Characterization of Multi-Albumin Pegylated Complexes Synthesized Using "Click" Chemistry as Drug Delivery Systems

Jonathan Alejandro Hill
Loyola University Chicago, jahill5@hotmail.com

Recommended Citation
https://ecommons.luc.edu/luc_diss/2813

This Dissertation is brought to you for free and open access by the Theses and Dissertations at Loyola eCommons. It has been accepted for inclusion in Dissertations by an authorized administrator of Loyola eCommons. For more information, please contact ecommons@luc.edu.
Creative Commons License
This work is licensed under a Creative Commons Attribution-Noncommercial-No Derivative Works 3.0 License.
Copyright © 2017 Jonathan Alejandro Hill
LOYOLA UNIVERSITY CHICAGO

CHARACTERIZATION OF
MULTI-ALBUMIN PEGYLATED COMPLEXES
SYNTHESIZED USING “CLICK” CHEMISTRY
AS DRUG DELIVERY SYSTEMS

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM IN CHEMISTRY AND BIOCHEMISTRY

BY

JONATHAN A. HILL

CHICAGO, IL

AUGUST 2017
ACKNOWLEDGEMENTS

To the following people, I express my undying thanks and gratitude for their guidance, patience, support, and assistance throughout this process.

Thank you, Dr. Kenneth Olsen, for everything you have done to help me grow as a student, as a researcher, and as a person.

To my committee, Drs. Miguel Ballicora, Dali Liu, and Yi Gao, thank you for your advice and assistance along the way.

Without the help of Jennifer Hendrickson, Matthew Rhodes, Dzenita Huskic, Jacob Parker, and Ross Carpino, none of this would have been possible.

To my wife, my parents, my siblings, and my friends, thank you for your constant encouragement and support in everything I do.
To my wife, Alisa, for all of her love and support.
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................................................................................ iii

LIST OF TABLES .................................................................................................................... viii

LIST OF FIGURES ................................................................................................................... ix

LIST OF ABBREVIATIONS ....................................................................................................... xi

CHAPTER I: INTRODUCTION .................................................................................................. 1

Albumin-Based Nanoparticles ............................................................................................... 1

nab™ Technology and Abraxane® ......................................................................................... 3

Albumin-Drug Conjugates ...................................................................................................... 5

Serum Albumin ........................................................................................................................ 6

Physiology ................................................................................................................................ 6

Clinical Applications ............................................................................................................... 7

Structural Basis for Ligand Binding ......................................................................................... 8

Binding sites ............................................................................................................................. 9

Fatty acid sites ......................................................................................................................... 10

Cys34 ..................................................................................................................................... 11

The N-terminus and the Multi-metal binding site .................................................................. 11

Drug Site 2 (DS2) ................................................................................................................... 11

Drug Site 1 (DS1) ................................................................................................................... 14

Albumin Metabolism .............................................................................................................. 15

Human vs. Bovine Serum Albumin ......................................................................................... 17

Amino Acid Correlation .......................................................................................................... 18

Structural Homology of DS1 and DS2 .................................................................................... 19

Polyethylene Glycol (PEG) ..................................................................................................... 20

PEG-Protein Nanoparticles ...................................................................................................... 21

PEG Metabolism ..................................................................................................................... 23

Toxicity .................................................................................................................................... 23

Advantages of PEGylation ...................................................................................................... 24

“Click” Chemistry .................................................................................................................. 26

Azide-Alkyne Cycloaddition (AAC) ....................................................................................... 27

Cu(I)-catalyzed AAC (CuAAC) ............................................................................................... 27

Strain-promoted AAC (SPAAC) ............................................................................................. 28

Thiol-ene Reactions ................................................................................................................. 29

Summary .................................................................................................................................. 30

CHAPTER II: STATEMENT OF RESEARCH ........................................................................ 32

CHAPTER III: MODIFICATION OF BOVINE SERUM ALBUMIN WITH MAL-PEG₈ AS
A DRUG DELIVERY SYSTEM .............................................................................................. 35

Introduction ............................................................................................................................. 35
**LIST OF TABLES**

Table 1. Contribution of the Major Classes of Serum Proteins to Osmotic Pressure. 7

Table 2. Example Ligands for Drug Site I and Drug Site II. 13

Table 3. Current Market-Available PEGylated Protein Products. 22

Table 4. Association Constants ($K_a$) and Number of Binding Sites (n) for BSA$_x$-te-PEG$_8$ for Sulfamethoxazole and Naproxen. 50

Table 5. Nomenclature for the Conjugation of BSA onto PEG$_8$ via Strain-Promoted Azide-Alkyne Cycloaddition. 63

Table 6. Association Constants ($K_a$) and Number of Binding Sites (n) for BSA$_x$-123t-PEG$_8$ and BSA$_x$-PEG$_4$-123t-PEG$_8$ for Sulfamethoxazole and Naproxen. 78

Table 7. Nomenclature for PEGylation of Bovine Serum Albumin via Thiol-Maleimide and Strain-Promoted Azide-Alkyne Cycloaddition Reactions. 89

Table 8. Temperatures of Denaturation and Aggregation for PEGylated BSA Species. 94
LIST OF FIGURES

Figure 1. Crystal structure of human serum albumin. 9
Figure 2. A selection of albumin binding sites. 10
Figure 3. Structure of Drug Site II in human serum albumin. 12
Figure 4. Structure of Drug Site I in human serum albumin. 14
Figure 5. Comparison of Drug Site II between human and bovine serum albumin. 19
Figure 6. Comparison of Drug Site I between human and bovine serum albumin. 20
Figure 7. Diagram of a PEGylated protein nanoparticle. 25
Figure 8. Cu(I)-catalyzed azide-alkyne cycloaddition. 27
Figure 9. Strain-promoted azide-alkyne cycloaddition. 29
Figure 10. Thiol-ene reaction. 30
Figure 11. Cys34, Drug Site I, and Drug Site II of bovine serum albumin. 37
Figure 12. Diagram of BSA$_x$-te-PEG$_8$. 38
Figure 13. Thiol-maleimide conjugation of bovine serum albumin onto PEG$_8$. 39
Figure 14. Purification of thiol-maleimide PEGylated BSA via size exclusion chromatography. 44
Figure 15. Circular dichroic spectra of FAF-BSA and BSA$_x$-te-PEG$_8$. 45
Figure 16. Electrophoretic separation of species of bovine serum albumin PEGylated via thiol-maleimide click chemistry. 46
Figure 17. Analytical ultracentrifugation analysis of BSA$_x$-te-PEG$_8$. 48
Figure 18. Fluorimetric spectra and non-linear regression of BSA$_x$-te-PEG$_8$ in the presence of naproxen. 51
Figure 19. Fluorimetric spectra and non-linear regression of BSA$_x$-te-PEG$_8$ in the presence of sulfamethoxazole.

Figure 20. Diagram of BSA$_x$-R-123t-PEG$_8$.

Figure 21. Conjugation of bovine serum albumin onto PEG$_8$ via strain-promoted azide-alkyne cycloaddition.

Figure 22. Optimization of the PEGylation procedure for DIBO-activated FAF-BSA.

Figure 23. Circular dichroic spectra of FAF-BSA and strain-promoted azide-alkyne cycloaddition PEGylated products.

Figure 24. Purification of BSA$_x$-R-123t-PEG$_8$ via size exclusion chromatography.

Figure 25. Electrophoretic separation of species of bovine serum albumin PEGylated via strain-promoted azide-alkyne click chemistry.

Figure 26. Analytical ultracentrifugation analysis of BSA$_x$-123t-PEG$_8$.

Figure 27. Analytical ultracentrifugation analysis of BSA$_x$-PEG$_4$-123t-PEG$_8$.

Figure 28. Fluorimetric spectra and non-linear regression of BSA$_x$-123t-PEG$_8$ and BSA$_x$-PEG$_4$-123t-PEG$_8$ in the presence of naproxen.

Figure 29. Fluorimetric spectra and non-linear regression of BSA$_x$-123t-PEG$_8$ and BSA$_x$-PEG$_4$-123t-PEG$_8$ in the presence of sulfamethoxazole.

Figure 30. Change in absorbance as a function of temperature for the determination of $T_{agg}$.

Figure 31. Temperature-induced changes in circular dichroic spectra.

Figure 32. Change in helicity as a function of temperature for the determination of $T_m$.

Figure 33. Helicity after pasteurization in the absence and presence of naproxen.

Figure 34. Non-linear regression analyses of fluorimetric assays of PEGylated bovine serum albumin before and after pasteurization.
LIST OF ABBREVIATIONS

123t - 1,2,3-triazole
AAC - Azide-alkyne cycloaddition
AUC - Analytical ultracentrifugation
βME - β-mercaptoethanol
BSA - Bovine serum albumin
BSAₙ-123t-PEG₈ - Multiple BSA attached to PEG₈ via 123t linkage
BSAₙ-PEG₄-123t-PEG₈ - Multiple BSA attached to PEG₈ via 123t linkage with PEG₄ spacer
BSAₙ-R-123t-PEG₈ - Multiple BSA attached to PEG₈ via 123t linkage with an unspecified spacer
BSAₙ-R-PEG₈ - Multiple BSA attached to PEG₈ via an unspecified linkage
BSAₙ-te-PEG₈ - Multiple BSA attached to PEG₈ via thioether linkage
CD - Circular dichroism
CrEL - Cremophor® EL
CuAAC - Cu(I)-catalyzed azide-alkyne cycloaddition
DDS - Drug delivery system
DIBO-BSA - BSA activated by DIBO-NHS
DIBO-NHS - Dibenzocyclooctyne-N-hydroxysuccinimidy l ester with no additional PEG spacer
DIBO-PEG₄-BSA - BSA activated by DIBO-PEG₄-NHS
DIBO-PEG₄-NHS - Dibenzocyclooctyne-PEG₄-N-hydroxysuccinimidy l ester
<p>| <strong>DIBO-R-BSA</strong> | - BSA activated by DIBO-R-NHS |
| <strong>DIBO-R-NHS</strong> | - Dibenzocyclooctyne-N-hydroxysuccinimidyl ester with an unspecified spacer |
| <strong>DS1</strong> | - Drug Site I; Sudlow's Site I |
| <strong>DS2</strong> | - Drug Site II; Sudlow's Site II |
| <strong>FA5</strong> | - Fatty acid binding site 5 |
| <strong>FAF-BSA</strong> | - Fatty acid-free BSA |
| <strong>FcRn</strong> | - Neonatal Fc receptor |
| <strong>Fluor-NHS</strong> | - Fluorescein-5-EX N-hydroxysuccinimide ester |
| <strong>FPLC</strong> | - Fast protein liquid chromatography |
| <strong>FrInt</strong> | - Fractional intensity |
| <strong>GdmCl</strong> | - Guanidinium chloride |
| <strong>gp18</strong> | - 18 kDa glycoprotein |
| <strong>gp30</strong> | - 30 kDa glycoprotein |
| <strong>gp60</strong> | - Albondin; 60 kDa glycoprotein |
| <strong>HSA</strong> | - Human serum albumin |
| <strong>IgG</strong> | - Immunoglobulin G |
| <strong>K_a</strong> | - Association constant |
| <strong>Mal-PEG₈</strong> | - Maleimido-functionalized PEG₈ |
| <strong>MOPS/NaCl</strong> | - 50 mM 3-(N-Morpholino)propanesulfonic acid /0.15 M NaCl pH 7.2 buffer |
| <strong>n</strong> | - Number of binding sites |
| <strong>N₃-PEG₈</strong> | - Azido-functionalized PEG₈ |
| <strong>nab™</strong> | - nanoparticle albumin-based |</p>
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAT</td>
<td>N-acetyl-L-tryptophan</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimidyl ester</td>
</tr>
<tr>
<td>NP</td>
<td>Nanoparticle</td>
</tr>
<tr>
<td>NPX</td>
<td>Naproxen</td>
</tr>
<tr>
<td>NTS</td>
<td>N-terminus</td>
</tr>
<tr>
<td>PC-DAC™</td>
<td>Preformed Conjugate-Drug Affinity Complex</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PEG₉(k)</td>
<td>(k)Da polyethylene glycol</td>
</tr>
<tr>
<td>PEG₈</td>
<td>8-armed polyethylene glycol</td>
</tr>
<tr>
<td>PTX</td>
<td>Paclitaxel</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEC</td>
<td>Size exclusion chromatography</td>
</tr>
<tr>
<td>SMZ</td>
<td>Sulfamethoxazole</td>
</tr>
<tr>
<td>SPAAC</td>
<td>Strain-promoted azide-alkyne cycloaddition</td>
</tr>
<tr>
<td>T_{agg}</td>
<td>Temperature of aggregation</td>
</tr>
<tr>
<td>te</td>
<td>Thioether</td>
</tr>
<tr>
<td>T_{m}</td>
<td>Temperature of denaturation</td>
</tr>
</tbody>
</table>
CHAPTER I
INTRODUCTION
Since the inception of nanotechnology in the 1980s, nanoparticles (NP) have been explored as more efficient and less toxic drug delivery systems (DDS). Many of these approaches have used organic polymers as their core structures such as cyclodextrin nanosponges (Torne, Darandale, Vavia, Trotta & Cavalli, 2013) and biodegradable polymers (Vila, Sánchez, Tobío, Calvo & Alonso, 2002) while others synthesized homologs of naturally occurring molecules such as liposomes (Batist et al., 2001) and self-assembling peptides (Boopathy & Davis, 2014). Another group of NPs have used protein cores around which to build their DDSs. Perhaps the most intriguing are albumin-based NPs.

Albumin provides an excellent core around which to design a DDS. It is biodegradable, nontoxic, and easily customizable due to a large number of accessible functional groups on its surface. More importantly, albumin is a natural mode of transport for both endogenous and exogenous hydrophobic molecules (Elsadek & Kratz, 2012; Elzoghby, Samy & Elgindy, 2012; Kratz, 2008; Peters, 1996).

**Albumin-Based Nanoparticles**
Several approaches have successfully produced nano-scale albumin-based DDSs. Discussed below are just a few to demonstrate the breadth of methodologies developed.

Desolvation, developed by the Langer group, uses organic solvents such as ethanol and acetone to dehydrate drug-loaded albumins. Sedimented protein is polymerized via a cross-
linking agent such as glutaraldehyde to provide stability before re-hydration (Langer et al., 2008; Weber, Coester, Kreuter & Langer, 2000). Human serum albumin (HSA) NPs were prepared using desolvation techniques and loaded with the chemotherapeutic doxorubicin. The NPs were further modified by the addition of trastuzumab as a targeting mechanism. Targeted NPs showed a cell viability of 20.1%, suggesting successful uptake and drug release (Anhorn, Wagner, Kreuter, Langer & von Briesen, 2008).

Spray-drying is accomplished by passing a protein solution through a wire mesh at elevated temperatures to generate a powder. Smooth, spherical bovine serum albumin (BSA) NPs were successfully prepared at temperatures between 80-120°C in the presence of Tween®-80. The morphology of the NPs was dependent on the surfactant content. Tween-80, often used as an excipient in protein formulations, produced the most homogenous and spherical NPs. NP size was most dependent on mesh size. The particle size increased as mesh size increased. A 4.0 µm mesh generated NPs 733 nm in diameter while those from a 7.0 µm mesh were 2609 nm (Lee, Heng, Ng, Chan & Tan, 2011).

Thermal gelation can be viewed as a two-phase process. Albumins are thermally denatured and then non-covalently linked via electrostatic and hydrophobic/hydrophilic interactions (Boye, Alli & Ismail, 1996). This process was successfully applied to the loading of BSA-dextran and BSA-dextran-chitosan NPs with doxorubicin. Covalent BSA-dextran conjugates were held at 80°C in the presence of chitosan. The resulting NPs consisted of chitosan and dextran arms stretching out from an albumin core. Administration of doxorubicin-loaded NPs to cancerous mice showed an increased survivability rate as compared to free doxorubicin indicating an increased cell permeability for the conjugate (Qi, Yao, He, Yu & Huang, 2010). Each of these systems has a distinct advantage in that they each provide a degree of fine control
over the final particle size via alteration of easily monitored parameters (pH, reaction time, solvent content, reagent concentration, equipment used).

The drawbacks of each approach provide cause for concern. Reliance on organic solvents and surfactants in the production of materials to be administered to living beings must be minimized to avoid toxicity. Thermal denaturation and a highly-altered surface from extensive cross-linking may elicit an immune response as well as potentially alter biological activity. Necessarily high temperatures place limitations on potential drug candidates.

*nab™ Technology and Abraxane®*

Abraxane®, a paclitaxel (PTX) formulation, is the most successful albumin-based NPs to date. Approved in 2005 by the US Food and Drug Administration for the treatment of metastatic breast cancer, it is recognized as the first protein-based NP on the market. American Biosciences, Inc., now Celgene Corporation, developed a method for producing nanoparticle albumin-based (*nab™*) carriers of which Abraxane was the first application (Desai, 2008).

The passage of an aqueous stream of HSA and hydrophobic drug through a high-pressure jet forms the *nab* NP. Albumins adsorb to one another to which drugs preferentially bind. Upon administration, the 130-nm shell dissociates (Elsadek & Kratz, 2012; Elzoghby et al., 2012; Kratz, 2008).

Abraxane is the trade name for the complexation of PTX and *nab* albumin. This product was developed as a response to the problems associated with Taxol®, the original formulation for stand-alone PTX. In the absence of *nab*, PTX requires Cremophor® EL (CrEL), a poly-ethoxylated castor oil, for solubilization. This necessitates infusion times of up to three hours and pre-medication with corticosteroids and antihistamines to reduce the chance of hypersensitivity reactions. Additionally, PTX delivery was hindered by micellar CrEL formation. Upon
administration of Taxol, CrEL aggregates, creating a hydrophobic environment into which PTX readily diffuses. CrEL toxicity can be quite severe as it presents as neutropenia and sometimes permanent neuropathy (Hawkins, Soon-Shiong & Desai, 2008).

The much simpler formulation of Abraxane, consisting of only HSA and PTX, allows for shorter infusion times (30 minutes), no pre-medicating, a reduced risk of hypersensitivity reactions, and no toxicity (Hawkins et al., 2008). Studies have reported 33% increase of intracellular PTX concentrations with Abraxane (Gradishar et al., 2005). Dissociation of Abraxane upon administration results in accumulation of albumins into tumor cells via both the passive targeting phenomenon of the enhanced permeation and retention effect and active targeting via albondin (gp60) endocytosis. It was determined that cancerous cells are able to acquire up to 17% of a single dose of $^{111}$In-labeled albumin, indicating a degree of preference by tumors for albumin (Stehle et al., 1999). By combining albumin’s two-fold tumor targeting with the ability of Abraxane to house multiple PTX, an increased drug concentration was unsurprising.

This same housing ability allows for Abraxane to be administered at a 50% higher dose than Taxol. PTX introduced into the bloodstream via the Taxol is unbound and free to interact with both cancerous and healthy tissues. nab technology provides a means of sequestering the chemotherapeutic until it is inside its intended target. This sequestration results in decreased toxicity, higher dosage rates, and a higher therapeutic window for Abraxane.

The wider application of nab technology is questionable. It has proven to be an effective chemotherapy due to its high drug load, solubility, and improved administration. The gp60 targeting mechanism is not universal for all drugs and the dissolution of Abraxane would require a targeting mechanism for each individual protein to be as effective for non-chemotherapeutics.
Additionally, dissolution of the *nab* NPs in the bloodstream results in the infusion of a large quantity of individual albumin molecules which may greatly impact the osmotic pressure.

**Albumin-Drug Conjugates**

The techniques described result in polymerized albumins which provide a framework to which the drug is non-covalently attached. Albumin-drug conjugates remain monomeric and provide a means of solubilizing and increasing circulation time for bound ligands. Two approaches have been employed to produce these conjugates. In the first, a drug is covalently attached to albumin via a surface residue. In the second, the drug is covalently attached to a ligand of albumin. Upon administration of this complex, the ligand binds to a high affinity site of endogenous albumin, effectively non-covalently binding the drug.

The first albumin-drug conjugate to reach phase I/II clinical studies was a complex with the antimitotic cancer drug methotrexate. Similar to PTX, methotrexate suffered from poor solubility and severe toxicity associated with the drug itself and components of the formulation. For the conjugate, methotrexate was covalently attached to HSA via lysine residues (Stehle, Sinn, Wunder, Schrenk, Schütt et al., 1997). Clinical results for this complex were somewhat promising in that three out of 17 patients showed a positive response to therapy. Other factors, such as stomatitis, limited the dosage rates, and potentially limited the efficacy of treatment (Kratz, 2008).

Long-lasting insulin (Levemir®) for the treatment of both type 1 and type 2 diabetes is a prime example of the second type of albumin-drug conjugate. Novo Nordisk, Inc. developed a method for producing recombinant human insulin in which the C-terminus had been shortened by elimination of the B-chain terminal threonine. This excision allowed access to an adjacent lysine which was covalently attached to myristic acid. Upon administration, the insulin-myristic
acid conjugate binds to albumin, resulting in an increase in circulatory half-life from four to six minutes for native recombinant insulin to five to seven hours for the conjugate (Elsadek & Kratz, 2012; Keating, 2012).

Albumin-drug conjugates are attractive in their reasonably simple production protocols, requiring no modifications of the protein outside of the attachment of the drug. While attachment to the albumin provides an answer for solubilization, it does not necessarily protect the ligand from the host. In the event of an immune response, the drug is susceptible to attack from immunogenic agents which could result in early elimination, degradation of the therapeutic, or dissection of the conjugate. If the drug were to be cleaved from the albumin before it reaches the site of action, a potentially toxic molecule would be released into the bloodstream to interact with healthy tissues.

**Serum Albumin**

**Physiology**

The robust nature of albumin, a highly-conserved protein found in the serum of all mammals, has singled it out as a potential DDS component. It is a 66 kDa monomer produced by the liver in quantities up to 12 g/day with a circulatory half-life of 20 days. Albumin can be found in high quantities in skin, muscle, liver, and the gut as well as almost all bodily fluids (Lundblad, 2012; Peters, 1996). Its high production rate and long half-life make albumin the most abundant protein in the serum.

Albumin is able to play a diverse set of roles in the body. Arguably its most important function, albumin is the body’s main control on osmotic pressure (Table 1). Determined by the number of particles in a system, not their size, osmotic pressure is the driving force between
tissues and blood. Despite albumin being many times smaller than fibrinogen, it is found at such a high concentration that it accounts for 79% of the osmotic pressure (Guyton & Hall, 2006).

Table 1. Contribution of the Major Classes of Serum Proteins to Osmotic Pressure (Guyton & Hall, 2006).

<table>
<thead>
<tr>
<th>Protein Class</th>
<th>Average MW (kDa)</th>
<th>Serum Concentration (mM)</th>
<th>Osmotic Pressure (mm Hg)</th>
<th>% Total Osmotic Pressure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>66</td>
<td>0.68</td>
<td>21.8</td>
<td>79</td>
</tr>
<tr>
<td>Globulins</td>
<td>140</td>
<td>0.17</td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>400</td>
<td>0.0075</td>
<td>0.2</td>
<td>1</td>
</tr>
</tbody>
</table>

This dependence on albumin as an osmotic regulator must be taken into account when designing a DDS. A drastic increase in albumin concentration associated with a high dose of albumin-bound drug would be accompanied by a drastic increase in osmotic pressure. This spike would cause down-regulation of albumin gene-expression to compensate (Pietrangelo, Panduro, Chowdhury & Shafritz, 1992). Decreased levels of native, unmodified albumin may lead to a build-up of fatty acids, heavy metals, drugs, and their metabolites.

**Clinical Applications**

Albumin was first explored as a therapeutic agent during World War II. Albumin solutions were administered as plasma expanders to wounded soldiers to minimize the effects of blood loss on the battlefield. The first purification method involved multiple rounds of low-temperature precipitations with ethanol to separate the protein components of blood. Albumin was the fifth precipitate in the process and was originally deemed “Cohn Fraction V” (Cohn et al., 1946; Peters, 1996). The peptide sequence of BSA was first published by Brown (1975) while that of HSA was later independently reported by the Brown and Kostka labs (Behrens, Spiekerman & Brown, 1975; Meloun, Morávek & Kostka, 1975). Since these initial works,
albumin has become a staple of hospitals and clinics. Unmodified albumin is still used as a plasma expander to aid in post-operative recovery and for treatment of shock and burns. It is also used to treat patients suffering from hypoalbuminemia, specifically those with acute liver failure. In these cases, the liver is unable to produce the requisite amount of albumin and the body requires supplementation to maintain normal functionality (Lundblad, 2012). It has also been developed as a protein-based resin for sealing wounds by CryoLife, Inc. BioGlue® contains compartments of aqueous glutaraldehyde and BSA solutions. These are mixed in the syringe tip during application and allowed to bond in situ. The resin is a matrix of highly cross-linked albumin strong enough to hold tissues together during the healing process and safe enough to be used internally ("CryoLife: BioGlue Instructions for Use," 2017).

**Structural Basis for Ligand Binding**

Albumins contain approximately 585 amino acids arranged in a highly helical heart-shaped tertiary structure (Figure 1). Three similar domains (I, II, and III) are each subdivided into subdomains A and B. Each domain is composed of 10 helices with six in subdomain A and four in subdomain B. Each subdomain is connected by a long loop. Conformational flexibility is conferred by helical bending and subdomain orientation is controlled by the movement of the loops. These types of movements open and close the various binding sites dotted throughout the structure.

While albumin is extremely flexible, the core structure is maintained via 17 disulfide bridges. Subdomain A is stapled together by four of these while two bridges stabilize subdomain B. The sole exception is Subdomain IA, which contains only three cystine bridges (Bujacz, 2012).
Figure 1. Crystal structure of human serum albumin. (a) Space-filling model and (b) Ribbon structure highlighting the three domains and heart-shaped structure. Domains I (blue), II (yellow), and III (red) are held together by 17 disulfide bridges (gray). PDB ID: 1E7I (Bhattacharya, Grüne & Curry, 2000). All crystal structure images were prepared using UCSF Chimera (Pettersen et al., 2004) and rendered with POV-Ray ("Persistence of Vision Pty. Ltd.: Persistence of Vision (TM) Raytracer," 2004).

**Binding sites.** In addition to its role in controlling osmotic pressure, another crucial function of albumin is its ability to bind a wide variety of ligands including fatty acids, heavy metals, drugs, and drug by-products (Figure 2). Discussed here are just a few of the many binding sites on albumin.
Figure 2. A selection of albumin binding sites. Fatty Acids are represented as yellow hydrophobic tails with red hydrophilic heads. PDB ID: 1E7I (Bhattacharya et al., 2000).

**Fatty acid sites.** Several sites have been identified for long- and medium-chain fatty acids (Ashbrook, Spector & Fletcher, 1972; Ashbrook, Spector, Santos & Fletcher, 1975; Bhattacharya et al., 2000). A preference for saturated, long-chain fatty acids was determined by examining fatty acid content and association constants. Oleic (18:1, 33%), palmitic (16:0, 25%) and linoleic acid (18:2, 20%) made up a majority of the bound fatty acid content of HSA. Five others accounted for another 12.5% and ranged between C14-C20 and zero to four degrees of unsaturation (A. Saifer & Goldman, 1961). Examining a series of fatty acids ranging from C6-C18 and zero to two degrees of unsaturation confirmed this preference for saturated, long-chain fatty acids. Affinity increased with carbon count and decreased as double bond content increased (Spector, 1975). As in the case of Levemir, this binding site allows for tailoring of a drug conjugate to take advantage of an innate function of albumin to form a DDS *in vivo*. The lack of modifications to the protein allow it to maintain functionality and avoid degradation.
Cys34. A single free thiol found at residue 34 has been reported to participate in covalent interactions with a variety of drugs and their metabolites, including bucillamine derivatives (Narazaki, Hamada, Harada & Otagiri, 1996), nitrogen mustards (Noort, Hulst & Jansen, 2002), and the reactive acetaminophen metabolite, N-acetyl-p-benzoquinoneimine (LeBlanc, Shiao, Roy & Sleno, 2014). This residue is on the surface of the molecule, slightly buried in a shallow cleft between two α-helices positioned well away from the two main drug binding sites. The accessibility of Cys34 provides a great deal of control over conjugation of albumin to other molecules. Given that it is the only sulfhydryl available, this allows for chemoselective, covalent surface modification.

The N-terminus and the Multi-metal binding site. Many species of albumin, specifically HSA and BSA, employ the X-Y-His motif at the N-terminus (NTS). This configuration allows for high affinity binding of Cu(II) on the order of 1 pM (Rózga, Sokołowska, Protas & Bal, 2007). This strong association between the NTS and Cu(II) must be taken into account during DDS development. Cu(I) is a catalyst for a set of biorthogonal reactions involving azides and alkynes which will be discussed in more detail in other sections.

A secondary metal interaction site has been found. Deemed the Multi-metal binding site, this area is known to interact with Cu(II), Ni(II), Zn(II), and Cd(II), however, none of these species bind with high affinity. Typically, metal cations are not bound until the NTS has been fully saturated (Bal, Christodoulou, Sadler & Tucker, 1998). Due to low affinities and little impact on the NTS, this site has been largely ignored in favor of a better understanding of the high-affinity NTS interaction with Cu(II).

Drug Site II (DS2). The two main drug binding sites of albumin were initially characterized by Sudlow et al. (1975, 1976). One site has been deemed Sudlow Site II or Drug
Site II. It is a small, compact single chamber with a patch of polar residues at the entrance to the cavity (Figure 3). Tyr411, Arg410 and Ser489 line the mouth of the site, serving as the only polar residues in the entire space.

Figure 3. Structure of Drug Site II in human serum albumin. Polar residues (green) lining the mouth of the cavity are the only polar features of DS2. PDB ID: 2VDB (Lejon, Cramer & Nordberg, 2008).

A lack of hydrophilicity in the pocket is mirrored in its ligands which are small hydrophobics with a terminal polar group (Table 2) (Yamasaki, Chuang, Maruyama & Otagiri, 2013). The hydrophilic head, typically a carboxylic acid, is used to anchor the molecule in the pocket. This also leads to a high degree of stereospecificity for ligand orientation. By no means a flexible site, DS2 has shown the ability to make slight adjustments to accommodate side-chain phenyl groups (Ghuman et al., 2005; Yamasaki et al., 2013).
Table 2. Example Ligands for Drug Site I and Drug Site II (Ghuman et al., 2005; Q. Wang, Zhang & Ji, 2014; Yamasaki et al., 2013).

<table>
<thead>
<tr>
<th>DS1 Ligands</th>
<th>DS2 Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>Warfarin</td>
<td>Naproxen</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>Ibuprofen</td>
</tr>
<tr>
<td>Oxymetazoline</td>
<td>Clofibric acid</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>Diclofenac</td>
</tr>
<tr>
<td>CMOF</td>
<td>6-MNA</td>
</tr>
<tr>
<td>Iopromide</td>
<td>Iopanoic acid</td>
</tr>
<tr>
<td>Iloprost</td>
<td>Etoileptic</td>
</tr>
<tr>
<td>Bicalutamide</td>
<td>Diflunisal</td>
</tr>
<tr>
<td>Iopromide</td>
<td>3-Indoxyl sulfone</td>
</tr>
</tbody>
</table>
Drug Site I (DS1). Drug Site I or Sudlow Site I is a large, multi-chamber, mostly apolar pocket with a few polar features that are key to the binding of ligands (Figure 4). A polar environment is formed at the entrance to the cavity by Lys195, Lys199, Arg218, and Arg222. Three residues are buried deep into the bottom of the site which help to orient molecules (Tyr150, His242, Arg257). Ile264 splits the large chamber, giving a total of three subsites for binding. Additionally, Trp214 resides near the mouth of the cavity. While this may participate in hydrogen bonding with ligands and play a role sterically, it also serves as an innate fluorescent probe for studying drug interactions (Ghuman et al., 2005; Yamasaki et al., 2013).

Figure 4. Structure of Drug Site I in human serum albumin. Polar residues (pink) at the entrance and back (orange) of the cavity are able to hydrogen bond with ligands. Ile264 (purple) divides the chamber into smaller subsites. Trp214 (red) provides an intrinsic probe for ligand binding. PDB ID: 2VDB (Lejon et al., 2008).

DS1 has been called a “large and flexible region.” It is able to bind a wide range of ligands, often more than one at a time, and is not stereospecific (Kragh-Hansen, 1988). Molecules that show a high affinity for DS1 are typically large, flat, and aromatic with at least one terminal and one internal polar functional group (Table 2). Flat molecules are preferred for
DS1 due to Leu238 and Ala291 which are able to snugly fit the molecule into place. Terminal polar moieties are needed to interact with the entryway residues while the internal polar group interacts with the buried polar residues (Ghuman et al., 2005; Yamasaki et al., 2013).

While each of these sites exhibits preferential binding for certain types of molecules, they are by no means exclusive for those molecules. Once the preferred site is filled, additional molecules are often able to bind with lower affinities to other sites. In the case of naproxen, crystal structures revealed three separate binding sites. DS2 was the preferred site as it was the only site consistent between bovine, equine, and leporine serum albumins. Additional naproxen were found in a fatty acid site and DS1 (Bujacz, Zielinski & Sekula, 2014).

**Albumin Metabolism**

Albumin has a half-life of 20 days, passing through circulation thousands of times before being lysosomally degraded. The pathways used to evade early degradation, delivery of its ligand payload, and ultimate catabolism provide useful knowledge for the informed design of DDSs.

The Megalin/Cubilin complexes have been implicated as role players in saving albumin from elimination through the kidneys. Cubilin, an extracellular protein, utilizes the transmembrane protein megalin to shuttle albumin into the intracellular space. Mice missing one or both of these proteins showed decreased albumin reabsorption, strongly suggesting a role in albumin recycling (Christensen, Birn, Storm, Weyer & Nielsen, 2012).

Similar conclusions were drawn in the case of the neonatal Fc receptor (FcRn). Immunoglobulin G (IgG) binds FcRn as a means to cross the placental barrier. Co-elution of the IgG-FcRn complex unexpectedly coupled with albumin was the first indication of interaction. Studies with FcRn deficient mice found a 40% decrease in albumin serum concentration, owing
to the elimination of albumin rather than the transmembrane movement provided by FcRn. Under acidic conditions, such as those experienced throughout the lysosomal pathway, FcRn binds with high affinity to both albumin and IgG and escorts them to a more neutral environment (Kim et al., 2006; Larsen, Kuhlmann, Hvam & Howard, 2016; Merlot, Kalinowski & Richardson, 2014). FcRn clearly plays a key role in the long half-life of albumin by way of rescuing the protein from degradation.

Glycoproteins of 30 kDa (gp30) and 18 kDa (gp18) have been shown to play a role in albumin catabolism. While native albumin appears able to escape elimination through the kidneys via the Megalin/Cubilin complex and FcRn pathways, damaged albumins are tagged by gp30 and gp18. The affinities of these proteins for altered albumins are 1000-fold stronger than for native albumin. Alterations are often due to oxidative damage or non-enzymatic glycosylation. Upon binding, the sequestered albumin is ushered to sites of degradation (Larsen et al., 2016; Schnitzer, Sung, Horvat & Bravo, 1992). The albumin is lysosomally catabolized and the amino acids re-enter the cycle of protein synthesis (Stehle, Sinn, Wunder, Schrenk, Stewart et al., 1997).

The most extensively studied albumin receptor is gp60. This receptor is common in continuous endothelia from various tissues. It selectively binds albumin and with the help of caveolin-1, endocytoses the protein (Schnitzer, 1992). In healthy cells, albumin uses this pathway to migrate between the bloodstream and tissue (Merlot et al., 2014).

This is not the case in cancerous cells. The fate of albumin and the mechanism of chemotherapy release are not fully known. Studies focused on the cellular uptake of Abraxane found that the endocytosis of albumin was accompanied by a spike in the concentration of osteonectin. It is postulated that osteonectin binds to albumin, tagging it for degradation which
releases paclitaxel in the process (Desai, 2008; Hawkins et al., 2008; Tiruppathi, Song, Bergenfeldt, Sass & Malik, 1997).

In addition to gp60 endocytosis, the enhanced permeation and retention effect must be taken into account when considering cancerous cells. This is the passive trapping of macromolecules in tumor tissues. The cells are growing rapidly and the lymphatic system is unable to maintain sufficient drainage which limits the ability of molecules to exit the tumor cells. Macromolecules, especially albumins, build up in the cells and are ultimately catabolized for protein synthesis and energy.

Studies have explored the ability of modified albumins to be endocytosed into tumor cells. One study covalently attached a low molecular weight protamine fragment to chemotherapeutic-loaded BSA NPs to facilitate transfer across the blood-brain barrier (Lin et al., 2016). Despite the modification, BSA was still successfully taken into tumor cells.

Both pathways have been exploited as ingress for chemotherapeutics into cancerous tissues. The advantages are two-fold. The chance of toxicity due to free ligand is greatly reduced when bound to albumin which also provides an innate targeting mechanism.

**Human vs. Bovine Serum Albumin**

To date, most clinical-use albumin formulations have been HSA-based. However, BSA provides an alternative to address many concerns associated with a human blood-borne protein.

The main concern with using any human-derived blood product is the possibility of disease. Human immunodeficiency virus, Hepatitis, Creutzfeldt-Jakob Disease, and West Nile Virus are all potential pathogens that may be transferred with blood products. Despite the stringent protocols employed to eliminate and avoid these blood-borne diseases, the stigma of a formulation containing a human blood-based protein could decrease patient compliance.
Additionally, the supply and cost of HSA is a cause for concern. The two main avenues for gathering HSA are donations and recombinant technology. Donation based materials are often in limited supply and can cause a fluctuation in availability. Recombinant human albumin has been developed with rice (He et al., 2011) and yeast (Recombumin® from Albumedix Ltd.) (Bosse et al., 2005). These have shown comparable safety, tolerability, pharmacokinetics, and pharmacodynamics to native HSA but are quite expensive.

As a by-product of the cattle industry, BSA provides a much more cost-effective and readily available substitute for HSA. A BLAST® search shows 76% homology between HSA and BSA, suggesting a high degree of structural similarity.

**Amino Acid Correlation**

Comparison of amino acid composition shows strong correlations for key residue types. The solubility of albumin is tied to the number of external charged amino acids. Approximately 31% of the residues on both species are charged, confirming similar solubilities.

A single free thiol can be found in both albumins. Of the 35 cysteine residues, 34 are paired to make disulfide bridges that stabilize the tertiary structure of the three domains. This unpaired cysteine allows for more controlled surface modification at a single, specific site.

Functionalization via surface modifications is a key attractant to albumin as a DDS. Lysine residues are often the sites targeted as their terminal amino groups are able to react with a variety of functional groups, allowing flexibility in the type of conjugation chemistry used. Both species contain 59 lysines, each of which is a potential location to anchor a targeting molecule, covalent drug conjugate, cross-linker, or polyethylene glycol chain.
Structural Homology of DS1 and DS2

A closer look at the Sudlow sites reveals a strong contrast between species. The polar residues of DS2 in HSA are present in BSA as residues Tyr410, Arg409, and Ser488 (Figure 5). Conservation of the key residues implies similar binding capabilities and preferences for similar molecules.

Figure 5. Comparison of Drug Site II between human and bovine serum albumin. (a) DS2 of HSA. PDB ID: 2VDB (Lejon et al., 2008). (b) DS2 of BSA. PDB ID: 4OR0 (Bujacz et al., 2014). Polar residues (green) are conserved between species.

As in HSA, DS1 of BSA contains a single tryptophan, Trp213. The polar features buried in the cavity of DS1 are consistent between HSA and BSA (Tyr149, His241, and Arg256). Some discrepancies are found at the entrance to DS1. The polar cluster of HSA is closely mimicked in BSA (Arg194, Arg198, Arg217, and Lys221) (Figure 6). However, the substitution of arginines for lysines may cause differences in binding capacities. The distal guanidinium group of arginine allows for more hydrogen-bonding and stronger ligand interactions. The delocalized charge, however, weakens the bonds with individual molecules (Li, Vorobyov & Allen, 2013).
Sterically, the guanidinium R-group is bulkier than the terminal amine of lysine which may hinder entry and exit from DS1 in BSA. Despite these discrepancies, interchange of these two proteins should be possible.

![Comparison of Drug Site I between human and bovine serum albumin.](image)

**Figure 6.** Comparison of Drug Site I between human and bovine serum albumin. (a) DS1 of HSA. PDB ID: 2VDB (Lejon et al., 2008). (b) DS1 of BSA. PDB ID: 4OR0 (Bujacz et al., 2014). External (pink) and internal (orange) polar residues are well-conserved between species. Additionally, Trp (red) provides a native fluorophore in both forms of albumin.

**Polyethylene Glycol (PEG)**

Polyethylene glycol (PEG), a water-soluble polymer, is used in a wide variety of industries for a wide variety of applications from control of the viscosity of printer ink to an anti-foaming agent in food preparation to laxatives as pre-operative patient preparation. In laboratory use, it has been employed as a protein precipitating agent as well as a means to facilitate cell fusion (Zalipsky & Harris, 1997).

PEG is available in a wide range of sizes and functional groups. Entire companies have been built around the production and customization of PEGs tailored for specific uses ("Creative PEGworks: PEG Products," 2017; "JenKem Technology: PEG Products," 2017). PEGs from as
small as 400 Da (PEG400) to as large as 40,000 Da (PEG40k) have been examined as possible surfactants and DDS components.

**PEG-Protein Nanoparticles**

PEG has played a role in the development of several successful protein-based NPs (Table 3) (Haag & Kratz, 2006; "US Food and Drug Administration: Drug Approval Packages," 2017; Veronese, Mero & Pasut, 2009). The regulatory approval of the first PEGylated enzymes, Adagen® and Oncaspar®, in the 1990s was the turning point for the change of perspective towards PEG as a building block for future DDSs rather than a mere additive to formulations (Duncan & Veronese, 2009). As a result, more research has been done on the metabolism, toxicity, and benefits of PEGylation.

<table>
<thead>
<tr>
<th>Trade Name</th>
<th>PEG MW (count)</th>
<th>Protein</th>
<th>Indication</th>
<th>Company</th>
<th>Approval Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adagen®</td>
<td>5 kDa (11-17)</td>
<td>Adenosine deaminase</td>
<td>Severe combined immunodeficiency disease</td>
<td>Sigma Tau Pharmaceuticals Inc.</td>
<td>1990</td>
</tr>
<tr>
<td>Oncaspar®</td>
<td>5 kDa (69-82)</td>
<td>Asparaginase</td>
<td>Leukemia</td>
<td>Sigma Tau Pharmaceuticals Inc.</td>
<td>1994</td>
</tr>
<tr>
<td>PEG-INTRON®</td>
<td>12 kDa</td>
<td>Interferon α2b</td>
<td>Hepatitis C</td>
<td>Schering-Plough Corporation</td>
<td>2001</td>
</tr>
<tr>
<td>Neulasta®</td>
<td>20 kDa</td>
<td>Granulocyte-colony stimulating factor</td>
<td>Neutropenia</td>
<td>Amgen Inc.</td>
<td>2002</td>
</tr>
<tr>
<td>PEGASYS®</td>
<td>40 kDa</td>
<td>Interferon α2a</td>
<td>Hepatitis C</td>
<td>F. Hoffmann-La Roche Ltd</td>
<td>2002</td>
</tr>
<tr>
<td>Somavert®</td>
<td>5 kDa (4-6)</td>
<td>Growth hormone antagonist</td>
<td>Acromegaly</td>
<td>Pharmacia &amp; Upjohn Company</td>
<td>2003</td>
</tr>
<tr>
<td>Macugen®</td>
<td>20 kDa (2)</td>
<td>Anti-VEGF aptamer</td>
<td>Age-related macular degeneration</td>
<td>Valeant Pharmaceuticals International Inc.</td>
<td>2004</td>
</tr>
<tr>
<td>Mircera®</td>
<td>30 kDa</td>
<td>Erythropoietin</td>
<td>Anemia associated with chronic kidney disease</td>
<td>F. Hoffmann-La Roche Ltd</td>
<td>2007</td>
</tr>
<tr>
<td>Cimzia®</td>
<td>40 kDa (2)</td>
<td>Anti-TNF Fab’</td>
<td>Rheumatoid arthritis and Crohn’s disease</td>
<td>UCB, Inc.</td>
<td>2008</td>
</tr>
</tbody>
</table>
**PEG Metabolism**

Heavy PEGs (MW > 1000 Da) are minimally absorbed by the gastrointestinal tract. For this reason, the oral administration of PEG conjugates is ineffective. The harsh environment of the digestive system limits the types of conjugates that can be administered orally. PEG-protein complexes risk damage due to enzymatic degradation before entering the bloodstream. For these reasons, most PEGylated drugs are administered via injection or intravenously.

Increasing PEG molecular weight corresponds to an increased circulatory half-life. In a study monitoring the distribution of $^{125}$I-labeled PEG between MWs of 6,000 Da (PEG6k) and 190,000 Da (PEG190k), urinary clearance decreased as molecular weight increased. PEG6k had a half-life of 18 minutes following intravenous administration. PEG190k, nearly 30 times larger, had a half-life of approximately 24 hours (Yamaoka, Tabata & Ikada, 1994). The size of the PEG chain clearly has an effect on time retained by the body.

Evidence was found that PEG-protein conjugates were first broken down by degradation of the protein followed by cleavage from the PEG backbone. Once separated, PEG is passed through the kidneys while proteins are catabolized under normal pathways, producing non-toxic metabolites (R. Webster et al., 2009). The safety of the remaining PEG must be examined to determine the suitability of these conjugates as DDSs.

**Toxicity**

Toxicity has only been associated with extremely low molecular weight PEGs. Fatalities have been reported in burn patients after treatment with an antimicrobial cream containing 95% PEG300. It was found that *in vivo*, PEG400 and smaller were oxidized by alcohol dehydrogenase into toxic diacid and hydroxyl acid metabolites in the blood, poisoning the patients. The rate of oxidation decreased greatly with increased PEG size. The smallest PEG studied, ethylene glycol
(MW 62 Da), was degraded 32 times faster than the largest, octaethylene glycol (MW 370 Da) (Herold, Keil & Bruns, 1989). Another study administered PEG400 and smaller to mice and rats. Doses of 10 $\text{mL/kg}$ or less proved fatal (Bartsch, Sponer, Dietmann & Fuchs, 1976). Intravenous administration of PEG1400 and larger showed no toxicity or negative effects in rabbits and dogs (Working, Newman, Johnson & Cornacoff, 1997).

**Advantages of PEGylation**

As Table 3 details, several proteins have been successfully attached to PEGs of various sizes. The benefits of PEGylation are most strongly tied to the hydrophilicity of PEG itself. This hydrophilicity, due to the oxygen content of the PEG backbone, means that PEG and its conjugates are highly soluble and highly hydrated in aqueous environments. It is estimated that PEG is able to adsorb three to five water molecules per ethoxylene subunit, thus greatly increasing the hydrodynamic volume of the backbone. Increased hydrodynamic volume results in a molecule that appears too large to pass through kidney filtration (Figure 7). Avoidance of elimination via normal pathways leads to increased circulatory half-life (Plesner, Fee, Westh & Nielsen, 2011; Veronese et al., 2009).
Figure 7. Diagram of a PEGylated protein nanoparticle. Covalent conjugation of PEG chains onto the surface of a protein greatly increases the hydrodynamic volume of the NP, conferring increased circulation lifetime and immunogenic masking.

Avoidance of degradation by immunogenic agents also increases the circulation half-lives of these NPs. Chicken Immunoglobulin Y was administered to mice in native and PEGylated forms. Protein conjugated with 3-13 PEG5k or PEG20k showed immune response levels drop to as low as 1.6% detection (Gefen et al., 2013). PEGylation is able to mask bound protein from immunogenic response as the antibodies are unable to sense the protein core due to steric hindrance from the PEG backbones. This helps to protect the NP against damage and degradation.

PEGylation confers a number of benefits to the attached protein core. However, PEGylation may also be deleterious to the NP. The attachment of PEG is typically done through covalent bonds with surface residues. Eliminating these charges and masking the protein surface may lead to poor target and ligand recognition which, in turn, could greatly alter the biological activity of the protein.

The effects of PEGylation on albumin stability were monitored using PEG chains ranging from PEG5k to PEG60k attached to BSA at Cys34 via the thiol-ene reaction. Circular dichroism
studies showed no loss of secondary structure after PEGylation. Upon application of heat, it was found that attachment of PEG, slightly lowered the temperature of denaturation of 82.5°C for unmodified BSA by 2-3°C. There was no direct correlation between the change of denaturation temperature and PEG molecular weight. There does, however, appear to be a connection between molecular weight and the temperature of aggregation. For PEGs between 10-40 kDa, aggregation began at 81°C while unmodified BSA began to aggregate at 71°C (Plesner et al., 2011). It appears that the stability of BSA is slightly compromised but the PEG backbone helps to stave off aggregation.

It should be noted that while attaching many strands of small molecular weight PEG has been successful, it has been proven to be more beneficial to use less PEGs with a total weight equal to these small PEGs. The Williams group demonstrated that attaching two to five strands of PEG30k to superoxide dismutase was more effective than 7-15 strands of PEG5k in preserving biological activity, increasing lifetime and reducing immunogenicity (M. G. P. Saifer, Somack & Williams, 1994).

“Click” Chemistry

“Click” chemistry denotes a series of reactions which are modular and give high yields. Reaction conditions are simple and insensitive to water and oxygen. The stereospecificity of these reactions is what makes them so attractive for bionconjugation. The final product is due completely to the functional groups reacted. Groups attached to these reactive moieties may enhance the reaction but may not change the final product (Kolb, Finn & Sharpless, 2001).

The basis for click chemistry begins with a modification of the Huisgen reaction between azides and alkynes. This reaction was largely ignored because of the need for high temperatures and pressures. The use of a Cu(I) catalyst as a way to run these reactions under less extreme
conditions paved the way for the rapid rise in popularity of click chemistry (Rostovtsev, Green, Fokin & Sharpless, 2002; Tornøe, Christensen & Meldal, 2002). These reactions and their applications have been extensively reviewed by Kolb et al. (2001) and Thirumurugan et al. (2013).

**Azide-Alkyne Cycloaddition (AAC)**

**Cu(I)-catalyzed AAC (CuAAC).** The Cu(I)-mediated reaction between azides and alkynes (Figure 8) is the prototypical click reaction. It is fast, high-yielding, and stereospecific, giving only 1,4-disubstituted 1,2,3-triazoles. Reaction conditions do not require elevated temperatures or pressures. 

![Figure 8. Cu(I)-catalyzed azide-alkyne cycloaddition.](image)

The reaction of a terminal azide with a terminal alkyne in the presence of Cu(I) with a compliment of ligand, reducing agent and suppressor reagent results in a single 1,4-disubstituted 1,2,3-triazole product. Kinetics may be enhanced by incorporating an electron-withdrawing group at the R₃ position.

The employment of a copper catalyst is accompanied by a variety of complimentary reagents. The accelerating ligand chelates the copper and helps maintain the proper coordination environment. The catalyst is more cost-effective as a Cu(II) salt and reduce which is reduced *in situ* by an agent such as ascorbate. However, this reduction often results in reactive oxygen species which may modify amino acid side chains. To counteract this, aminoguanidine is included (Hong, Presolski, Ma & Finn, 2009).

Despite the high solubility and stability of these reagents at physiological pH, this form of AAC is not ideal for production of albumin-based DDSs. As previously discussed, the N-
terminus in many albumins is able to bind Cu(II) with an affinity of 1 pM. The bound Cu(II) will be difficult to remove during work-up of the resulting NPs. All traces of copper must be removed before introduction into a patient as additional, undesirable heavy metals are toxic to the host. Cu(II) has been implicated in neurodegenerative diseases, including prion diseases and Alzheimer’s disease (Rózga et al., 2007). While the copper catalyst and its compliment of reagents are readily available and cost-effective, simpler AAC reactions have been developed.

**Strain-promoted AAC (SPAAC).** Copper-free click chemistry circumvents the need for a Cu(I) catalyst by incorporating the alkyne into a strained ring such as a cyclooctyne or cyclononyne (Figure 9a) (Agard, Prescher & Bertozzi, 2004). With a simple, unsubstituted cyclooctyne, the reaction kinetics are considerably slower than those of CuAAC. Attachment of electron withdrawing groups onto the ring adjacent to the alkyne has been used to increase reaction rates. Difluoro- (Figure 9b) and Dibenzocyclooctynes (Figure 9c) returned rates back to those of CuAAC without the need for a Cu(I) catalyst and have become the first choice for SPAAC reactions (Ning, Guo, Wolfert & Boons, 2008; van Berkel et al., 2007).
Azides and alkynes are biorthogonal as both are virtually absent from nature. The sole exceptions are azide-containing natural products found in toxic red algae on the Gulf Coast of Florida. Additionally, both are virtually non-reactive with any naturally-occurring functional group (Baskin & Bertozzi, 2009). This, coupled with the reduced toxicity due to the elimination of the copper catalyst, make SPAAC an excellent candidate for conjugation chemistry with albumin.

**Thiol-ene Reactions**

Thiol-ene reactions, while involving neither an azide nor an alkyne, are another class of click chemistry (Figure 10). All of the criteria are met in that the reaction is simple and the product formed is stereospecific and gives high yields (Lowe, 2010). This reaction is especially applicable to proteins with exposed cysteine residues. The free thiol may be chemoselectively
reacted with an alkene-containing linker to attach drugs or targeting molecules (Chalker, Bernardes, Lin & Davis, 2009).

![Thiol-ene reaction](image)

Figure 10. Thiol-ene reaction. (a) A thiol from an exposed cysteine may be reacted with an alkene resulting in a thioether linkage. (b) A common form of the -ene used in these reactions is the maleimide functional group.

The most common functional group employed for protein modification at cysteines is the maleimide. Incorporation of the electron-deficient moiety into the molecule to be attached to the protein allows for fast and easy modification under physiological conditions. For example, a maleimide-methotrexate prodrug was developed as a treatment for arthritis (Fiehn, Kratz, Sass, Müller-Ladner & Neumann, 2008). Upon intravenous administration, this prodrug binds to Cys34 of HSA in vivo. The conjugate is later enzymatically cleaved, releasing a bioactive derivative of methotrexate. The maleimide-cysteine reaction is rapid and selective, greatly increasing the efficacy of the methotrexate treatment.

**Summary**

BSA provides an intriguing option to build a nano-scale DDS. It is highly soluble, with a plethora of binding pockets, and a large number of surface residues which can be used for customization without the need for recombinant methodologies. Its structural similarities with HSA suggests a similar degree of effectiveness can be achieved when incorporated into any of the published NP methodologies.
A multitude of approaches have been attempted at formulating a successful albumin-based DDS. Each was successful at solubilizing a hydrophobic pharmaceutical in the presence of albumin but flaws in each must be addressed to move forward. Only one albumin-based NP has successfully made it to market. Abraxane, while effective, still requires an infusion of a large dose of albumin into the bloodstream, potentially affecting the osmotic pressure and subsequently the production of native albumin.

Several PEGylated proteins have been approved, but none are DDSs. PEGylation has proven to be advantageous as it confers increased hydrodynamic volume which leads to increased circulation lifetimes and decreased immune response. However, PEGylation may slightly destabilize the protein at elevated temperatures and steric hindrance due to the attached PEGs may negatively affect biological activity and cellular recognition.

The biorthogonality of SPAAC and the selectivity of the thiol-ene reactions allow for easy modification of surface residues of BSA. Due to the high affinity between the N-terminus of albumins and Cu(II), traditional CuAAC cannot be used. A strained cyclooctyne further modified by an electron-withdrawing group provides an even easier and simpler alternative.

A NP that can successfully harness the advantages of albumin, avoid the pitfalls encountered in other production methods, and gain the advantages of PEGylation with simple surface modifications would prove beneficial for the advancement of nanotechnology as DDSs.
CHAPTER II
STATEMENT OF RESEARCH

The aim of this research is to develop a multi-use albumin-based nanoparticle for drug delivery in order to address the concerns associated with current methodologies and available products. The most pressing concerns are the cost and risk of pathogens associated with human-sourced blood proteins, the negative effect on osmotic pressure due to a large dose of albumins, the potential immune response and loss of biological activity due to heavy modification of these albumins, and the need for organic solvents for preparation of these products.

Most market available albumin formulations use human serum albumin. When sourced from blood donations, supplies are limited and the risk of potential contamination with pathogens such as human immunodeficiency virus and hepatitis must be considered. Recombinant albumin provides a reliable but expensive way to avoid these human pathogens. An alternative approach is to use bovine serum albumin (BSA). This species of albumin is cheaper and more readily available than its human counterpart. The two are structurally similar. Specifically, key residues of Drug Sites 1 and 2 are conserved, suggesting similar binding capabilities.

Covalent attachment of polyethylene glycol (PEG) chains to proteins has been shown to greatly increase circulatory half-life and reduce immunogenic response. In this research, several BSAs will be attached to an 8-arm polyethylene glycol backbone (BSA₈-R-PEG₈) using two types of reactions, resulting in different “R” group linkers between protein and backbone. Use of a branched PEG backbone minimizes the effect on osmotic pressure. Upon administration of a
single dose of drug, the total number of albumins introduced into the bloodstream will be greatly reduced with BSA$_x$-R-PEG$_8$ as opposed to conventional albumin nanoparticles.

In the first part of this work, the lone, unpaired sulfhydryl of BSA at Cys34 will be reacted with a maleimido-functionalized PEG$_8$ (Mal-PEG$_8$) resulting in a thioether (te) linkage. This eliminates the need for organic solvents entirely, requires no additional modifications to the protein and provides a chemoselective conjugation method. Yields, secondary structure, speciation, and ligand binding capabilities will be determined for this form of PEGylated BSA (BSA$_x$-te-PEG$_8$).

Secondly, BSA$_x$-R-PEG$_8$ will be prepared using strain-promoted azide-alkyne cycloaddition. BSA, “activated” at a single superficial lysine with either a short- or long-chain Dibenzocyclooctyne-N-hydroxysuccinimidy ester (DIBO-NHS; DIBO-PEG$_4$-NHS), will be PEGylated via reaction with complimentary terminal azides (N$_3$-PEG$_8$). BSA and PEG$_8$ will be conjugated via a 1,2,3-triazole (123t). This approach requires a single modification of the albumin and a minimal amount of organic solvent necessary to dissolve each activating agent. PEGylated BSA activated with the short alkyne (BSA$_x$-123t-PEG$_8$) will be used to optimize the PEGylation methodology. BSA$_x$-123t-PEG$_8$ and PEGylated BSA activated with the longer alkyne (BSA$_x$-PEG$_4$-123t-PEG$_8$) will be characterized in terms of secondary structure, speciation, and ligand-binding capabilities.

The third area of research examines the effect of these methods of PEGylation on the stability of BSA. Monitoring the temperatures of denaturation and aggregation of PEGylated BSA has shown a reduction in thermal stability of the attached protein. Published works have examined complexes containing a single albumin conjugated with multiple PEG backbones, resulting in a higher degree of modification. These complexes showed a slight decrease in the
The temperature of denaturation is accompanied by an increase in the temperature of aggregation. PEGylation of BSA creates three new complexes: BSA$_x$-te-PEG$_8$, BSA$_x$-123t-PEG$_8$, and BSA$_x$-PEG$_4$-123t-PEG$_8$. These complexes require a single point of modification in order to attach multiple albumins to a single PEG backbone. Less modification of albumin may have less effect on the thermal stability of the final product.

Traditional pasteurization of albumin requires N-acetyl-L-tryptophan and sodium caprylate to stabilize the protein during the heating process. Both of these additives have shown a preference for DS2, creating competition for potential ligands. In an effort to eliminate these stabilizers, PEGylated BSA will be pasteurized in the absence of both reagents. The effects of pasteurization on the helicity and ligand binding capabilities of PEGylation by both thiol-maleimide and strain-promoted azide-alkyne cycloaddition reactions in conjunction with the pre-loading of a DS2 ligand will be monitored.

Every drug delivery system must be non-toxic, non-immunogenic, cost-effective, easy to produce, and applicable to a variety of ligands. By addressing the concerns associated with previous approaches, these qualities can be achieved.
CHAPTER III
MODIFICATION OF BOVINE SERUM ALBUMIN WITH
MAL-PEG₈ AS A DRUG DELIVERY SYSTEM

Introduction
Serum albumin has become an attractive option in the development of drug delivery systems largely due to its role as a vehicle for hydrophobic molecules. Human serum albumin (HSA) has been studied extensively for this purpose but the cost of production and risk of pathogenic transfer are considerable. Bovine serum albumin (BSA) provides a more cost-effective and readily available alternative while maintaining key structural features, including two drug binding sites, DS1 and DS2.

Crystallographic data has shown that DS1 and DS2 are well-conserved between HSA and BSA (Bujacz, Zielinski & Sekula, 2014; Lejon, Cramer & Nordberg, 2008). The polar environment at the mouth of DS1 is the sole difference between binding sites of these species. In HSA, the polar cluster consists of residues K195, K199, R218, and R222 while in BSA, the entrance is coated in mostly arginines (R194, R198, R217, and K221). Arginine, while still capable of hydrogen-bonding, has a more dispersed positive charge and a larger functional group. Sterics as well as the delocalization of the positive charge weaken interactions with individual polar molecules (Li, Vorobyov & Allen, 2013). Despite this difference, it is believed that DS1 and DS2 on BSA will house similar molecules as HSA.

A single, free cysteine at position 34 is also conserved across species. In the body, Cys34
plays a key role as an antioxidant (Anraku, Chuang, Maruyama & Otagiri, 2013; Roche, Rondeau, Singh, Tarnus & Bourdon, 2008) and is also capable of binding various ligands (LeBlanc, Shiao, Roy & Sleno, 2014; Narazaki, Hamada, Harada & Otagiri, 1996; Noort, Hulst & Jansen, 2002). In terms of drug delivery system development, the most attractive aspect of Cys34 is that it provides an accessible, chemoselective site for surface modification.

Cysteine has been selectively modified using a variety of chemistries (Abbas, Xing & Loh, 2014; Abegg et al., 2015; Mehtala, Kulczar, Lavan, Knipp & Wei, 2015) in order to increase the circulatory half-lives of a variety of protein-based therapies (Léger et al., 2004; Plesner, Fee, Westh & Nielsen, 2011; Stoddart et al., 2008). CJC-1134-PC is an application of ConjuChem’s Preformed Conjugate-Drug Affinity Complex (PC-DAC™) technology for the treatment of type 2 diabetes. This complex consists of maleimido-functionalized Exendin-4, a homolog of glucagon-like peptide 1, covalently attached to recombinant human albumin via interaction with Cys34. Exendin-4 has a short half-life, requiring twice daily injections to maintain therapeutic levels. Conjugation onto albumin extends circulatory lifetime while maintaining biological potency (Baggio, Huang, Cao & Drucker, 2008).

In the case of PC-DAC, the therapeutic agent is left exposed to the environment in order to interact with cell surface targets. However, drug molecules need to be removed from the environment. Direct conjugation of a drug to Cys34 provides no protection for the ligand, subjecting the bound drug to possible degradation or alteration. The binding pockets of albumin provide shelter for these molecules while keeping Cys34 free for surface modifications.

Cys34 is found in a shallow cleft at the surface of albumin in Domain IA (Figure 11). DS2, found entirely in Domain IIIA, is likely unaffected by conjugation at a single residue in Domain I. DS1, however, is found at the interface of Domains IB and IIA. Modification at Cys34
may cause conformational changes which may alter the binding characteristics of DS1.

![Figure 11. Cys34, Drug Site I, and Drug Site II of bovine serum albumin. (a) Cys34 (brown) is located in Domain I (blue), adjacent to DS1 (pink) in Domain II (yellow). DS2 (green) in Domain III (red) is far removed from Cys34. (b) It is slightly buried in a shallow cleft on the surface of the protein. PDB ID: 4OR0 (Bujacz et al., 2014). All crystal structure images were prepared using UCSF Chimera (Pettersen et al., 2004) and rendered with POV-Ray ("Persistence of Vision Pty. Ltd.: Persistence of Vision (TM) Raytracer," 2004).](image)

A common modification for protein-based drug delivery systems is covalent attachment of a polyethylene glycol (PEG) backbone. PEG-protein conjugates often consist of multiple PEG chains covalently attached at several locations on the surface of a single protein core (Nischan & Hackenberger, 2014; Veronese & Pasut, 2005). This approach results in a potential problem for albumin-based drug delivery systems. Albumin is a key regulator in osmotic pressure. Administration of a large dose of individually PEGylated albumins may lead to down-regulation of albumin production (Pietrangelo, Panduro, Chowdhury & Shafritz, 1992). Down-regulation would result in accumulation of materials normally transported by albumin, such as toxic metabolites and heavy metals.

A multi-albumin complex would allow for a higher drug dose with introduction of fewer
albumins (Figure 12). Osmotic pressure is controlled by the number of particles, not their individual sizes. In the case of serum proteins, with a MW of 400 kDa, fibrinogen is among the largest but accounts for only 1% of total osmotic pressure due to low concentration (0.0075 mM). Albumin, however, is several times smaller (MW 66 kDa) but is found at a much higher concentration (0.68 mM) and accounts for 79% of total osmotic pressure (Guyton & Hall, 2006). For example, the same therapeutic dose of eight individual albumins pre-loaded with a drug could be achieved with administration of a single eight-albumin particle, lessening the effects on osmotic pressure of treatment.

Figure 12. Diagram of BSA₈-te-PEG₈. Thioether (te) linkages connect eight BSA molecules to a single PEG₈ backbone via reaction between terminal maleimides and native Cys34 residues.
In this study, BSA will be attached to maleimido-functionalized PEG₈ (Mal-PEG₈) via thiol-ene reaction (Figure 13). A thioether (te) linkage will connect BSA to the PEG₈ backbone via reaction between a terminal maleimide and native Cys34. The resulting BSA₄-te-PEG₈ (Figure 12) will be characterized in terms of yield, secondary structure, speciation, and ligand binding.

Figure 13. Thiol-maleimide conjugation of bovine serum albumin onto PEGs. The free thiol of Cys34 reacts with Mal-PEG₈ to give a thioether linkage between protein and PEG backbone.

Materials and Methods

Materials

Lyophilized BSA (Product Number A7906), sulfamethoxazole (SMZ; S7507), naproxen (NPX; N8280), and all reagents not detailed were purchased from Sigma-Aldrich.

Methods

All experiments were carried out in 50 mM 4-Morpholinepropanesulfonic acid/0.15 M NaCl pH 7.2 (MOPS/NaCl) buffer unless otherwise noted. Fast protein liquid chromatography (FPLC) separations were performed on an ÄKTAprime plus chromatography system (Amersham Biosciences; 11001313) with MOPS/NaCl mobile phase. Resulting fractions were concentrated with 30 kDa MW cut-off centrifuge filters (Sartorius; VS2022). BSA concentrations were calculated using absorbance values obtained on an Agilent 8453 UV-visible Spectroscopy System at 280 nm with an extinction coefficient of 43.824 mM⁻¹ cm⁻¹ and a MW of 66,430 Da.
Fatty acid removal. Fatty acid-free BSA (FAF-BSA) was prepared via an activated charcoal method (Chen, 1967). In short, BSA was dissolved in MOPS/NaCl. The solution was acidified to pH 3 and stirred on ice with 20-40 mesh particle size activated charcoal in order to remove any bound fatty acids. Charcoal was pelleted at 10,000 rpm and the supernatant was filtered through 0.2 µm cellulose filters to remove any remaining carbon. The de-fatted albumin solution was returned to pH 7.2 before further purification. Dimers were removed by size exclusion chromatography (SEC) on a pre-packed 120 mL 60 cm x 16 mm S300 FPLC column (GE Healthcare; 17-1167-01).

PEGylation of FAF-BSA. The final product consisted of multiple BSA molecules attached to a PEG₈ backbone via a thioether (te) linkage formed by the reaction of terminal maleimides of PEG₈ and the sulphhydryl of Cys34 (BSA₅-te-PEG₈). 40 kDa Mal-PEG₈ (Creative PEGworks; PSB-864) was dissolved in MOPS/NaCl and added to 1 mM FAF-BSA at 8:1 FAF-BSA:Mal-PEG₈. The solution was allowed to incubate at room temperature for one week with constant stirring. PEGylated FAF-BSA was purified via a two-phase SEC separation. The bulk of unreacted FAF-BSA was removed by way of an S300 FPLC column, the remainder via a 320 mL 60 cm x 26 mm S500 column (GE Healthcare; 28-9356-07). Chromatograms from the S300 separation were processed in PrimeView 5.0 software to determine yields as the percentage of total area count.

Circular dichroism (CD). The effects on secondary structure of the PEGylation procedure were determined by CD. BSA₅-te-PEG₈ were assayed in 50 mM MOPS pH 7.2. Studies were done on an Olis DSM 20 CD Spectrophotometer. Spectra were collected at 30°C.
over the range of 190-260 nm with a step-size of 1 nm. Helicity was calculated with DichroWeb (Whitmore & Wallace, 2008) and the analysis program CDSSTR (Sreerama & Woody, 2000). Inclusion of a crystal structure for HSA (Wardell et al., 2002) allowed for use of reference set SP175 (Janes, 2009; Lees, Miles, Wien & Wallace, 2006).

**SDS-PAGE.** Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as an initial characterization of BSA$_x$-te-PEG$_8$ species. The assay was performed on a PhastSystem (Amersham Biosciences; 18-1018-23) with a 4-15% gradient PhastGel (GE Healthcare; 17-0678-01), Coomassie R 350 stain (GE Healthcare; 17-0518-01), and high molecular weight markers (GE Healthcare; 17-0615-01).

**Analytical ultracentrifugation (AUC).** AUC was carried out in a Beckman Coulter ProteomeLab XL-A analytical ultracentrifuge using standard two sector cells with quartz windows in four- or eight-hole rotors. All sedimentation velocity experiments were performed at 50,000 rpm with a preset scan number of 100 per cell. The reference sector was filled with 450 µL of MOPS/NaCl and the sample sector was filled with 430 µL of sample with an absorbance at 280 nm between 0.75 and 1.1. Path length was 1 cm. Buffer density was measured at 20.0°C in a Mettler/Paar Calculating Density Meter DMA 55A and the buffer viscosity was measured in an Anton Parr AMVn Automated Microviscometer. Data were analyzed using the program Sedfit (Schuck, 2000; "Sedfit," 2017). Molecular weights and sedimentation coefficients ($S_{20,w}$) were determined using the continuous c(s) mode in the Sedfit program. Native monomeric BSA yielded an $S_{20,w}$ value of 4.04 S which agreed with published values (Shulman, 1953; Taylor, 1952; Zhao et al., 2015). The c(s) plots were deconvoluted using non-linear least-squares fitting algorithms available in the Origin (v. 9.0) software package (OriginLab Corporation, Northampton, MA). Data were fit to multiple Gaussian components, increasing the number of
components until residual plots (data-fit) were random and the chi square value for the fit was minimal. Values reported are percentages of the total area count of peaks corresponding to PEGylated species. These area counts were calculated via the trapezoidal formula.

**Fluorimetric analyses.** Binding studies were performed on a Photon Technology International fluorimeter using Felix software (version 1.1). SMZ, a DS1 ligand, and NPX, a DS2 ligand, were studied to determine if PEGylation affected ligand binding capabilities. The number of binding sites (n) and the association constant (K_a) were calculated for BSA_x-te-PEG_8. In each case, the fluorophore monitored was Trp213, found at the entrance to DS1 in Domain IIA.

For SMZ, 30 µM stock solutions of FAF-BSA and BSA_x-te-PEG_8 were prepared in 0.2 M Tris-HCl/0.1 M NaCl pH 7.4. SMZ was dissolved in methanol to a final stock concentration of 30 mM. Working SMZ stocks were prepared at 3 mM and 1 mM by dilution with Tris buffer. Monitoring emission between 300-380 nm upon excitation at 283 nm at 25°C, aliquots of working SMZ stocks were titrated into a 3 µM BSA solution. 1 mM working stock was used up to 20 µM SMZ. 3 mM working stock was used to raise the final concentration to 80 µM SMZ.

To determine n and K_a for NPX, 30 µM stock solutions of FAF-BSA and BSA_x-te-PEG_8 were prepared in 10 mM phosphate buffer pH 7.0. NPX was dissolved in methanol to a final stock concentration of 45 mM. A working NPX stock was prepared at 0.15 mM by dilution with phosphate buffer. Monitoring emission between 300-380 nm upon excitation at 295 nm at 25°C, aliquots of working NPX stocks were titrated into a 0.725 µM BSA solution to a final concentration of 3 µM NPX.

Blanks for each ligand at each concentration were necessary to account for any fluorescence due to the ligand itself. SMZ had almost no signal under experimental conditions.
NPX, however, showed strong emission with maxima at approximately 350 nm. Particularly at high concentrations, NPX signal obscured the BSA maxima which ranged from 344 to 350 nm.

Non-linear regression analyses were carried out using The Gnumeric Spreadsheet (version 1.10.16) ("The Gnome Project: The Gnumeric Spreadsheet: Free, Fast, Accurate - pick any three," 2011) and a modified Hill equation. In order to normalize samples, fractional intensities (FrInt) were used in place of raw intensities.

\[
\text{FrInt} = \left( \frac{(\text{FrInt}_{\text{max}} - \text{FrInt}_{\text{min}}) \cdot [\text{Ligand}]^n}{K_d^n + [\text{Ligand}]^n} \right) + \text{FrInt}_{\text{min}}
\]

\[
\text{FrInt} = \left( \frac{\text{Int}_{1 \mu\text{M Ligand}} - \text{Int}_{0 \mu\text{M Ligand}}}{\text{Int}_{\text{max} \mu\text{M Ligand}} - \text{Int}_{0 \mu\text{M Ligand}}} \right)
\]

\[
K_a = \frac{1}{K_d}
\]

**Results and Discussion**

**BSA\textsubscript{x}-te-PEG\textsubscript{8} Yield**

BSA\textsubscript{x}-te-PEG\textsubscript{8} was purified via two size exclusion FPLC columns (Figure 14). Integration of the S300 separation showed that the PEGylation procedure converted an average of 36.1 ± 0.6% of BSA into BSA\textsubscript{x}-te-PEG\textsubscript{8}. In the initial purification, a small peak eluted at approximately 40 mL suggests the presence of extremely high molecular weight material. This same material was buried in the S500 separation, resulting in peak broadening for BSA\textsubscript{x}-te-PEG\textsubscript{8} fractions.
Figure 14. Purification of thiol-maleimide PEGylated bovine serum albumin via size exclusion chromatography. (a) Initial purification was done on an S300. (b) Final purification was done an S500 column. FAF-BSA (---) and BSA₉-te-PEG₈ (red).
**Effects on Secondary Structure**

Helicity was maintained after the PEGylation procedure. BSA<sub>x</sub>-te-PEG<sub>8</sub> contained minima at 210 nm and 220 nm which are characteristic of α-helices (Figure 15). The secondary structure of FAF-BSA was 59.2 ± 2.2% α-helical. PEGylation resulted in a slight decrease in helicity to 56.3 ± 1.3%. Both values are in agreement with published values which range from 56-66% helical content for BSA (Das et al., 2017; Moriyama et al., 2008). Given that Cys34 is shallowly sheltered in a cleft between two helices, attachment of a large PEG chain may have caused some loss of helicity.

![Figure 15. Circular dichroic spectra of FAF-BSA and BSA<sub>x</sub>-te-PEG<sub>8</sub>. FAF-BSA (---) and BSA<sub>x</sub>-te-PEG<sub>8</sub> (red) contain minima at 210 nm and 220 nm characteristic of α-helices.](image-url)
BSA₃-te-PEG₈ Speciation

Electrophoretic separation of PEGylated BSA via SDS-PAGE revealed the presence of high molecular weight material (Figure 16). A faint band at 116 kDa corresponds to dimeric BSA and BSA₁-te-PEG₈ (MW 106 kDa). A second faint band at 170 kDa corresponds to BSA₂-te-PEG₈ (MW 172 kDa). A dark streak at MWs heavier than 220 kDa contains distinct banding, most likely corresponding to BSA₃-₄-te-PEG₈. Material retained in the stacking zone is made of five or more PEGylated BSAs as the molecular weight cut-off for proteins in the separating zone is 300 kDa. Smearing is most likely due to interaction of the PEG₈ backbone with the polyacrylamide gel.

![Figure 16](image)

Figure 16. Electrophoretic separation of species of bovine serum albumin PEGylated via thiol-maleimide click chemistry. Lane 1-2: BSA₃-te-PEG₈; Lane 3: FAF-BSA; Lane 4: High molecular weight marker.

Several species of PEGylated BSA were found via AUC (Figure 17). 70% of the total PEGylated area count was due to BSA₂₆-te-PEG₈. 11% corresponded to a single BSA attached
to a single PEG$_8$ backbone. The remaining 19% consisted of very high molecular weight species. Given that there are only eight arms on Mal-PEG$_8$ and only one free cysteine on BSA, the largest particle, BSA$_8$-te-PEG$_8$, would have a MW of 502 kDa. Peaks with a sedimentation coefficient greater than 15 S have calculated molecular weights well above this mark. It is believed that the extremely high molecular weight species are a combination of BSA$_{7,8}$-te-PEG$_8$ and PEGylated species of a side-reaction of Mal-PEG$_8$. 
<table>
<thead>
<tr>
<th>Peak</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>S_{20,w}</td>
<td>2.12</td>
<td>4.24</td>
<td>6.06</td>
<td>7.58</td>
<td>8.79</td>
<td>10.6</td>
<td>12.7</td>
<td>15.8</td>
<td>18.2</td>
<td>19.7</td>
<td>21.8</td>
<td>24.2</td>
</tr>
<tr>
<td>MW_{calc} (kDa)</td>
<td>35</td>
<td>82.7</td>
<td>nr</td>
<td>194</td>
<td>259</td>
<td>343</td>
<td>473</td>
<td>704</td>
<td>nr</td>
<td>nr</td>
<td>970</td>
<td>1160</td>
</tr>
<tr>
<td>MW_{theor} (kDa)</td>
<td>66</td>
<td>106</td>
<td>172</td>
<td>238</td>
<td>304</td>
<td>370</td>
<td>436</td>
<td>n/a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td># BSA conjugated</td>
<td>monomer</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>n/a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Area Count</td>
<td>n/a</td>
<td>11</td>
<td>9</td>
<td>14</td>
<td>17</td>
<td>15</td>
<td>15</td>
<td>19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 17. Analytical ultracentrifugation analysis of BSA_{x}-te-PEGs. % Area Count values are the percent of PEGylated species only. nr: not reported; n/a: not applicable.

Maleimides react readily with sulphhydryls, however, Mal groups may also react with the terminal amino group of lysine (Brewer & Riehm, 1967). At neutral pH, thiolation occurs 1000
times faster than the lysine interaction. Given the high concentration of the reaction mixture, the large number of superficial lysines, and the long incubation time, it is highly possible that multiple lysines reacted with one or more PEG₈ chains to create very high molecular weight oligomers.

Results were similar to hemoglobin-based nanoparticles developed with the same Mal-PEG₈ (K. D. Webster et al., 2017). Hemoglobin, a 64 kDa tetrameric protein, contains two free cysteines at position 93 of each β-subunit. Similar reaction conditions to those used in this study resulted in a heterogeneous mixture of PEG₈ complexed with predominantly three hemoglobins. As many as six hemoglobin tetramers were found to be conjugated to a single PEG₈ backbone. BSA, similar in size to hemoglobin, behaved in a similar fashion during PEGylation. A similar spread of conjugated protein was found, with a majority of PEGylated species falling between two and six BSA molecules per PEG₈ backbone.

**Ligand Binding Capabilities**

Fluorescent emission was monitored to determine the viability of DS1 and DS2 of PEG-conjugated BSA. NPX was used to probe DS2. Published crystal structures of de-fatted bovine, equine, and leporine serum albumins complexed with NPX indicate a preference for DS2 (Bujacz et al., 2014). Experimental values for NPX in the presence of FAF-BSA and BSAₓ-te-PEG₈ were consistent with published values (Table 4), suggesting that DS2 remains intact upon PEGylation. While the crystal structure reported by Bujacz et al. show three NPX molecules bound, DS2 has been the only high affinity site reported. Discrepancies in the n values are possibly due to de-fatted BSA used in this work while fatty acid-containing albumin was used in the published results.
Table 4. Association Constants ($K_a$) and Number of Binding Sites (n) for BSA$_x$-te-PEG$_8$ for Sulfamethoxazole and Naproxen.

<table>
<thead>
<tr>
<th>Sample</th>
<th>SMZ</th>
<th>NPX</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_a$ (x10$^3$ M$^{-1}$)</td>
<td>n</td>
</tr>
<tr>
<td>FAF-BSA</td>
<td>2.03 ± 0.18</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>BSA$_x$-te-PEG$_8$</td>
<td>0.19 ± 0.05</td>
<td>0.8 ± 0.03</td>
</tr>
<tr>
<td>Lit. values$^{1,2}$</td>
<td>3.55-15.6</td>
<td>0.9-1.0</td>
</tr>
</tbody>
</table>

$^1$SMZ values were published by Naik et al. (2009; 2015), Rajendiran & Thulasidhasan (2015), and Wang et al. (2014).

$^2$NPX values were reported by Banerjee et al. (2006), Bou-Abdallah et al. (2016), Fielding et al. (2005), and Honoré & Brodersen (1984). Bujacz et al. (2014) have reported a crystal structure (PDB ID: 4OR0) for BSA complexed with three NPX molecules.

Binding of NPX to FAF-BSA and BSA$_x$-te-PEG$_8$ showed similar trends (Figure 18). BSA in the absence of NPX had a maximum emission wavelength at 344 nm. With increasing amounts of NPX, a red shift resulted in a maximum at 350 nm. This is consistent with binding to a site adjacent to the fluorophore. In this case, the fluorophore studied was Trp213 found in Domain IIA at the mouth of DS1. As ligand binds to DS2, slight conformational changes exposed Trp213 to the solvent, causing a red shift.
Figure 18. Fluorimetric spectra and non-linear regression of BSA$_x$-te-PEG$_8$ in the presence of naproxen. (a) BSA$_x$-te-PEG$_8$ and (b) FAF-BSA spectra. BSA in the absence of NPX (---) had a maximum emission at 344 nm. Upon addition of NPX to a final concentration of 3 µM, maximum emission shifted to 350 nm and intensities increased. (c) Non-linear regression analyses for BSA$_x$-te-PEG$_8$ (red □) and FAF-BSA (black ◊). Experimental data is represented as symbols while calculated data points are lines of the same color.
DS1, however, appears to be greatly affected by the covalent modification of Cys34. FAF-BSA values were similar to published values for both $K_a$ and $n$ for SMZ studies (Table 4). However, BSA$_x$-te-PEG$_8$ demonstrated a greatly reduced affinity for SMZ. Spectra for both FAF-BSA and BSA$_x$-te-PEG$_8$ followed similar trends (Figure 19). Intensities decreased as SMZ concentration increased. The signal from Trp213 is quenched in the presence of the ligand though it is not tightly bound in BSA$_x$-te-PEG$_8$. Modification of Cys34 in Domain IA, clearly had an effect on binding to DS1 in Domains IB and IIA.
Figure 19. Fluorimetric spectra and non-linear regression of BSA$_{x}$-te-PEG$_{8}$ in the presence of sulfamethoxazole. (a) BSA$_{x}$-te-PEG$_{8}$ and (b) FAF-BSA spectra. BSA in the absence of SMZ (---) had a maximum emission at 346 nm. Intensities decreased with each addition of SMZ. (c) Non-linear regression analyses for BSA$_{x}$-te-PEG$_{8}$ (red □) and FAF-BSA (black ◊). Experimental data is represented as symbols while calculated data points are lines following the same color.
Conclusions

Mal-PEG₈ was successfully used as a scaffolding to combine multiple native BSA molecules into a single complex with drug binding capabilities. The reaction was simple to run, requiring only a relatively high molar ratio of FAF-BSA:Mal-PEG₈. A majority of PEGylated species contained 2-6 BSA molecules though some species of molecular weights greater than BSA₈-te-PEG₈ were found. While a larger spread of BSAₓ-te-PEG₈ species were generated, the complexes still showed the ability to effectively bind drugs to DS2, maintaining the key functionality of these albumin-based nanoparticles.
CHAPTER IV

CONJUGATION OF BOVINE SERUM ALBUMIN ONTO PEG$_8$ VIA STRAIN-PROMOTED AZIDE-ALKYNE CYCLOADDITION AS A DRUG DELIVERY SYSTEM

Introduction

The hydrophobicity of many drugs is a major hurdle for efficient delivery to target tissues. Drug delivery systems (DDS) aim to increase the bioavailability of pharmaceuticals by increasing the solubility and subsequently their efficacy. Increased solubility also reduces the risk of rapid elimination and *in vivo* degradation.

The ideal DDS must be non-immunogenic, easily eliminated, and applicable to a range of drugs. This versatility requires the ability to bind different types of ligands and allow for targeting of specific cell types. It must protect the ligand from degradation while protecting the host from toxicity by chaperoning bound drug to the site of action.

Incorporation of serum albumin into a DDS would address many of these points. Albumin serves as a natural vehicle for insoluble materials by binding these molecules in a variety of hydrophobic pockets found throughout its structure. Albumin-based DDSs have also been shown to greatly increase the aqueous solubility of drugs (Khoder et al., 2016). By sequestering a pharmaceutical, albumin provides protection against degradation, redox reactions, and immunogenic agents. Additionally, the drug is no longer available to freely move through the bloodstream and possibly damage healthy tissue, protecting the patient.
These hydrophobic cavities are able to non-covalently bind a wide variety of ligands, making albumin a versatile drug carrier. Fatty acids (Bhattacharya, Grüne & Curry, 2000), heavy metals (Bal, Christodoulou, Sadler & Tucker, 1998; Rózga, Sokółowska, Protas & Bal, 2007), and metabolites such as bilirubin (Zunszain, Ghuman, McDonagh & Curry, 2008) are just a few of the endogenous materials transported by albumin. The two main binding sites of extrinsic hydrophobic molecules are Drug Site I (DS1) and Drug Site II (DS2). Both are mostly apolar pockets with only a few hydrophilic residues. DS1, located at the interface between Domains IB and IIA, is a large, multi-chambered cavity which is able to accommodate multiple ligands of different sizes. DS2, found entirely in Domain IIIA, is less versatile and shows a higher degree of stereospecificity due to a much more compact structure. These binding sites are reviewed in greater detail by Ghuman et al. (2005) and Yamasaki et al. (2013).

Albumin also allows for specific cell targeting. Tumor cells have been shown to accumulate albumins passively through the enhanced permeation and retention effect and actively via albondin-mediated transcytosis (Desai, 2008; Hawkins, Soon-Shiong & Desai, 2008; Tiruppathi, Song, Bergenfeldt, Sass & Malik, 1997). This provides a natural targeting ability for chemotherapeutics and led to the approval of Abraxane®, an albumin-based DDS of paclitaxel for treatment of metastatic breast cancer (Desai, 2008).

Additionally, albumins contain up to 59 lysine residues. The terminal amino groups of these residues provide a potential site for surface modification with antibodies (Anhorn, Wagner, Kreuter, Langer & von Briesen, 2008), vitamins (Leamon & Low, 1991), and other targeting molecules.

One concern for using albumin as a drug-carrier is the effect on osmotic pressure upon introduction of a large number of free, individual albumins. Approximately 79% of osmotic
pressure is credited to albumin (Guyton & Hall, 2006). For this reason, serum albumin levels are tightly regulated by the body. A large infusion of free albumin would be accompanied by down-regulation of albumin production (Pietrangelo, Panduro, Chowdhury & Shafritz, 1992). This would lead to aggregation of fatty acids and other endogenous, insoluble biomaterials in blood and tissue.

Conjugation of multiple bovine serum albumin (BSA) molecules onto a single eight-armed polyethylene glycol backbone (BSA₈-R-123t-PEG₈) addresses this concern (Figure 20). The linker between PEG₈ and BSA in this diagram is a 1,2,3-triazole (123t) formed by strain-promoted azide-alkyne cycloaddition (SPAAC) with a variable R group spacer between protein and alkyne. Osmotic pressure is dependent on the number of molecules present, not their individual masses. Delivering the same dose of albumin-drug via BSA₈-R-123t-PEG₈ would have less effect on the osmotic pressure. For example, to deliver therapeutic levels of drug, PEG₈ complexed with eight BSA molecules would require one-eighth of the dose of unmodified albumin. Osmotic pressure would be affected by a single molecule as opposed to eight.
Figure 20. Diagram of BSA<sub>x-R-123t-PEG<sub>8</sub></sub>. PEGylation run to completion would result in eight BSAs attached to a single PEG<sub>8</sub> backbone. The linkers between PEG<sub>8</sub> and BSA are a 1,2,3-triazole (123t) formed by SPAAC with a variable R group spacer between protein and alkyne.

PEG is a biologically inert polymer commercially available in a wide variety of sizes and functional groups ("Creative PEGworks: PEG Products," 2017; "JenKem Technology: PEG Products," 2017). Inclusion of a PEG backbone into a DDS would increase solubility of the complex and provide additional protection of the attached BSA molecules from degradation and 

*in vivo* modifications.
Human serum albumin (HSA) has been the main albumin studied for delivery of synthetic hydrophobic molecules. HSA, being a human-sourced, blood-borne protein, presents an increased risk of pathogen transfer. Recombinant human albumins have been developed but are expensive to produce (Bosse et al., 2005; He et al., 2011). BSA provides a readily available and inexpensive alternative to its human counterpart. Crystallographic data has shown that many structural features, including DS1 and DS2, are well-conserved between HSA and BSA (Bujacz, Zielinski & Sekula, 2014; Lejon, Cramer & Nordberg, 2008). The polar environment at the mouth of DS1 is the sole difference between binding sites of these species. In HSA, the polar cluster consists of residues K195, K199, R218, and R222 while in BSA, the entrance is coated in mostly arginines (R194, R198, R217, and K221). While still capable of hydrogen-bonding, arginine has a more dispersed positive charge and a larger functional group. Sterics as well as the delocalization of the positive charge weaken interactions with individual polar molecules (Li, Vorobyov & Allen, 2013). Despite this difference, it is believed that DS1 and DS2 on BSA will house similar molecules as HSA.

While BSA is a natural product that shares a large number of similarities with endogenous HSA, BSA is still a foreign molecule and is at risk of being treated as such upon administration to humans. Studies have shown that oral and parenteral administration of unmodified BSA to humans resulted in production of BSA antibodies. In some cases, no immune response was invoked (Korenblat, Rothberg, Minden & Farr, 1968). In cross-species administration of proteins, covalently attached PEG has been shown to effectively mask the foreign molecules and greatly reduce immunogenic response (Gefen et al., 2013). A PEGylated complex such as BSA₅-R-123t-PEG₈ (Figure 20) may hide the foreign BSA molecules from immunogenic agents.
Studies have shown that PEGylated proteins are safely metabolized in two stages. First, protein components are degraded. In the case of albumin, glycoproteins gp18 and gp30 bind to albumin, tagging it for lysosomal catabolism (Larsen, Kuhlmann, Hvam & Howard, 2016; Schnitzer, Sung, Horvat & Bravo, 1992; Stehle, Sinn, Wunder, Schrenk, Stewart et al., 1997). The remaining PEG backbone is excreted through the kidneys (R. Webster et al., 2009). Safe metabolism is key for any DDS. Once the drug has been delivered, deleterious effects of the DDS or its metabolites are undesirable.

This BSA₅-R-123t-PEG₈ complex will be generated using “click” chemistry. The term denotes reactions that are modular, able to give near quantitative yields, and are stereospecific. These reactions are simple, easy to perform and insensitive to water and oxygen (Lowe, 2010).

SPAAC is a form of click chemistry in which a terminal azide is reacted with an alkyne incorporated into a cyclooctyl or cyclononyl ring. To further strain the already reactive cyclic structure, electron-withdrawing groups have been incorporated adjacent to the alkyne in order to make it even more reactive with the complimentary azide. The most commonly utilized groups are difluoro- and dibenzo- moieties (Ning, Guo, Wolfert & Boons, 2008; van Berkel et al., 2007). SPAAC allows for a simple and straightforward production method which does not require special equipment, high pressures, or elevated temperatures.

Additionally, SPAAC is a biorthogonal reaction which provides a stable, covalent linker between protein and PEG. Neither alkyne nor azide are reactive with common functional groups found in the body (Baskin & Bertozzi, 2009). This non-reactivity eliminates the possibility of side-reactions between residual, unreacted SPAAC groups and native proteins upon administration. The BSA₅-R-123t-PEG₈ complex will pass through a variety of environments as
it circulates through the body. The stable 1,2,3-triazole linker will reduce the chances of dissolution of the PEG complex before delivery of ligand (Massarotti et al., 2014).

Figure 21. Conjugation of bovine serum albumin onto PEG\textsubscript{8} via strain-promoted azide-alkyne cycloaddition. (a) BSA is first activated with DIBO-R-NHS at a single lysine. Activated BSA is attached to the PEG\textsubscript{8} backbone via SPAAC. “R” denotes a variable spacer incorporated into the activating reagent. (b) DIBO-NHS and (c) DIBO-PEG\textsubscript{4}-NHS were used to activate BSA.

In this study, dibenzocyclooctyl-functionalized BSA molecules (DIBO-R-BSA) will be conjugated onto an 8-arm PEG backbone functionalized with terminal azides (N\textsubscript{3}-PEG\textsubscript{8}) (Figure 21). BSA will be “activated” with one of two N-hydroxysuccinimidyld ester-functionalized DIBO reagents (DIBO-R-NHS) via one of BSA’s 59 native lysine residues (Cline & Hanna, 1988).

A short activator, Dibenzocyclooctyne-N-hydroxysuccinimidyld ester (DIBO-NHS), was used from the outset of this study. In an attempt to increase yields, the longer
Dibenzocyclooctyne-PEG₄-N-hydroxysuccinimidyl ester (DIBO-PEG₄-NHS) was added. It was hypothesized that once a few BSA molecules attached to the backbone, additional proteins would be unable to access the remaining PEG arms due to steric hindrance. The PEG₄ spacer provided an additional 14 Å between protein and alkyne. The reduced congestion at the attachment sites between DIBO and N₃ was expected to increase yields. Also, with easier access to the PEG backbone, heterogeneity of the product was expected to decrease, resulting in fewer species of BSAₓ-R-123t-PEG₈.

A 1:1 activation ratio of DIBO:BSA will be used and the PEGylation methodology will be optimized to give the highest yield of PEGylated protein. The degree of activation will also be determined using Fluorescein-NHS (Fluor-NHS) to mimic the interaction of BSA and the activating agents. The PEGylated complexes will then be characterized in terms of secondary structure, speciation, and ligand-binding capabilities.

**Materials and Methods**

**Materials**

Lyophilized BSA (Product Number A7906), DIBO-NHS (761524), DIBO-PEG₄-NHS (764019), Fluorescein-5-EX N-hydroxysuccinimide ester (Fluor-NHS; F9551), sulfamethoxazole (SMZ; S7507), naproxen (NPX; N8280), and all reagents not detailed were purchased from Sigma-Aldrich.

**Methods**

All experiments were carried out in 50 mM 4-Morpholinepropanesulfonic acid/0.15 M NaCl pH 7.2 (MOPS/NaCl) buffer unless otherwise noted. Fast protein liquid chromatography (FPLC) separations were performed on an ÄKTAprime plus chromatography system (Amersham Biosciences; 11001313) with MOPS/NaCl mobile phase. Resulting fractions were concentrated
with 30 kDa MW cut-off centrifuge filters (Sartorius; VS2022). Absorbance values were obtained on an Agilent 8453 UV-visible Spectroscopy System. BSA concentrations were calculated at 280 nm with an extinction coefficient of 43.824 mM⁻¹ cm⁻¹ and a MW of 66,430 Da (Hirayama, Akashi, Furuya & Fukuhara, 1990; "Thermo Scientific: Extinction Coefficients," 2013).

**Fatty acid removal.** Fatty acid-free BSA (FAF-BSA) was prepared via an activated charcoal method (Chen, 1967). In short, BSA was dissolved in MOPS/NaCl. The solution was acidified to pH 3 and stirred on ice with 20-40 mesh particle size activated charcoal in order to remove any bound fatty acids. Charcoal was pelleted at 10,000 rpm and the supernatant was filtered through 0.2 µm cellulose filters to remove any remaining carbon. The de-fatted albumin solution was returned to pH 7.2 before further purification. Dimers were removed by size exclusion chromatography (SEC) on a pre-packed 120 mL 60 cm x 16 mm S300 FPLC column (GE Healthcare; 17-1167-01).

**Activation of FAF-BSA with DIBO.** Conjugation of BSA molecules onto a 40 kDa N₃-PEG₈ backbone required two steps, activation and PEGylation (Figure 21). Table 5 details the nomenclature used to discuss these reactions.

Table 5. Nomenclature for the Conjugation of BSA onto PEG₈ via Strain-Promoted Azide-Alkyne Cycloaddition. Reaction with complimentary azide for all DIBO-functionalized BSA reactions results in a 1,2,3-triazole (123t).

<table>
<thead>
<tr>
<th>Activator</th>
<th>Activated BSA</th>
<th>PEG Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIBO-R-NHS (generic)</td>
<td>DIBO-R-BSA</td>
<td>BSAₓ-R-123t-PEG₈</td>
</tr>
<tr>
<td>DIBO-NHS (short)</td>
<td>DIBO-BSA</td>
<td>BSAₓ-123t-PEG₈</td>
</tr>
<tr>
<td>DIBO-PEG₄-NHS (long)</td>
<td>DIBO-PEG₄-BSA</td>
<td>BSAₓ-PEG₄-123t-PEG₈</td>
</tr>
</tbody>
</table>
Activation involves the attachment of a DIBO functional group onto BSA via reaction between an NHS-ester and the amino side-chain of lysine. DIBO-NHS and DIBO-PEG₄-NHS stocks were prepared in dry dimethyl sulfoxide and added to 1 mM FAF-BSA to give a final ratio of 1:1 DIBO-R-NHS:FAF-BSA. Introduction of DIBO-R-NHS was made in small aliquots with mixing between additions to avoid BSA denaturation due to the organic solvent. After two hours at room temperature, excess activator was removed by triple-rinsing the sample through 30 kDa centrifuge filters ("Thermo Scientific: NHS-Azide and NHS-Phosphine Reagents," 2014).

**PEGylation of DIBO-activated FAF-BSA.** N₃-PEG₈ (Creative PEGworks; PSB-884) was dissolved in MOPS/NaCl and added to 1 mM DIBO-activated FAF-BSA at 16:1 DIBO-BSA:N₃-PEG₈. The solution was allowed to incubate at room temperature for one week with constant stirring. PEGylated FAF-BSA was purified via a two-phase SEC separation. The bulk of unreacted FAF-BSA was removed by way of an S300 FPLC column, the remainder via a 320 mL 60 cm x 26 mm S500 column (GE Healthcare; 28-9356-07). Chromatograms from the S300 separation were processed in PrimeView 5.0 software to determine yields as the percentage of total area count.

**PEGylation optimization.** Optimization of the PEGylation methodology was determined by monitoring the amount of BSAₓ-123t-PEG₈ produced with varying PEGylation ratios, temperatures, and incubation times. PEGylation ratios were monitored at 4, 8, 16, 32, and 128:1 DIBO-BSA:N₃-PEG₈. Reactions were carried out at 4°C, room temperature, and 32°C for one week. The time trial was performed by determining the amount of product formed after two, five, and seven days of incubation. Weekly assays were performed over the course of an additional four weeks. The baseline conditions for all PEGylation reactions were 16:1 DIBO-BSA:N₃-PEG₈.
at room temperature for one week with constant stirring. Samples were passed through an S500 column and the amount of product was determined as the percentage of total area count.

**Fluorescent labeling.** The degree of activation was determined using Fluor-NHS to mimic the DIBO activators. Fluor-NHS has a MW of 590.56 $\text{g/mol}$ which is similar to both DIBO-NHS (MW 402.4) and DIBO-PEG$_4$-NHS (MW 649.69). The activation procedure was followed, substituting Fluor-NHS. Unreacted fluorophore was removed via triple rinse with 50 mM Tricine/0.15 M NaCl pH 8.5 through 30 kDa centrifugation filters. The following calculations used absorbance at 280 nm ($\lambda_{\text{max}}$ for BSA) and 495 nm ($\lambda_{\text{max}}$ for Fluor) to determine Fluor$_{\text{bound}}$:FAF-BSA ("Thermo Scientific: NHS-Fluorescein," 2016).

\[
\text{Molar} \quad \frac{\text{Fluor}}{\text{BSA}} = \frac{(\text{Abs}_{495} \times C)}{(\text{Abs}_{280} - (\text{CF} \times \text{Abs}_{495}))}
\]

\[
\text{CF} = \frac{\text{Abs}_{280} \text{ of unreacted Fluor}}{\text{Abs}_{495} \text{ of unreacted Fluor}}
\]

\[
C = \frac{(\text{MW}_{\text{BSA}} \text{ (Da)} \times E_{280}^{0.1\%})}{(\text{MW}_{\text{Fluor}} \times 195)}
\]

\[
195 = E_{495}^{0.1\%} \text{ of bound Fluor}
\]

\[
\text{MW}_{\text{Fluor}} = 475.47 \text{ Da (MW}_{\text{Fluor-NHS}} \text{ minus MW}_{\text{NHS}} \text{ which is lost upon reaction with lysine)}
\]

**Circular dichroism (CD).** The effects on secondary structure of the PEGylation procedure were determined by CD. BSA$_x$-123t-PEG$_8$ and BSA$_x$-PEG$_4$-123t-PEG$_8$ were assayed in 50 mM MOPS pH 7.2 at 30$^\circ$C on an Olis DSM 20 CD Spectrophotometer. Ellipticity was monitored between 190 nm and 260 nm. Helicity was calculated with DichroWeb (Whitmore & Wallace, 2008) and the analysis program CDSSTR (Sreerama & Woody, 2000). Inclusion of a
crystal structure for HSA (Wardell et al., 2002) allowed for use of reference set SP175 (Janes, 2009; Lees, Miles, Wien & Wallace, 2006).

**SDS-PAGE.** Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as an initial characterization of BSAX-R-123t-PEG8 species. The assay was performed on a PhastSystem (Amersham Biosciences; 18-1018-23) with a 4-15% gradient PhastGel (GE Healthcare; 17-0678-01), Coomassie R 350 stain (GE Healthcare; 17-0518-01), and high molecular weight markers (GE Healthcare; 17-0615-01).

**Analytical ultracentrifugation (AUC).** AUC was carried out in a Beckman Coulter ProteomeLab XL-A analytical ultracentrifuge using standard two sector cells with quartz windows in four- or eight-hole rotors. All sedimentation velocity experiments were performed at 50,000 rpm with a preset scan number of 100 per cell. The reference sector was filled with 450 µL of MOPS/NaCl and the sample sector was filled with 430 µL of sample with an absorbance at 280 nm between 0.75 and 1.1. Path length was 1 cm. Buffer density was measured at 20.0°C in a Mettler/Par Calculating Density Meter DMA 55A and the buffer viscosity was measured in an Anton Parr AMVn Automated Microviscometer. Data were analyzed using the program Sedfit (Schuck, 2000; "Sedfit," 2017). Molecular weights and sedimentation coefficients (S20,w) were determined using the continuous c(s) mode in the Sedfit program. Native monomeric BSA yielded an S20,w value of 4.04 S which agreed with published values (Shulman, 1953; Taylor, 1952; Zhao et al., 2015). The c(s) plots were deconvoluted using non-linear least-squares fitting algorithms available in the Origin (v. 9.0) software package (OriginLab Corporation, Northampton, MA). Data were fit to multiple Gaussian components, increasing the number of components until residual plots (data-fit) were random and the chi square value for the fit was
minimal. Values reported are percentages of the total area count of peaks corresponding to PEGylated species. These area counts were calculated via the trapezoidal formula.

**Fluorimetric analyses.** Binding studies were performed on a Photon Technology International fluorimeter using Felix software (version 1.1). SMZ, a DS1 ligand, and NPX, a DS2 ligand, were studied to determine if PEGylation affected ligand binding capabilities. The number of binding sites (n) and the association constant (K_a) were calculated for BSA_x-123t-PEG_8 and BSA_x-PEG_4-123t-PEG_8. In each case, the fluorophore monitored was Trp213, found at the entrance to DS1 in Domain IIA.

For SMZ, 30 µM stock solutions of FAF-BSA, BSA_x-123t-PEG_8, and BSA_x-PEG_4-123t-PEG_8 were prepared in 0.2 M Tris-HCl/0.1 M NaCl pH 7.4. SMZ was dissolved in methanol to a final stock concentration of 30 mM. Working SMZ stocks were prepared at 3 mM and 1 mM by dilution with Tris buffer. Monitoring emission between 300-380 nm upon excitation at 283 nm at 25°C, aliquots of working SMZ stocks were titrated into a 3 µM BSA solution. 1 mM working stock was used up to 20 µM SMZ. 3 mM working stock was used to raise the final concentration to 80 µM SMZ.

To determine n and K_a for NPX, 30 µM stock solutions of FAF-BSA, BSA_x-123t-PEG_8, and BSA_x-PEG_4-123t-PEG_8 were prepared in 10 mM phosphate buffer pH 7.0. NPX was dissolved in methanol to a final stock concentration of 45 mM. A working NPX stock was prepared at 0.15 mM by dilution with phosphate buffer. Monitoring emission between 300-380 nm upon excitation at 295 nm at 25°C, aliquots of working NPX stocks were titrated into a 0.725 µM BSA solution to a final concentration of 3 µM NPX.

Blanks for each ligand at each concentration were necessary to account for any fluorescence due to the ligand itself. SMZ had almost no signal under experimental conditions.
NPX, however, showed strong emission with maxima at approximately 350 nm. Particularly at high concentrations, NPX signal obscured the BSA maxima which ranges from 344 to 350 nm.

Non-linear regression analyses were carried out using The Gnumeric Spreadsheet (version 1.10.16) ("The Gnome Project: The Gnumeric Spreadsheet: Free, Fast, Accurate - pick any three," 2011) and a modified Hill equation. In order to normalize samples, fractional intensities (FrInt) were used in place of raw intensities.

\[
FrInt = \left( \frac{(FrInt_{\text{max}} - FrInt_{\text{min}}) \cdot [\text{Ligand}]^n}{K_d^n + [\text{Ligand}]^n} \right) + FrInt_{\text{min}}
\]

Results and Discussion

Methodology Optimization

The PEGylation portion of the polymerization procedure was optimized by varying the PEGylation ratios, temperature, and reaction time. FAF-BSA was activated at a 1:1 mole ratio with DIBO-NHS, triple-rinsed with MOPS/NaCl, and concentrated to 1 mM with 30 kDa centrifuge filters before PEGylation. Samples were evaluated for BSA_\text{x}-123t-PEG_8 content as the percent of the total area count after separation on an S500 column.
Figure 22. Optimization of the PEGylation procedure for DIBO-activated FAF-BSA. (a) PEGylation ratio and temperature were varied to produce the highest yields. (b) A time trial of PEGylation at 1:1 activation with DIBO-NHS, 16:1 DIBO-BSA:N₃-PEG₈ at room temperature.

PEGylation ratio proved to be the most effective control over product formation (Figure 22). Varying PEGylation ratios from 4:1 DIBO-BSA:N₃-PEG₈ to 128:1 showed a maximum of 31% BSAₕ-123t-PEG₈ product formed at 16:1. When this ratio was pushed to a higher excess of DIBO, the lowest yield of 4% was obtained at 128:1. This could be due to dilution of the N₃-PEG₈, resulting in less interaction between azide and alkyne. When in excess of N₃-PEG₈, a similarly low 9% is obtained. At 16:1, the optimal balance between DIBO-BSA:N₃-PEG₈ ratio and individual reactant concentrations was found.
Temperature proved to be the least effective control. Assays performed at room temperature generated the highest yields. Slightly less product was formed at a lower temperature (4°C) with similar results at an elevated temperature (37°C). The negligible difference in yields suggests an indifference to temperature.

One week of reaction time was sufficient to generate a considerable amount of product. Yields trended upwards over five weeks. While reacting for longer periods generated more product, majority of the BSA\textsubscript{x}-123t-PEG\textsubscript{8} was formed within the first seven days. Given an already extensive reaction time, additional weeks of incubation were deemed unnecessary for only slightly more product.

**Effects on Secondary Structure**

Helicity content for BSA has been reported between 56-66% (Das et al., 2017; Moriyama et al., 2008). Characteristic minima at 210 nm and 220 nm were seen in all spectra of PEGylated BSA (Figure 23). Unmodified FAF-BSA contained 59.2 ± 2.2% α-helix. Neither BSA\textsubscript{x}-123t-PEG\textsubscript{8} (63.4 ± 2.1%) nor BSA\textsubscript{x}-PEG\textsubscript{4}-123t-PEG\textsubscript{8} (59.3 ± 3.5%) showed any loss in secondary structure.
Figure 23. Circular dichroic spectra of FAF-BSA and strain-promoted azide-alkyne cycloaddition PEGylated products. FAF-BSA (---); BSA\textsubscript{x}-123\textsubscript{t}-PEG\textsubscript{8} (blue); BSA\textsubscript{x}-PEG\textsubscript{4}-123\textsubscript{t}-PEG\textsubscript{8} (orange).

Degree of Activation

A 1:1 activator:FAF-BSA mole ratio was necessary to reduce the risk of over-activation resulting in a single BSA making multiple connections to a single or multiple N\textsubscript{3}-PEG\textsubscript{8} backbones. The degree of activation was quantified using Fluor-NHS that would mimic the interactions between FAF-BSA and either activating agent. By monitoring the absorbance maxima at 280 nm and 495 nm, an average of 0.75 ± 0.04 Fluor per BSA was calculated. By controlling the mole ratio of activator:FAF-BSA, the odds of multiple attachments was greatly decreased.
BSA$_x$-R-123t-PEG$_8$ Yields

While the risk of over-PEGylation was limited, so too was the yield of PEGylated protein. By integrating chromatograms from the S300 separation (Figure 24a), yields were calculated for both DIBO reagents. The hypothesis that an additional 14 Å would be sufficient to greatly increase yields was proven incorrect. The shorter DIBO-NHS produced an average of 23.6% BSA$_x$-123t-PEG$_8$ (range: 19.5-31.0%). The longer DIBO-PEG$_4$-NHS generated only slightly more product (average: 25.3%, range: 19.1-36.5%). The additional length of the extended alkyne activating agent did not result in substantially higher yields but does suggest that further separation of the bulky BSA from the bulky backbone of PEG$_8$ may enhance yields.
Figure 24. Purification of BSA$_\text{x}$-R-123t-PEG$_8$ via size exclusion chromatography. (a) Initial purification was done on an S300. (b) Final purification was done an S500 column. FAF-BSA (---); BSA$_\text{x}$-123t-PEG$_8$ (blue); BSA$_\text{x}$-PEG$_4$-123t-PEG$_8$ (orange).
BSA<sub>r</sub>-R-123t-PEG<sub>8</sub> Speciation

Purified BSA<sub>r</sub>-123t-PEG<sub>8</sub> and BSA<sub>r</sub>-PEG<sub>4</sub>-123t-PEG<sub>8</sub> were run through an SDS-PAGE 4-15% gradient gel. A band at approximately 116 kDa corresponds to a combination of dimeric BSA and BSA<sub>r</sub>-PEG<sub>8</sub> conjugates (Figure 25). High molecular weight bands in the separation zone and a heavy band retained in the stacking zone were seen for both BSA<sub>r</sub>-123t-PEG<sub>8</sub> and BSA<sub>r</sub>-PEG<sub>4</sub>-123t-PEG<sub>8</sub>. This suggests a minimum of three to four species of PEGylated FAF-BSA were formed with either activating agent. The bands likely correspond to PEG<sub>8</sub> +2 FAF-BSA (172 kDa), +3 FAF-BSA (238 kDa), and +4 FAF-BSA (304 kDa). Smearing in the separating zone was due to the interaction of the PEG backbone with the polyacrylamide gel as well as variability of the specific lysine residue activated by the DIBO reagents. Manufacturer specifications quote the upper limit of separation for these gels at 300 kDa for non-PEGylated proteins. It is inferred that material retained in the stacking zone consists of a minimum of five FAF-BSA molecules attached to the PEG<sub>8</sub> backbone with a MW of approximately 370 kDa.
Figure 25. Electrophoretic separation of species of bovine serum albumin PEGylated via strain-promoted azide-alkyne click chemistry. Lane 1: FAF-BSA; Lane 2-3: BSA_{x}-123t-PEG_{8}; Lane 4-5: BSA_{x}-PEG_{d}-123t-PEG_{8}; Lane 6: High molecular weight marker.

The speciation of purified PEGylated BSA was examined by AUC. The majority of the PEGylated species generated with both activators were complexes containing BSA_{2-4}. These species accounted for 55% of BSA_{x}-123t-PEG_{8} (Figure 26) and 65% of BSA_{x}-PEG_{d}-123t-PEG_{8} (Figure 27). BSA_{5-7}-R-123t-PEG_{8} conjugates were present in much lower concentrations, making up 31% and 14% of short and long activator species, respectively. The shorter DIBO-BSA resulted in 8% BSA_{1} species while the longer DIBO-PEG_{d}-BSA generated 19%. The peaks found at S_{20,w} values greater than 15 S are thought to be a mixture of BSA_{8-R}-123t-PEG_{8} species and complexes formed from over-activated protein.
Figure 26. Analytical ultracentrifugation analysis of BSA<sub><i>x</i></sub>-123t-PEG<sub><i>y</i></sub>. % Area Count values are the percent of PEGylated species only. nr: not reported; n/a: not applicable.
Figure 27. Analytical ultracentrifugation analysis of BSA-x-PEG_{4}-123t-PEG_{8}. % Area Count values are the percent of PEGylated species only. nr: not reported; n/a: not applicable.

AUC results corresponded well with the SDS-PAGE analysis. Each purified sample contained BSA_{1}-R-123t-PEG_{8} with a much higher degree of BSA_{2:4}-R-123t-PEG_{8}. Higher
molecular weight material was present but a lower concentration. The longer DIBO-PEG₄-NHS generated only slightly more PEGylated species than DIBO-NHS based on SEC data. However, the distribution of those species was smaller, generating a more homogenous final product.

**Ligand Binding Capabilities**

To compare the effects of PEGylation on the ability of conjugated BSA to bind ligands, fluorescent emission intensity of Trp213 was monitored. The ability of PEGylated species to bind ligands in DS2 was probed with NPX. Crystal structures of de-fatted bovine, equine, and leporine serum albumins complexed with NPX have been reported (Bujacz et al., 2014). In each species of serum albumin, NPX preferentially binds to DS2. A second molecule of NPX binds to fatty acid binding site 6. In leporine serum albumin, a tertiary site for NPX is found in a superficial cleft in subdomain IIIA while in BSA, the tertiary binding site is DS1. Experimental values for NPX in the presence of FAF-BSA, BSAₓ-123t-PEG₈, and BSAₓ-PEG₄-123t-PEG₈ were consistent with published values (Table 6). NPX was tightly bound by all species of BSA.

Table 6. Association Constants (Kₐ) and Number of Binding Sites (n) for BSAₓ-123t-PEG₈ and BSAₓ-PEG₄-123t-PEG₈ for Sulfamethoxazole and Naproxen.

<table>
<thead>
<tr>
<th>Sample</th>
<th>SMZ</th>
<th>NPX</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kₐ (x10⁻³ M⁻¹)</td>
<td>n</td>
</tr>
<tr>
<td>FAF-BSA</td>
<td>2.03 ± 0.18</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>BSAₓ-123t-PEG₈</td>
<td>0.39 ± 0.29</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>BSAₓ-PEG₄-123t-PEG₈</td>
<td>0.15 ± 0.06</td>
<td>0.8 ± 0.05</td>
</tr>
<tr>
<td>Lit. values¹²</td>
<td>3.55-15.6</td>
<td>0.9-1.0</td>
</tr>
</tbody>
</table>

¹ SMZ values were published by Naik et al. (2009; 2015), Rajendiran & Thulasidhasan (2015), and Wang et al. (2014).

² NPX values were reported by Banerjee et al. (2006), Bou-Abdallah et al. (2016), Fielding et al. (2005), and Honoré & Brodersen (1984). Bujacz et al. (2014) have reported a crystal structure (PDB ID: 4OR0) for BSA complexed with three NPX molecules.
Binding of NPX to FAF-BSA, BSA_{x}-123t-PEG_{8}, and BSA_{x}-PEG_{4}-123t-PEG_{8} showed similar trends (Figure 28). BSA in the absence of NPX had a maximum emission wavelength at 344 nm. With increasing amounts of NPX, a red shift resulted in a maximum at 350 nm. This is consistent with binding to a site adjacent to the fluorophore. In this case, the fluorophore studied was Trp213 found in Domain IIA at the mouth of DS1. As ligand binds to DS2, slight conformational changes exposed Trp213 to the solvent, causing a red shift.
Figure 28. Fluorimetric spectra and non-linear regression of $\text{BSA}_x$-123t-PEG$_8$ and $\text{BSA}_x$-PEG$_4$-123t-PEG$_8$ in the presence of naproxen. Spectra for (a) $\text{BSA}_x$-PEG$_4$-123t-PEG$_8$ and (b) FAF-BSA in the presence of increasing concentrations of NPX. $\text{BSA}_x$-123t-PEG$_8$ spectra followed similar trends as both FAF-BSA and $\text{BSA}_x$-PEG$_4$-123t-PEG$_8$. (c) Non-linear regression analyses for FAF-BSA (black $\blacklozenge$), $\text{BSA}_x$-123t-PEG$_8$ (blue $\bigtriangleup$), and $\text{BSA}_x$-PEG$_4$-123t-PEG$_8$ (orange $\circ$). Experimental data is represented as symbols while calculated data points are lines.
DS1, however, appears to be greatly affected by the covalent attachment of PEG₈. FAF-BSA values were similar to published values for both $K_a$ and $n$ for SMZ studies (Table 6). However, both BSAₓ-123t-PEG₈ and BSAₓ-PEG₄-123t-PEG₈ demonstrated a greatly reduced affinity for SMZ. Spectra for all forms of BSA followed similar trends (Figure 29). Intensities decreased as SMZ concentration increased. The signal from Trp213 was quenched in the presence of the ligand though it was not tightly bound in either form of PEGylated BSA.
Figure 29. Fluorimetric spectra and non-linear regression of BSA\textsubscript{x}-R-123t-PEG\textsubscript{8} in the presence of sulfamethoxazole. Spectra for (a) BSA\textsubscript{x}-123t-PEG\textsubscript{8} and (b) FAF-BSA in the presence of increasing concentrations of SMZ. BSA\textsubscript{x}-PEG\textsubscript{4}-123t-PEG\textsubscript{8} spectra followed similar trends as both FAF-BSA and BSA\textsubscript{x}-123t-PEG\textsubscript{8}. (c) Non-linear regression analyses for FAF-BSA (black ◊), BSA\textsubscript{x}-123t-PEG\textsubscript{8} (blue △), and BSA\textsubscript{x}-PEG\textsubscript{4}-123t-PEG\textsubscript{8} (orange ○). Experimental data is represented as symbols while calculated data points are lines.
Similar results were seen for PEGylation at Cys34 with maleimido-PEG$_8$. Upon modification of this single residue in Domain IA, the affinity for SMZ was reduced to 0.19 x 10$^3$ M$^{-1}$. It was hypothesized that conformational changes induced by PEGylation at Cys34 altered the structure of DS1 which is found in Domains IB and IIA. The conditions employed for SPAAC PEGylation allow for selective modification of lysines, but do not preferentially alter specific lysines on the surface of BSA. Given the results from the modification of Cys34, it is possible that lysines in Domain I are preferentially altered by DIBO-R-NHS activators.

**Conclusions**

The conjugation of BSA onto a PEG$_8$ backbone via SPAAC proved successful using both forms of DIBO activator. The PEGylation process proved to be most dependent on the ratio of DIBO-BSA:N$_3$-PEG$_8$ with an optimal reaction ratio of 16:1.

The shorter DIBO-NHS PEGylated an average of 23% FAF-BSA. The extended DIBO-PEG$_4$-NHS yielded only slightly more product at 25%. The additional 14 Å was of little assistance in terms of yield. The longer activating agent did, however, produce a more homogenous final product, generating a majority of BSA$_{2-4}$-R$_{123t}$-PEG$_8$ species.

In terms of ligand binding capabilities, neither activating agent greatly affected BSA’s ability to bind NPX, a model DS2 ligand. DS1 was affected by PEGylation as evidenced by greatly reduced association constants.

The PEGylation procedure proved to be successful regardless of the activating agent. High molecular weight complexes of FAF-BSA were still capable of binding ligands to one of the main drug binding sites. These complexes show promise as a possible drug delivery method.
CHAPTER V
STUDIES ON THERMAL STABILITY AND
THE EFFECTS OF PASTEURIZATION ON
PEGYLATED MULTI-ALBUMIN COMPLEXES

Introduction

PEGylation has become a common method of increasing circulatory lifetimes of protein-based therapies. Due to increased hydrodynamic volume conferred by the attachment of a polyethylene glycol (PEG) backbone, the PEGylated complexes appear several times larger than molecular weight would suggest (Plesner, Fee, Westh & Nielsen, 2011; Veronese, Mero & Pasut, 2009). Increased size allows the particle to escape elimination and remain in circulation for longer periods of time.

In order to increase solubility and provide protection for hydrophobic drugs, serum albumins have been incorporated into drug carriers (Anhorn, Wagner, Kreuter, Langer & von Briesen, 2008; Desai, 2008). Bovine serum albumin (BSA) contains a multitude of binding sites which are tailored for endogenous molecules including fatty acids and heavy metals (Bhattacharya, Grüne & Curry, 2000; Rózga, Sokolowska, Protas & Bal, 2007). Fatty acid binding pockets, have been found to bind drugs but are typically secondary or tertiary options. The primary hydrophobic cavities for drug binding were initially characterized by Sudlow et al. (1975, 1976). Deemed Drug Sites I and II (DS1 and DS2), these sites are able to house a variety of ligands (Ghuman et al., 2005; Yamasaki, Chuang, Maruyama & Otagiri, 2013).
DS1 is found at the interface of Domains IB and IIA while DS2 resides in Domain IIIA. Access to these sites is controlled via flexing of the local tertiary structure.

BSA contains three highly helical domains (I, II, and III), each divided into two subdomains (A and B) which are connected by long loops. Conformational flexibility of each domain is conferred by helical bending and subdomain orientation is controlled by movement of the loops. These types of movements open and close the various binding sites dotted throughout the structure.

While the exterior of albumin is somewhat flexible, the interior of the protein is rigidly maintained via 17 disulfide bridges. Subdomain A is stapled together by four of these while two bridges stabilize subdomain B. An exception is Subdomain IA, which contains only three bridges (Bujacz, 2012). A single free cysteine at position 34 is found on the surface of Domain I. This superficial residue allows for selective, covalent attachment of a PEG chain.

Modifications of cysteines are often made via thiol-maleimide reactions, resulting in a thioether linkage (Anhorn et al., 2008; Manjula et al., 2003; K. D. Webster et al., 2017). This class of reaction has become attractive due to typically simple reaction conditions as it can readily proceed in an aqueous environment and requires neither catalyst nor high temperatures.

In addition to this free cysteine, 59 lysines are scattered throughout BSA. Terminal amino groups allow for modification with a number of different functional groups. One of the more common reactions is via an N-hydroxysuccinimide (NHS) ester (Cline & Hanna, 1988). The reactivity between NHS and lysine provides a convenient way to anchor another functional group to the protein.

The abundance and easy access to these lysines allows for attachment of a foreign moiety without the need for recombinant techniques. Strain-promoted azide-alkyne cycloaddition
(SPAAC), a class of “click” chemistry, results a highly stable 1,2,3-triazole linker. Additionally, both azide and alkyne are biorthogonal in that they are absent from nature and non-reactive with naturally-occurring functional groups (Agard, Prescher & Bertozzi, 2004; Baskin & Bertozzi, 2009). By using the NHS-lysine reaction, a strained alkyne may be covalently attached to BSA. PEG functionalized with the complimentary azido moiety may then be reacted with the alkyne-activated BSA to link the two molecules.

PEGylation, while providing many benefits to the modified protein, have also been shown to affect thermal stability, which may be described by the temperatures of denaturation ($T_m$) and aggregation ($T_{agg}$). $T_m$ is the temperature at which a protein is 50% unfolded (K. W. Olsen, 1994). $T_{agg}$ describes the ability of both native and non-native forms of a protein to form oligomerize (Plesner et al., 2011; Wetzel et al., 1980). Both are important parameters to consider during the development of protein-based drug delivery systems as they factor into shelf-life and terminal sterilization.

In the case of BSA, typical $T_m$ is approximately 82°C as determined by differential scanning calorimetry (Plesner et al., 2011). Attachment of a single linear 40 kDa PEG chain at Cys34, lowered $T_m$ to 80°C. $T_{agg}$, on the other hand, increased upon PEGylation. Unmodified BSA aggregated at 71°C while the 40 kDa PEG-BSA species had a $T_{agg}$ of 82°C.

Steam sterilization, or autoclaving, is the most common approach used in pharmaceutical production. Materials are heated to 121°C at 1.5 bar for a minimum of 15 minutes. These conditions are likely to denature protein components. For this reason, protein-based formulations are sterilized aseptically, an approach where each component is sterilized individually before preparation of the final product (Baez & Assaf-Anid, 2008; Yaman, 2001).
Albumin-based components are pasteurized at 60°C for ten hours. In order to maintain helicity, the stabilizing reagents N-acetyl-L-tryptophan (NAT) and sodium caprylate are included at mole ratios of 5.5:1 stabilizer:albumin (Anraku et al., 2004; Christiansen & Skotland, 2010; Farcet, Kindermann, Modrof & Kreil, 2012; Gellis et al., 1948).

High concentrations of these stabilizers have been implicated in the reduced efficacy of albumin solutions administered to patients with impaired liver function. For these patients, albumin, which is produced in the liver, is typically found at extremely low concentrations. Exogenous albumin, which has been pasteurized with NAT and caprylate, is often administered intravenously to compensate. Both reagents have been shown to have an affinity for DS2, a site also shared by metabolites commonly formed at high levels in patients with liver disease (Kawai et al., 2017).

In healthy individuals, NAT and caprylate bind to albumin. Albumin production is unimpaired and routine transport of NAT, caprylate, and other metabolites is unaffected. In impaired individuals, elevated levels of these toxins are often found due to direct competition with stabilizers for a single high affinity binding site (Stange et al., 2011).

These additives also pose a potential problem for production of albumin-based drug delivery systems. Their preference for DS2 results in competition between NAT, caprylate, and the drug molecule being administered. For naproxen (NPX), a known DS2 ligand, affinity was 40 times lower in pharmaceutical-grade albumin solutions (i.e. pasteurized with NAT and caprylate) than in research-grade albumin (i.e. un-pasteurized) (H. Olsen, Andersen, Nordbo, Kongsgaard & Bormer, 2004).

In this study, the effects of PEGylation on the thermal stability of BSA were examined. Three PEGylated BSA complexes were prepared. In the first case, native Cys34 of BSA was
used as a site of direct attachment of an eight-armed PEG backbone with terminal maleimides (Mal-PEG₈) resulting in a thioether linker. For the two others, BSA were attached to PEG₈ via SPAAC click chemistry. BSA was “activated” with either a short- or long-chain dibenzocyclooctyne-functionalized N-hydroxysuccinimidy ester (DIBO-NHS; DIBO-PEG₄-NHS). Activated BSA were attached to an azido-functionalized PEG₈ backbone (N₃-PEG₈) resulting in a 1,4-disubstituted, 1,2,3-triazole. Changes in the absorbance at 300 nm as a function of temperature were used to calculate \( T_{agg} \) while \( T_m \) was determined for each PEG species by circular dichroism (CD). PEGylated BSA, pre-loaded with NPX, were pasteurized in the absence of NAT and caprylate to determine if a combination of PEGylation and bound ligand would allow for omission of these stabilizing compounds.

**Materials and Methods**

**Materials**

Lyophilized BSA (Product Number A7906), DIBO-NHS (761524), DIBO-PEG₄-NHS (764019), NPX (N8280), and all reagents not detailed were purchased from Sigma-Aldrich.

**Methods**

All experiments were carried out in 50 mM 4-Morpholinepropanesulfonic acid/0.15 M NaCl pH 7.2 (MOPS/NaCl) buffer unless otherwise noted. Fast protein liquid chromatography (FPLC) separations were performed on an ÄKTApure plus chromatography system (Amersham Biosciences; 11001313) with MOPS/NaCl mobile phase. Resulting fractions were concentrated with 30 kDa molecular weight cut-off centrifuge filters (Sartorius; VS2022). Absorbance values were obtained on an Agilent 8453 UV-visible Spectroscopy System at 280 nm. Concentrations of BSA were calculated with an extinction coefficient of 43.824 mM⁻¹ cm⁻¹ and a molecular weight

**Fatty acid removal.** Fatty acid-free BSA (FAF-BSA) was prepared via an activated charcoal method (Chen, 1967). In short, BSA was dissolved in MOPS/NaCl. The solution was acidified to pH 3 and stirred on ice with 20-40 mesh particle size activated charcoal in order to remove any bound fatty acids. Charcoal was pelleted at 10,000 rpm and the supernatant was filtered through 0.2 µm cellulose filters to remove any remaining carbon. The de-fatted albumin solution was returned to pH 7.2 before further purification. Dimers were removed by size exclusion chromatography (SEC) on a pre-packed 120 mL 60 cm x 16 mm S300 FPLC column (GE Healthcare; 17-1167-01).

**PEGylation of FAF-BSA.** Table 7 details the nomenclature used to discuss these reactions. Cys34, requiring no further activation, was attached to PEG₈ with a thioether (te) linkage formed by reaction with Mal-PEG₈. For SPAAC reactions, the complimentary azide for all DIBO-functionalized BSA reactions was N₃-PEG₈, resulting in a 1,2,3-triazole (123t).

<table>
<thead>
<tr>
<th>Activator</th>
<th>Activated BSA</th>
<th>PEG₈</th>
<th>PEG Product</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n/a</td>
<td>n/a</td>
<td></td>
</tr>
<tr>
<td>Thiol-Maleimide</td>
<td></td>
<td>Mal-PEG₈</td>
<td>BSAₓ-te-PEG₈</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strain-Promoted Azide-Alkyne Cycloaddition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DIBO-R-NHS (generic)</td>
<td>DIBO-R-BSA</td>
<td>BSAₓ-R-123t-PEG₈</td>
<td></td>
</tr>
<tr>
<td>DIBO-NHS (short)</td>
<td>DIBO-BSA</td>
<td>N₃-PEG₈</td>
<td>BSAₓ-123t-PEG₈</td>
</tr>
<tr>
<td>DIBO-PEG₄-NHS (long)</td>
<td>DIBO-PEG₄-BSA</td>
<td></td>
<td>BSAₓ-PEG₄-123t-PEG₈</td>
</tr>
</tbody>
</table>
**PEGylation of Cys34.** 40 kDa Mal-PEG₈ (Creative PEGworks; PSB-864) was dissolved in MOPS/NaCl and added to 1 mM FAF-BSA at 8:1 FAF-BSA:Mal-PEG₈. The solution was allowed to incubate at room temperature for one week with constant stirring.

**PEGylation with SPAAC.** Conjugation of BSA molecules onto a 40 kDa N₃-PEG₈ backbone required two steps, activation and PEGylation. Activation involves the attachment of a DIBO functional group onto BSA via reaction between an NHS-ester and the amino side-chain of lysine. DIBO-NHS and DIBO-PEG₄-NHS stocks were prepared in dry dimethyl sulfoxide and added to 1 mM FAF-BSA to give a final ratio of 1:1 DIBO-R-NHS:FAF-BSA. Introduction of DIBO-R-NHS was made in small aliquots with mixing between additions to avoid BSA denaturation due to the organic solvent. After two hours at room temperature, excess activator was removed by triple-rinsing the sample through 30 kDa centrifuge filters ("Thermo Scientific: NHS-Azide and NHS-Phosphine Reagents," 2014).

N₃-PEG₈ (Creative PEGworks; PSB-884) dissolved in MOPS/NaCl was added to 1 mM DIBO-activated FAF-BSA at 16:1 DIBO-R-BSA:N₃-PEG₈. The solution was allowed to incubate at room temperature for one week with constant stirring.

**Purification of PEGylated BSA.** PEGylated FAF-BSA was purified via a two-phase SEC separation. The bulk of unreacted FAF-BSA was removed by way of an S300 FPLC column, the remainder via a 320 mL 60 cm x 26 mm S500 column (GE Healthcare; 28-9356-07).

**Spectroscopic evaluation of aggregation.** T_{agg} was determined by monitoring absorbance at 300 nm as samples were heated from 30°C to 100°C over two hours. Samples were initially assayed in MOPS/NaCl. Due to a lack of aggregation of the PEGylated samples, the buffer was further modified with 1.5 M Guanidinium chloride (GdmCl) and 0.4% (v/v) 2-Mercaptoethanol (βME). The maximum temperature was held for an additional 20 minutes to
ensure the maximum absorbance had been reached. $T_{agg}$ was calculated following the two-state method detailed by Olsen (1994).

Additionally, the absorbance at 300 nm was monitored for samples prepared in MOPS/NaCl/0.4% βME/1.5 M GdmCl at 30°C for 2 hours 20 minutes. This ensured that unfolding was not due solely to the high concentrations of denaturing reagents.

**Circular dichroic evaluation of denaturation.** The effect of PEGylation on the thermal stability of each of these complexes was determined by CD. Samples were diluted to 0.15 mg/mL in 50 mM MOPS pH 7.2 and heated from 30°C to 90°C in an Olis DSM 20 CD Spectrophotometer. Spectra were collected at 30, 40, 50, 60, 63, 66, 70, 80, and 90°C over the range of 190-260 nm with a 1 nm step-size. Helicity was calculated with DichroWeb (Whitmore & Wallace, 2008) and the analysis program CDSSTR (Sreerama & Woody, 2000). Inclusion of a crystal structure for human serum albumin (Wardell et al., 2002) allowed for use of reference set SP175 (Janes, 2009; Lees, Miles, Wien & Wallace, 2006). Helicity at each temperature was compared to that of 30°C to calculate the % Remaining Helicity. A plot of % Remaining Helicity as a function of temperature was used to calculate $T_m$.

**Pasteurization studies.** To determine the necessity for the stabilizing agents NAT and caprylate in pasteurization, PEGylated BSA samples were diluted to 0.15 mg/mL in 50 mM MOPS pH 7.2 and incubated at 60°C for ten hours in the presence and absence of NPX. A 150 mM NPX stock prepared in methanol was further diluted to a working stock concentration of 0.5 mM with MOPS buffer. Samples were loaded at a mole ratio of 3:1 BSA:NPX prior to incubation. Control samples were held at 4°C for the duration of all experiments. All samples were brought to room temperature before further analysis.
Circular dichroic evaluation of secondary structure. CD spectra of pasteurized samples were collected at 30°C under the conditions described above. For neat solutions of NPX, no signal was observed for NPX under these conditions.

Fluorimetric evaluation of naproxen binding. Binding studies were performed on a Photon Technology International fluorimeter using Felix software (version 1.1). NPX was studied to determine if pasteurization in the absence of stabilizing agents affected ligand binding in DS2. The number of binding sites (n) and the association constant (K_a) were calculated.

30 kDa centrifuge filters were used to concentrate pasteurized BSA and exchange MOPS buffer for 10 mM phosphate buffer pH 7.0. 30 µM BSA stock solutions were prepared in phosphate buffer. NPX was dissolved in methanol to a final stock concentration of 45 mM. A working NPX stock was prepared at 0.15 mM by dilution with phosphate buffer. Monitoring emission between 300-380 nm upon excitation at 295 nm at 25°C, aliquots of working NPX stocks were titrated into a 0.725 µM BSA solution to a final concentration of 3 µM NPX.

NPX blanks at each concentration were necessary to account for any fluorescence due to the ligand itself. NPX showed strong emission with maxima at approximately 350 nm. Particularly at high concentrations, NPX signal obscured the BSA maxima which ranges from 344 to 350 nm.

Non-linear regression analyses were carried out using The Gnumeric Spreadsheet (version 1.10.16) ("The Gnome Project: The Gnumeric Spreadsheet: Free, Fast, Accurate - pick any three," 2011) and a modified Hill equation. In order to normalize samples, fractional intensities (FrInt) were used in place of raw intensities.
\[
\text{FrInt} = \frac{(\text{FrInt}_{\text{max}} - \text{FrInt}_{\text{min}}) \cdot [\text{Ligand}]^n}{K_d^n + [\text{Ligand}]^n} + \text{FrInt}_{\text{min}}
\]

\[
\text{FrInt} = \frac{(\text{Int}_1 \mu M \text{Ligand} - \text{Int}_0 \mu M \text{Ligand})}{(\text{Int}_{\text{max}} \mu M \text{Ligand} - \text{Int}_0 \mu M \text{Ligand})}
\]

\[
K_a = \frac{1}{K_d}
\]

**Results and Discussion**

**Temperature of Aggregation (T_{agg})**

Initial experiments were performed in MOPS/NaCl (Figure 30a). However, neither FAF-BSA nor any of the PEGylated species peaked under these conditions. In order to further weaken the secondary structure of BSA, 0.4% (\(^{\nu}/\nu\)) \(\beta\)ME was added to MOPS/NaCl to reduce the 17 disulfide bridges of BSA. FAF-BSA and BSA\(_{x}\)-123t-PEG\(_8\) denatured and aggregated under these conditions but BSA\(_{x}\)-te-PEG\(_8\) and BSA\(_{x}\)-PEG\(_4\)-123t-PEG\(_8\) still did not peak (Figure 30b). MOPS/NaCl/0.4% (\(^{\nu}/\nu\)) \(\beta\)ME was further modified with 1.5 M GdmCl. With a high concentration of a strong chaotrope and reducing agent, all species of BSA peaked (Figure 30c). The decrease in signal after maximum absorbance was due to aggregates precipitating and settling. Samples incubated at 30°C for the full run-time showed no change in absorbance at 300 nm, indicating that denaturation was not due solely to the presence of high concentrations of GdmCl and \(\beta\)ME.

FAF-BSA had the lowest T_{agg} of 72.5°C (Table 8). BSA\(_{x}\)-123t-PEG\(_8\) (91.8°C) and BSA\(_{x}\)-PEG\(_4\)-123t-PEG\(_8\) (92.9°C) had significantly higher T_{agg} values with similar peak shapes. BSA\(_{x}\)-te-PEG\(_8\) reached the 50% mark at a higher temperature (77.5°C) than FAF-BSA but the signal began increasing at a lower temperature.
Table 8. Temperatures of Denaturation and Aggregation for PEGylated BSA Species.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$T_{\text{agg}}$ ($^\circ\text{C}$)</th>
<th>$T_{\text{m}}$ ($^\circ\text{C}$)</th>
<th>$\Delta T_{\text{m}}$ ($^\circ\text{C}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAF-BSA</td>
<td>72.5 ± 5.3</td>
<td>81.2 ± 4.1</td>
<td>0</td>
</tr>
<tr>
<td>BSAx-te-PEG$_8$</td>
<td>77.5 ± 5.3</td>
<td>77.7 ± 0.9</td>
<td>-3.5</td>
</tr>
<tr>
<td>BSA$_\chi$-123t-PEG$_8$</td>
<td>91.8 ± 2.0</td>
<td>69.4 ± 3.6</td>
<td>-11.8</td>
</tr>
<tr>
<td>BSA$_\chi$-PEG$_4$-123t-PEG$_8$</td>
<td>92.9 ± 2.6</td>
<td>76.0 ± 3.2</td>
<td>-5.2</td>
</tr>
</tbody>
</table>

The broadening of the BSA$_\chi$-te-PEG$_8$ peak was attributed to the dissolution of the thioether linker in the presence of high concentrations of excess thiol in the form of βME. Studies have explored the dissociation of antibody-drug conjugates due to this phenomenon (Alley et al., 2008; Shen et al., 2012). Following intravenous administration to mice, the thioether linkage reverted back to thiol-containing antibody and maleimido-functionalized drug, which covalently bound to Cys34 of native serum albumin. To stabilize the thioether, bromination of the maleimide moiety and hydrolysis of the succinimide to succinic acid have been explored and provide intriguing options (Fontaine, Reid, Robinson, Ashley & Santi, 2015; Smith et al., 2015).

The shape of BSA$_\chi$-te-PEG$_8$ in Figure 30c is due to the gradual dissolution of the thioether and subsequent denaturation and aggregation of the released BSA. The triazole of BSA$_\chi$-R-123t-PEG$_8$ species provides a thermally stable linker and results in a sharper peak.
Figure 30. Change in absorbance as a function of temperature for the determination of $T_{agg}$. (a) Aggregation was minimal in MOPS/NaCl. (b) Addition of βME to the buffer resulted in aggregation of some species. (c) All samples aggregated at measurable temperatures in 50 mM MOPS/0.15 M NaCl/0.4% βME/1.5 M GdmCl pH 7.2. FAF-BSA (black); BSA$_x$-te-PEG$_8$ (red); BSA$_x$-123t-PEG$_8$ (blue); BSA$_x$-PEG$_4$-123t-PEG$_8$ (orange). Dashed lines correspond to 50% aggregation.
Temperature of Denaturation ($T_m$)

CD spectra for all species of BSA showed characteristic minima at 210 nm and 220 nm at 30°C, indicating highly $\alpha$-helical protein (Figure 31). The helicity for each species ranged between 59-63% which is in agreement with published values of 56-66% (Das et al., 2017; Moriyama et al., 2008). As temperature increased, these valleys became less defined and helicity decreased.

Figure 31. Temperature-induced changes in circular dichroic spectra. Definition of valleys at 210 and 220 nm of FAF-BSA was lost as temperature increased. BSA$_x$-te-PEG$_8$, BSA$_x$-123t-PEG$_8$, and BSA$_x$-PEG$_4$-123t-PEG$_8$ followed this same trend.
T_m was calculated as the temperature at which 50% of the original helical content was lost. For FAF-BSA and all three types of PEGylated complexes, helicity slowly decreased up to 60°C where the values dropped rapidly to minima at 90°C (Figure 32).

Figure 32. Change in helicity as a function of temperature for the determination of T_m. (a) FAF-BSA; (b) BSA_x-te-PEG_8; (c) BSA_x-123t-PEG_8; (d) BSA_x-PEG_4-123t-PEG_8. n/a: not applicable.

Unmodified FAF-BSA had the highest T_m at 81.2°C (Table 8). This is in agreement with the published value of 82.5°C (Plesner et al., 2011).

The effect seen with PEGylated species on T_m is not due to the type or size of PEG backbone used as evidenced by BSA_x-te-PEG_8. For modifications at Cys34, neither a straight-chain nor branched PEG caused a significant change in denaturation temperature. Attachment of
a branched 40 kDa PEG\textsubscript{8} backbone resulted in a decrease in T\textsubscript{m} by 3.5°C, which is similar to the published 2°C decrease with a straight chain 40 kDa Mal-PEG. As reported by Plesner et al. (2011), the decrease in T\textsubscript{m} of PEGylated BSA was approximately 2°C for all species of linear 5-60 kDa PEG, regardless of the molecular weight of the PEG chain.

The drastic change in lysine-modified PEGylated BSA may be related to the proximity of DIBO to the surface of the protein. Modification of a lysine with the extended DIBO-PEG\textsubscript{4}-NHS activator resulted in a decrease in T\textsubscript{m} by 5.2°C while the largest difference of 11.8°C was seen with the shorter DIBO-NHS activator. DIBO, being a bulky, hydrophobic group, is capable of altering the surface chemistry of BSA. This is likely the case for BSA\textsubscript{x}-123t-PEG\textsubscript{8}, where the activating agent lacks a spacer. In BSA\textsubscript{x}-PEG\textsubscript{4}-123t-PEG\textsubscript{8}, the added space between DIBO and the surface of BSA lessened the effect of hydrophobic interactions. Addition of a nonpolar patch onto the exterior of BSA greatly affected solvent interaction and may have promoted denaturation.

**Effects of Pasteurization on Secondary Structure and Ligand Binding**

The effects of pasteurization without stabilizing reagents was monitored by CD and NPX binding studies. In the absence of NPX, up to 18% of total helicity was lost (Figure 33). BSA\textsubscript{x}-PEG\textsubscript{4}-123t-PEG\textsubscript{8} lost only 7%, further strengthening the argument that increasing the distance between protein and PEG\textsubscript{8} may further stabilize the complex.

Helicity was maintained after addition of NPX at 4°C. Upon pasteurization, all samples consistently lost 7-10% of the original helicity. NPX has been found to bind with an association constant (\(K_a\)) on the order of \(10^7\) M\(^{-1}\). Addition of a tightly bound ligand proved to stabilize all of these species of BSA. This is most significant for BSA\textsubscript{x}-te-PEG\textsubscript{8} as helicity was restored to levels comparable to BSA\textsubscript{x}-R-123t-PEG\textsubscript{8} species.
Figure 33. Helicity after pasteurization in the absence and presence of naproxen. Samples held at 4°C in the absence of NPX were treated as controls and set to 100% helicity (---). All other values were calculated against these controls.
A significant amount of helicity was lost upon pasteurization of BSA species without ligand bound. Ligand binding capabilities of these samples was determined using NPX to probe the high affinity site DS2.

Prior to pasteurization, all samples were able to tightly bind NPX with $K_a$ values in the range of $10^7 \text{ M}^{-1}$ which correspond well with published values (Figure 34). After pasteurization, these values decreased by several fold for all species of BSA except BSA$_{x}$-123t-PEG$_8$. This suggests major structural changes that greatly affect DS2.
Before pasteurization

<table>
<thead>
<tr>
<th>Sample</th>
<th>$K_a$ (x10^7 M$^{-1}$)</th>
<th>n</th>
<th>After pasteurization</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAF-BSA</td>
<td>0.40 ± 0.13</td>
<td>0.4 ± 0.1</td>
<td>0.09 ± 0.05</td>
</tr>
<tr>
<td>BSA$_x$-te-PEG$_8$</td>
<td>1.99 ± 0.17</td>
<td>0.9 ± 0.3</td>
<td>0.15 ± 0.00</td>
</tr>
<tr>
<td>BSA$_x$-123t-PEG$_8$</td>
<td>0.79 ± 0.61</td>
<td>2.4 ± 1.1</td>
<td>0.57 ± 0.26</td>
</tr>
<tr>
<td>BSA$_x$-PEG$_4$-123t-PEG$_8$</td>
<td>3.55 ± 1.6</td>
<td>0.9 ± 0.2</td>
<td>0.03 ± 0.00</td>
</tr>
<tr>
<td>Lit. values$^1$</td>
<td>0.12-3.70</td>
<td>0.6-3</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Lit. values$^1$ were reported by Banerjee et al. (2006), Bou-Abdallah et al. (2016), Fielding et al. (2005), and Honoré & Brodersen (1984). Bujacz et al. (2014) have reported a crystal structure (PDB ID: 4OR0) for BSA complexed with three NPX molecules. n/a: not applicable.

Figure 34. Non-linear regression analyses of fluorimetric assays of PEGylated bovine serum albumin before and after pasteurization. FAF-BSA (black ◇); BSA$_x$-te-PEG$_8$ (red □); BSA$_x$-123t-PEG$_8$ (blue △); BSA$_x$-PEG$_4$-123t-PEG$_8$ (orange ○).
Conclusions

Attachment of a PEG chain to BSA clearly affected the thermal stability of the protein. PEGylation, while lowering $T_m$, increased $T_{agg}$. Use of a short activating agent resulted in alteration of the surface of BSA due to interaction with the hydrophobic DIBO group, resulting in a marked decrease in $T_m$. Added space between the protein surface and the DIBO group lessened the effect of the nonpolar DIBO.

Mal-PEG$_8$ attached at the lone free thiol of BSA showed an increase in $T_{agg}$ but peak broadening may be due to the dissolution of the thioether linker. Both BSA$_x$-123t-PEG$_8$ and BSA$_x$-PEG$_4$-123t-PEG$_8$ effectively blocked aggregation, as seen by considerably higher $T_{agg}$.

PEGylation and pre-loading of complexes with a high affinity ligand are insufficient measures for maintenance of helicity during pasteurization. Sterilization of PEG species in the absence of ligand resulted in significant loss of helicity along with reduction of ligand binding capabilities. Pre-loading PEGylated BSA species mitigated these losses but still resulted in some loss of helicity. A combination of effects is needed to stabilize the complex. Binding NPX prior to pasteurization helps to prevent denaturation while PEGylation if more effective at preventing aggregation.
CHAPTER VI
GENERAL CONCLUSIONS AND FUTURE RESEARCH

General Conclusions

Three different species of PEGylated BSA have been developed using thiol-maleimide and strain-promoted azide-alkyne cycloaddition “click” chemistries. All three products were mixtures of predominantly BSA$_{2-4}$-R-PEG$_8$ with some amount of unmodified BSA and higher molecular weight species. BSA$_x$-PEG$_4$-123t-PEG$_8$ was the most homogeneous sample, containing the fewest higher molecular weight species while BSA$_x$-te-PEG$_8$ was the least homogeneous, containing a large number of higher molecular weight species. The wider distribution of BSA$_x$-te-PEG$_8$ was unexpected due to the presence of a single PEGylation site at Cys34. Formation of higher molecular weight species was attributed to the side-reaction of Mal-PEG$_8$ with native lysines.

All species of BSA$_x$-R-PEG$_8$ demonstrated similar ligand binding capabilities. DS2 was unaffected as association constants for naproxen were within the range of reported values for unmodified BSA. Interactions between DS1 and sulfamethoxazole, however, were greatly weakened by PEGylation. While the number of binding sites for each remained in agreement with the literature, association constants were several times smaller for PEGylated BSA than unmodified FAF-BSA.

The difference in effects of PEGylation on these binding sites was likely due to the placement of the PEG backbone. In the case of BSA$_x$-te-PEG$_8$, the main site of modification was
Cys34 in Domain IA. DS1 is located at the interface of Domains IB and IIA. Conformational changes due to PEGylation in the same domain altered the cavity of DS1 and greatly reduced binding capabilities. DS2, on the other hand, is located in Domain IIIA. Modifications made at Cys34 appear to have little to no effect on ligand binding to DS2.

For BSA\textsubscript{x}-123t-PEG\textsubscript{8} and BSA\textsubscript{x}-PEG\textsubscript{4}-123t-PEG\textsubscript{8}, the exact site of modification is unknown as both activating agents were attached by an NHS-lysine reaction. BSA contains 59 lysines. 20 appear in Domains I and III while 19 are found in Domain II. Given the high number and even distribution of potential sites of modification, a preference for a given domain is not readily apparent. However, similar drug binding profiles between BSA\textsubscript{x}-te-PEG\textsubscript{8} and BSA\textsubscript{x}-R-123t-PEG\textsubscript{8} of this study suggest a preference for modification of Domain I.

Temperature of unfolding was most affected by activation with the short DIBO-NHS. Both BSA\textsubscript{x}-te-PEG\textsubscript{8} and BSA\textsubscript{x}-PEG\textsubscript{4}-123t-PEG\textsubscript{8} showed similar denaturation curves to unmodified FAF-BSA. BSA\textsubscript{x}-123t-PEG\textsubscript{8} was the least thermally stable of the complexes. This was attributed to the proximity of the hydrophobic DIBO group to the surface of BSA altering solvent interaction. Moving the DIBO further from the protein surface, as in BSA\textsubscript{x}-PEG\textsubscript{4}-123t-PEG\textsubscript{8}, or complete elimination of DIBO (BSA\textsubscript{x}-te-PEG\textsubscript{8}), restored T\textsubscript{m} to similar values for that of unmodified FAF-BSA.

The reverse was found for the temperature of aggregation. BSA\textsubscript{x}-R-123t-PEG\textsubscript{8} species aggregated at much higher temperatures than either FAF-BSA or BSA\textsubscript{x}-te-PEG\textsubscript{8}. While Cys34 modified FAF-BSA had a higher T\textsubscript{agg}, the signal began increasing at a much lower temperature than FAF-BSA, likely due to the dissolution of the thioether linker. This instability was potentially due to the high concentration of excess thiol from 2-Mercaptoethanol in the denaturing buffer.
PEGylation and pre-loading of a high affinity DS2 ligand proved insufficient to allow for omission of stabilizing agents used in the pasteurization process. A significant decrease in helicity was seen for all PEGylated BSA species following pasteurization in the absence of naproxen. When sterilized with naproxen bound, a moderate loss of helicity was seen for all species of BSA. A combination of PEGylation and pre-loading the complex with a tightly bound ligand reduced the denaturing effects of pasteurization but did not completely eliminate them.

**Future Research**

The future of this project may be aimed at answering four questions: 1) Can a homogeneous BSA$_x$-R-PEG$_8$ product be generated with higher yields? 2) Can a fatty acid which does not compete for DS2 be substituted for caprylate? 3) Can these PEGylated species effectively release drugs? and 4) Does PEGylation affect cellular uptake?

The formation of three different forms of PEGylated BSA has been verified but the production process may still be improved. Yields and homogeneity of the products are potentially tied to the distance between BSA and the PEG$_8$ backbone. An additional 14 Å between BSA and PEG$_8$ was gained by incorporation of a PEG$_4$ linker into the activating agent. This modification produced slightly higher yields and a more homogeneous final product. Extension of the PEG arms is the easiest way to increase this distance. The largest available eight-armed PEG is 40 kDa, each branch having an average molecular weight of 5 kDa which corresponds to 113 subunits. By extending these branches, the congestion around the PEG backbone will be relieved. Less steric hindrance would allow for BSA molecules to have easier access to the terminal functional groups. The amount of PEGylated product generated would increase while the number of different species formed would decrease.
Caprylate, one of the stabilizing agents used in pasteurization, is a saturated eight-carbon fatty acid (8:0) with an affinity for DS2 (Kawai et al., 2017). Oleate (18:1), palmitate (16:0), and linoleate (18:2) account for the majority of bound fatty acids to human serum albumin (A. Saifer & Goldman, 1961). One of these compounds may prove to be an alternative stabilizing agent.

BSA contains seven fatty acid binding sites (Bhattacharya, Grüne & Curry, 2000; Rizzuti, Bartucci, Sportelli & Guzzi, 2015; Simard et al., 2005). Fatty acid site 5 (FA5) resides in Domain III and contains hydrophilic features (Y400 and K524) at the entrance to the cavity which interact with the polar head group of bound fatty acids. This cavity has been shown to bind long chain fatty acids with the highest affinity of the seven sites (Rizzuti et al., 2015; Simard et al., 2005).

DS2 of BSA is also located in Domain III with polar residues at Y410, R409, and S488. Use of a single long chain fatty acid which preferentially binds to FA5 may provide a substitute for caprylate in the pasteurization process. This would reduce competition for DS2, however, the proximity of FA5 to DS2 may alter binding profiles. By binding to a site away from DS2, thermal stability may be enhanced while maintaining ligand binding capabilities.

Also of interest is the ability to release bound drugs. Reported results show that these PEGylated BSA species are able to bind ligands, if only in DS2. If addition of a DS2 ligand is required before sterilization, the storage stability of the drug-BSA complex must be evaluated to avoid dissociation of the complex before administration. Diffusion-based experiments monitoring the effects of pH, temperature, time, etc. will provide information as to the shelf-life of these formulated products.

Gp60 has been identified as a cell surface receptor for albumin and plays a key role in the efficacy of the non-PEGylated albumin-based therapy Abraxane® (Desai, 2008; Schnitzer, 1992).
Albumin-based nanoparticles prepared via conjugation of BSA with either polystyrene (Wang, Tiruppathi, Cho, Minshall & Malik, 2011) or PEGylated liposomes (Thöle, Nobmann, Huwyler, Bartmann & Fricker, 2002) were successfully endocytosed into cells *in vitro*. This suggests that the degree of PEGylation in BSA$_x$-te-PEG$_8$, BSA$_x$-123t-PEG$_8$, and BSA$_x$-PEG$_4$-123t-PEG$_8$ will not necessarily inhibit gp60-mediated transcytosis. In order to evaluate the viability as drug delivery systems, the ability of cells to endocytose these PEGylated complexes must be assessed.

These PEGylated species utilize more cost-effective components, require no specialized equipment, and modify the albumin core at a bare minimum of sites. By maintaining albumin close to its native form while conferring the benefits of PEGylation, potential alternatives to current drug delivery systems and protein-based therapies have been developed.
REFERENCE LIST


VITA

Jonathan Alejandro Hill was born and raised in Lumberton, North Carolina. He earned a Bachelor of Science in Chemistry in 2005 from the University of North Carolina at Greensboro. After graduation, he accepted a position as an Analytical Chemist at Syngenta Crop Protection, LLC in Greensboro, NC. In 2011, his academic career was resumed by pursuing his doctorate at Loyola University Chicago. This work is the culmination of those efforts.