-eda, M.; Hasegawa, M.; Hattori, K.; Kuboniwa, H.; Kojima, T.; Orita, T.; Tomonou, K.; Yamazaki, T.; Ochi, N., *O*-linked sugar chain of human granulocyte colony-stimulating factor protects it against polymerization and denaturation allowng it to retain its biological activity. *J. Biol. Chem.* **1990**, *265*, 11432-11435.
(179) Duplay, P.; Bedouelle, H.; Fowler, A.; Zabin, I.; Saurin, W.; Hognung, M., Sequences of the *malE* gene and of its product, the maltose-binding protein of *Escherichia coli* K12. *J. Biol. Chem.* **1984**, *259*, 10606-10613.
(180) Kapust, R. B.; Waugh, D. S., *Escherichia coli* maltose-binding protein is uncommonly effective at promoting the solubility of polypeptides to which it is fused. *Protein Sci.* **1999**, *8*, 1668-1674.

(181) Sachdev, D.; Chirgwin, J. M., Solubility of proteins isolated from inclusion bodies is enhanced by fusion to maltose-binding protein or thioredoxin. *Protein Expr. Purif.* **1998**, *12*, 122-132.
(182) Bach, H.; Mazor, Y.; Shaky, S.; Shoham-Lev, A.; Berdichevsky, Y.; Gutnick, D. L.; Benhar, I., *Escherichia coli* maltose-binding protein as a molecular chaperone for recombinant intracellular cytoplasmic single-chain antibodies. *J. Mol. Biol.* **2001**, *312*, 79-93.
(183) Kim, C. K.; Lee, C. H.; Lee, S.-B.; Oh, J.-W., Simplified large-scale refolding, purification, and characterization of recombinant human granulocyte-colony stimulating factor in *Escherichia coli*. *PLoS One* **2013**, *8*, e80109.
(184) Singh, S. M.; Panda, A. K., Solubilization and refolding of bacterial inclusion body proteins. *J. Biosci. Bioeng.* **2005**, *99*, 303-310.
(185) Shahravan, S. H.; Qu, X.; Chan, I.-S.; Shin, J. A., Enhancing the specificity of the enterokinase cleavage reaction to promote efficient cleavage of a fusion tag. *Protein Expr. Purif.* **2008**, *59*, 314-319.
(186) Corrigan, T., Hunt for a genetically engineered, rationally designed, stealth peptide to prevent non-specific protein interactions, University of Washington, 2018.
(187) Pace, C. N.; Scholtz, J. M., A helix propensity scale based on experimental studies of peptides and proteins. *Biophys. J.* **1998**, *75*, 422-427.
(188) Zhang, Y.; Huo, M.; Zhou, J.; Xie, S., PKSolver: an add-in program for pharmacokinetic and pharmacodynamic data analysis in Microsoft Excel. *Comput. Methods Programs Biomed.* **2010**, *99*, 306-314.
(189) Broyer, R. M.; Grover, G. N.; Maynard, H. D., Emerging synthetic approaches for protein-polymer conjugations. *Chem. Commun.* **2011**, *47*, 2212-2226.
(190) Hoffman, A. S., Bioconjugates of intelligent polymers and recognition proteins for use in diagnostics and affinity separations. *Clinical Chem.* **2000**, *46*, 1478-1486.
(191) Imran ul-haq, M.; Lai, B. F. L.; Chapanian, R.; Kizhakkedathu, J. N., Influence of architecture of high molecular weight linear and branched polyglycerols on their biocompatability and biodistribution. *Biomaterials* **2012**, *33*, 9135-9147.
(192) Oh, E. J.; Kim, J.-W.; Kong, J.-H.; Ryu, S. H.; Hahn, S. K., Signal transduction of hyaluronic acid-peptide conjugate for formyl peptide receptor like 1 receptor. *Bioconjug. Chem.* **2008**, *19*, 2401-2408.
(193) Chen, X.; Henschke, L.; Wu, Q.; Muthoosamy, K.; Neumann, B.; Weil, T., Site-selective azise incorporation into endogenous RNase A via a "chemistry" approach. *Org. Biomol. Chem.* **2013**, *11*, 353-361.
(194) Chen, D.; Disotuar, M.; Xiong, X.; Wang, Y.; Chou, D. H.-C., Selective N-terminal functionalization of native peptides and proteins. *Chem. Sci.* **2017**, *8*, 2717-2722.
(195) Jain, N.; Smith, S. W.; Ghone, S.; Tomczuk, B., Current ACD linker chemistry. *Pharm. Res.* **2015**, *32*, 3526-3540.
(196) Dimasi, N.; Fleming, R.; Zhong, H.; Bezabeh, B.; Kinneer, K.; Christine, R. J.; Fazenbaker, C.; Wu, H.; Gao, C., Efficient preparation of site-specific antibody-drug conjugates using cysteine insertion. *Mol. Pharm.* **2017**, *14*, 1501-1516.
(197) Stimmel, J. B.; Merrill, B. M.; Kuyper, L. F.; Moxham, C. P.; Hutchins, J. T.; Fling, M. E.; Kull, F. C., Jr., Site-specific conjugation on serine → cysteine variant monoclonal antibodies. *J. Biol. Chem.* **2000**, *275*, 30445-30450.

(198) Gentle, I. E.; De Souza, D. P.; Baca, M., Direct production of proteins with N-terminal cysteine for site-specific conjugation. *Bioconjugate Chem.* **2004**, *15*, 658-663.
(199) Qiu, H.; Honey, D. M.; Kingsbury, J. S.; Park, A.; Boudanova, E.; Wei, R. R.; Pan, C. Q.; Edmonds, T., Impact of cysteine variants on the structure, activity, and stability of recombinant human α-galactosidase A. *Protein Sci.* **2015**, *24*, 1401-1411.
(200) Carrico, I. S.; Carlson, B. L.; Bertozzi, C. R., Introducing genetically encoded andehydes into proteins. *Nature Chem. Biol.* **2007**, *3*, 321-322.
(201) Liu, C. C.; Schultz, P. G., Adding New Chemistries to the Genetic Code. *Annu. Rev. biochem.* **2010**, *79*, 413-444.
(202) Xu, M.-Q.; Evans, T. C., Jr., Intein-mediated ligation and cyclization of expressed proteins. *Methods* **2001**, *24*, 257-277.
(203) Tanaka, T.; Kamiya, N.; Nagamune, T., N-terminal glycine-specific protein conjugation catalyzed by microbial transglutaminase. *FEBS Letters* **2005**, *579*, 2092-2096.
(204) Chen, X.; Wu, Y.-W., Selective chemical labeling of proteins. *Org. Biomol. Chem.* **2016**, *14*, 5407-5439.
(205) van Hest, J. C. M.; Tirrell, D. A., Protein-based materials, toward a new level of structural control. *Chem. Commun.* **2001**, *0*, 1897-1904.
(206) Yuen, K. C. J.; Conway, G. S.; Popovic, V.; Merriam, G. R.; Bailey, T.; Hamrahian, A. H.; Biller, B. M. K.; Kipnes, M.; Moore, J. A.; Humphriss, E.; Bright, G. M.; Cleland, J. L., A long-acting human growth hormone with delayed clearance (VRS-317): results of a double-blind, placebo-controlled, single ascending dose study in growth hormone-deficient adults. *J. Clin. Endocrinol. Metlab* **2013**, *98*, 2595-2603.
(207) Veronese, F. M.; Pasut, G., PEGylation, successful approach to drug delivery. *Drug Discov. Today* **2005**, *10*, 1451-1458.
(208) Natalello, A.; Ami, D.; Collini, M.; D'Alfonso, L.; Chirico, G.; Tonon, G.; Scaramuzza, S.; Schrepfer, R.; Doglia, S. M., Biophysical characterization of Met-G-CSF: effects of different site-specific mono-pegylations on protein stability and aggregation. *PLoS ONE* **2012**, *7*, e42511.
(209) Amblard, M.; Fehrentz, J.-A.; Martinez, J.; Subra, G., Methods and protocols of modern solid phase peptide synthesis. *Mol. Biotech.* **2006**, *33*, 239-254.
(210) Malakhov, M. P.; Mattern, M. R.; Malakhova, O. A.; Drinker, M.; Weeks, S. D.; Butt, T. R., SUMO fusions and SUMO-specific protease for efficient expression and purification of proteins. *J. Struct. Funct. Genomics* **2004**, *5*, 75-86.
(211) Kamionka, M., Engineering of therapeutic proteins production in *Escherichia coli*. *Curr. Pharm. Biotechnol.* **2011**, *12*, 268-274.
(212) Wang, Z.; Li, H.; Guan, W.; Ling, H.; Wang, Z.; Mu, T.; Schuler, F. D.; Fang, X., Human SUMO fusion systems enhance protein expression and solubility. *Protein Expr. Purif.* **2010**, *73*, 203-208.
(213) Sørensen, H. P.; Mortensen, K. K., Soluble experssion of recombinant proteins in the cytoplasm of *Escherichia coli*. *Microb. Cell Fact.* **2005**, *4*, 1.
(214) Marblestone, J. G.; Edavettal, S. C.; Lim, Y.; Lim, P.; Zuo, X.; Butt, T. R., Comparison of SUMO fusion technology with tradition gene fusion systems: Enhanced epression and solubility with SUMO. *Protein Sci.* **2005**, *15*, 182-189.
(215) Zuo, X.; Li, S.; John, H.; Mattern, M. R.; Tran, H.; Shoo, J.; Tan, R.; Weiss, S. R.; Butt, T. R., Enhanced expression and purification of membrane proteins by SUMO fusion in *Escherichia coli*. *J. Struct. Funct. Genomics* **2005**, *6*, 103-111.

Appendix A

Author qualifications

A.1 Education

University of Washington — Seattle, WA
Doctor of Philosophy, Chemical Engineering — June 2019 (anticipated)

University of Washington — Seattle, WA
Master of Science, Chemical Engineering — June 2015

California Institute of Technology — Pasadena, CA
Bachelor of Science, Chemical Engineering — June 2013

A.2 Publications

Galvan, D. D.; Liu, E. J.; Sun, F.; Hou, C.; Parekh, V.; Wu, P.; Yu, Q., Enrichment and characterization of bacteria with combined dielectrophoresis and surface-enhanced Raman scattering in a microfluidic system. *Anal. Chem.* **2019**, *In Revision*.

Zhang, P.; Liu, E. J.; Tsao, C.; Chen, Y.; Kasten, S. A.; Otto, R.; Cerasoli, D. M.; Jain, P.; Sun, F.; Li, W.; Hung, H.; Yuan, Z.; Ma, J.; Corrigan, T.; Bigley, A. N.; Rauschel, F. M.; Jiang, S., Nanoscavenger provides long-term prophylactic protection against nerve agents. *Sci. Transl. Med.* **2019**, *11*, eaau7091.

Galvan, D. D.; Parekh. V.; Liu, E. J.; Liu, E.; Yu, Q., Sensitive bacterial detection via dielectrophoretic enhanced mass transport using surface plasmon resonance biosensors. *Anal. Chem.* **2018**, *90*, 14635-14642.

Liu, E. J.; Jiang, S., Expressing a monomeric organophosphate hydrolase as an EK fusion protein. *Bioconjugate Chem.* **2018**, *29*, 3686-3690.

Hung, H.; Jain, P.; Zhang, P.; Sun, F.; Sinclair, A.; Bai, T.; Li, B.; Wu, K.; Tsao, C.; Liu, E. J.; Sundaram, H. S.; Lin, X.; Farahani, P.; Fujihara, T.; Jiang, S., A coating-free nonfouling polymeric elastomer. *Adv. Mater.* **2017**, *29*, 1700617.

Liu, E. J.; Sinclair, A.; Keefe, A. J.; Nannenga, B. L.; Coyle, B. L.; Baneyx, F.; Jiang, S., EKylation: addition of an alternating-charge peptide stabilizes proteins. *Biomacromolecules* **2015**, *16*, 3357-3361.

A.3 Invited talks

Liu, E. J.; Sinclair, A.; Jiang, S., Using poly(EK) fusion proteins to enhance protein stability. American Institute of Chemical Engineers Annual Conference, San Francisco, CA, USA. 18 November 2016.

A.4 Poster presentations

Liu, E. J.; Jiang, S., Expression of EK fusion proteins to enhance protein kinetics and stability. 3rd International Conference on Bioinspired and Zwitterionic Materials, Tokyo, Japan. 18-20 October 2017.

Liu, E. J.; Sinclair, A.; Jiang, S., The addition of poly(EK) to protein drugs to enhance protein stability. 10th World Biomaterials Congress, Montréal, Canada. 19 May 2016.

A.5 Honors and awards

Biomaterials Science poster award, 3rd International Conference and Bioinspired and Zwitterionic Materials, Tokyo, Japan. 18-20 October 2017.

A.6 Outreach

Moderator, National Ocean Sciences Bowl 2009-present