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Green Chemistry Oxidative Modification of Peptoids Utilizing Bleach and TEMPO

Jesse Leland Roberts

University of Arkansas, Fayetteville

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Green Chemistry Oxidative Modification of Peptoids Utilizing Bleach and TEMPO

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Engineering

by

Jesse Leland Roberts
University of Arkansas
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University of Arkansas

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Dr. Shannon Servoss
Thesis Director

Dr. Jamie Hestekin
Committee Member

Dr. Wei Shi
Committee Member

Abstract

Biotherapeutic drugs, derived from biological molecules such as proteins and DNA, are becoming an integral and exceptionally critical aspect of modern medicine. Compared to common pharmaceutical drugs, biotherapeutics are much larger in size and have greater target specificity, allowing them to treat many chronic diseases ranging from cancer to rheumatoid arthritis. The major issue with protein based therapeutics is that they readily undergo proteolysis, or enzymatic degradation, when administered through subcutaneous injections. Traditionally, biotherapeutic modification procedures have centered on the use of PEG derivatives. This process, called PEGylation, is unfavorable due to the increases in molecular weights of the proteins and the heterogeneous mixture of products formed. Instead of PEG derivatives, we propose peptoids with N- methoxyethylglycine (NMEG) side chains to decrease proteolysis. NMEG groups are more advantageous than PEG derivatives due to their low molecular weight and ability to form homogeneous products. Our work focuses on increasing the protease resistance of target biotherapeutic proteins by cross-linking a NMEG-5 peptoid to a cytochrome c via reductive amination. In the presence of a reducing agent, an imine bond is formed through the reduction of the peptoid's aldehyde group and cytochrome c's primary amine groups. Due to the expensive and unstable nature of commercially available aldehyde side chains, a green chemistry method, using only sodium hypochlorite (bleach) and 2,2,6,6-Tetramethylpiperidinoxy (TEMPO, free radical), oxidized the peptoid's hydroxyl group into the desired aldehyde for cross linkage.

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1. Introduction

Biotherapeutic drugs derived from biological molecules are becoming an integral, and very critical aspect of modern medicine. The term “biotherapeutics” can represent a large class of treatments that are produced from cytokines, growth factors, hormones, antibodies, and other regulatory proteins or peptides [1, 2]. These therapeutics are typically produced or extracted using genetically engineered bacteria, yeast, fungi, and other cell types [3].

Biotherapeutic drugs have been used for decades to help treat multiple pathophysiological illnesses including cancer, hemophilia, infectious diseases, inflammatory and autoimmune diseases, and other rare diseases [4, 5, 6]. These larger biotherapeutic drugs are significantly favored over common pharmaceuticals due to their increased size, advantages in target specificity, and non-toxicity factors within the body [7]. The first biotherapeutic drug, high-quality human insulin, was derived from recombinant DNA and produced by Eli Lilly in 1982 [8]. Since, over 150 biotherapeutic medicines have been developed to improve treatment options and patient quality of life [9]. These advancements are critical in improving the accuracy and effectiveness of current treatment methods. For instance, since the 1970's the 10-year survival rate of cancer patients has nearly doubled from one in four patients to one in two patients [10]. Although drastically increased, it leaves room for further improvement, ultimately opening the door for research and the eventual use of biotherapeutics as a treatment option.

1.1. Biotherapeutic Proteins

The use of proteins as potential biotherapeutic agents is gaining interest at an intriguing rate. In recent years, many distinct proteins have been discovered, and can be linked to the underlying mechanistic pathways of several common diseases. Researchers have estimated that between 25,000 and 40,000 functional genes that code for these proteins have been discovered within the human genome [11]. Using alternative splicing of genes and posttranslational modifications of proteins, these human genes have the potential to code for the distinct proteins that are being found within disease mechanisms [12, 13, 14]. Biotherapeutics are typically classified into three main groups of proteins, based on their physiological properties and course of treatment. The first group is made up of peptides and small protein therapeutics including growth factors, hormones, and cytokines [15]. Two current therapeutics within this class are Epogen®, a form of erythropoietin protein commonly used to increase the body's production of red blood cells in anemia patients, and Neupogen®, a protein used to boost the body's production of white blood cells for neutropenia patients [16, 17].

The second group consists of non-immune therapeutic proteins including replacement enzymes, blood factors, anticoagulants, and other recombinant proteins [15]. The FDA approved drug Myozyme uses a recombinant human α -glucosidase enzyme for treatment in patients with Pompe Disease, an autosomal recessive myopathy that causes an abnormal storage of glycogen in tissues, resulting in premature fatalities [18, 19]. Tissue plasminogen activator (t-PA), one of the only successful treatment options for ischemic stroke victims, falls under this class of biotherapeutics. The naturally occurring protein, t-PA, serves as an

anticoagulant by converting the inactive proenzyme plasminogen into an active serine protease plasmin. In 1980, t-PA was first identified in melanoma cells, and later scientists were able to isolate and purify the protein, creating today's biotherapeutic [20].

The third class of biotherapeutic proteins includes therapeutic antibodies and Fc-like fusion proteins. Monoclonal antibodies (mAbs) have shown great success as biotherapeutics for the treatment of autoimmune diseases due to the robust and flexible nature of the immunoglobulin molecule and their highly specific antigen-binding capabilities [15]. Immunoglobulin G (IgG) serves as one of the main types of antibodies found in the blood and extracellular fluid, functioning as a control mechanism for infections within body tissues. Antibodies and Fc-like fusion proteins serve as practical and viable means for the treatment of cancers, autoimmune, and inflammatory diseases [21, 22]. All three classes of biotherapeutic proteins provide excellent insight into the future of medicine, thus many pharmaceutical and biotechnology companies are investing substantial resources for their discovery and development. However, as promising as these drugs may be, there are still limitations in the mode of action, manufacturing and characterization techniques, and drug delivery methods.

1.2. Biotherapeutic Drug Delivery

Although biotherapeutic proteins have proven successful as treatment options for various diseases, there are still complications with delivery. Traditional routes, including oral, submucosal (nasal), parenteral (injection), and transdermal (through the skin) [23], are not feasible due to enzyme degradation and low absorption efficiency [24]. The oral delivery of

biotherapeutic proteins faces issues with poor absorbance within the gastrointestinal system and chemical degradation due to harsh enzymes within the digestive system, resulting in the loss of activity and function [25]. Proteins are extremely sensitive, where even the smallest change in conformation can cause a complete loss of function [26]. It is important to note that the pH within the colon and ileum is much higher than any other region in the GI tract, so difficulties arise when developing pH-controlled therapeutics. These pH-sensitive drugs are prone to degradation within the colon's harsh environment [27].

Nasal drug delivery is of interest due to the high vascularity and permeability within the nasal mucosa [28, 29]. These desirable characteristics stem from the nasal cavity's large surface area, porous endothelial membrane, and highly vascularized epithelium [30]. Nasal drug delivery may be great for small molecules, but issues arise with high molecular weight compounds (above 1 kDa). There are also volume limitations in that the volume per dose that can be permeated across the membrane is restricted to 25-200 microliters [31]. As seen with other delivery methods, the body's immune defense mechanism bodes an even bigger issue. Mucociliary clearance is the most important physiological defense mechanism inside the nasal cavity. If the biotherapeutic causes any irritation in the nasal mucosa, then this mechanism will cause the drug to be rapidly diluted, increasing the clearance by forming nasal mucus that will be eliminated from the nose [32].

Subcutaneous injections and transdermal administration routes are challenging due to immunogenic potential and unwanted immune responses [33]. It has been reported that

subcutaneous degradation occurs with protein-based drugs due to the lymphatic transfer of these proteins when delivered parenterally [34]. The lymphatic system directly affects the absorption and distribution of therapeutic proteins after administration through T-cell responses initiated by skin-derived dendritic cells [35]. No matter the delivery route, any introduction of a foreign protein into the body has the potential to illicit an immune response, triggering the production of antibodies. The immune system is extremely sensitive, in that it can detect three-dimensional structural differences between the proteins native to the body and those being introduced [36]. For this reason, drug delivery systems and post-translational modifications are growing in interest to combat the immunogenicity issues of protein therapeutics.

Currently, most drug delivery systems (DDS) are within the colloidal size range (1-1000nm), and act to release the drug at a controlled rate for a prolonged period of time [37]. The drug is typically kept within a solid inner matrix that is layered by a permeable outer polymeric membrane through which the drug diffuses [38]. Research efforts have been focused on three main classes of DDS, including nanoparticles, liposome and other lipid-based carriers, and polymer-drug conjugates [39]. Poly(lactic-co-glycolic acid) (PLGA) nanoparticles are attractive as DDSs due to their biodegradability and biocompatibility, FDA approval in parenteral administration systems, well-described production and characterization methods, protections from drug degradation, sustained release capabilities, possibility to modify surface properties, and target specificity for desired organs or cells [40]. The ability to modify surface properties is an important property in combatting cellular immune response and increasing cellular uptake of the drug. Surface

charge plays a major role in the interaction of the DDS with the cell. Studies have shown that positive charged nanoparticles allow a higher extent of cellular uptake due to the ionic interactions with the negatively charge cell membrane [41, 42, 43]. Despite these desirable properties, PLGA-nanoparticles have their limitations when dealing with certain protein therapeutics. The synthesis process of these nanoparticles involves factors and processes that may destabilize the proteins. When loading the protein into the nanoparticle, a double emulsion method is currently required leading to the aggregation of most proteins.

Depending on the hydrophilicity of the protein, interactions between PLGA and the protein may also lead to the denaturing and aggregation of proteins [44]. It has been shown that immunogenicity can be minimized by ensuring stability, while limiting the formation of higher molecular weight protein aggregates [45]. Another issue associated with the use of nanoparticles is the complexity of cellular uptake and the unknown stability and cytotoxicity of the nanoparticles following metabolism. Evidence proves that the exocytosis of nanoparticles is drastically slower than endocytosis, but there is little information on the metabolism and long-term effects of these particles [46, 47].

Of the three main classes of drug delivery systems, liposomes and lipid-based carriers have already had a major impact on targeted therapeutic protein delivery. Liposomes are defined as phospholipid vesicles consisting of multiple lipid bilayers enclosing discrete aqueous spaces [48]. Liposomes and lipid-based carriers are advantageous as drug delivery systems due to their biocompatibility, ability to self-assemble, extended drug circulation time, and their ability to carry multiple drugs at once [49]. Unlike PLGA-nanoparticles, liposomes possess the ability to encapsulate both hydrophilic and hydrophobic protein

drugs. Hydrophilic protein therapeutics can be trapped in the aqueous center, and hydrophobic proteins can be encapsulated in the bilayer membrane [50]. Water-soluble drugs can be loaded onto the liposome or lipid carriers through passive or active loading, depending on the functional groups and chemical environment. Passive loading, involving the formation of liposomes within an aqueous solution of the drug is the simplest, but least efficient method due to the limited loading capacity and waste of solution [51]. Active loading is more efficient, taking advantage of the pH difference between the external and internal liposome environments to allow the passage of drugs with charged functional groups through the membrane. Non-water soluble drugs can be incorporated directly into the membrane of the liposome or lipid-carrier; however, the drug to lipid ratio of the membrane is important to the dexterity of the liposome [52, 53]. For anticancer drugs, the antitumor efficacy is directly related to the drug release rate, and previous research demonstrated that by varying the drug to liposome ratio, optimal drug release rates could be achieved [54]. However, as with other therapeutic drug delivery methods, liposomes and lipid-carriers are still susceptible to enzyme degradation and macrophages, primarily in the spleen and liver. Therefore, to be used as treatment options for cancer and inflammatory diseases (inflammatory bowel disease, rheumatoid arthritis, etc) it is important to develop long-circulating liposomes that avoid the reticulo-endothelial system (RES) [55]. To achieve this passive drug delivery, liposomal surface modifications must be made to provide a steric boundary to the liposome that prevents RES uptake and blocks degradative enzymes from attaching [56, 57]. The use of ganglioside, GM₁, to modify the liposome surface created “stealth” liposomes that were not readily taken up by the RES; ultimately, allowing the carrier to stay in circulation for a longer period of time [58]. A

second modification method, PEGylation, utilizes the addition of polyethylene glycol (PEG) groups onto the liposomal membrane surface to drastically improve the carrier's circulation time [59].

1.3. PEGylation

Since the 1970's, polyethylene-glycol (PEG) has been a highly-investigated polymer for the attachment and modification of biological macromolecules for multiple pharmaceutical applications. PEGylation is the covalent or noncovalent attachment of PEG polymers to macromolecules, most typically peptides, proteins, and antibody fragments [60]. Each individual PEG exemplifies many desired characteristics, including the absence of toxicity and immunogenicity, high water-solubility, and low mass-dependent elimination from the kidney [61, 62, 63]. Once conjugated, PEG sterically shields the protein's surface from degradative agents and RES uptake, decreasing the protein's immunogenic response, thus improving body-residence time. Along with the shielding effect, the increased molecular weight of the PEG-protein conjugate is advantageous in reducing renal clearance and altering biodistribution, also improving the residence time [64]. Renal clearance works by selectively filtering blood components through the glomerular filtration barrier (GFB). The GFB's permeability is often dependent on the size and charge of the blood components [65]. While most proteins are selectively retained in the blood by the GFB, certain low molecular weight proteins and degraded protein fragments can undergo rapid renal clearance [66, 67]. Thus, by increasing the protein's molecular weight and improving the resistance to degradative agents in the body, PEGylation serves as a viable modification method for prolonging the body-residence time of potential therapeutic proteins [68].

Initially, researchers were skeptical that PEG could be attached to large molecular weight proteins while maintaining biological activity; therefore, they directed their work solely on catalase and bovine serum albumin. For both molecules, PEGylation enhanced circulation times and eliminated immunological responses, while ultimately maintaining optimal protein activity and structural integrity [69, 70]. From these results, the efficacy of PEGylation in improving therapeutic drug delivery was deemed successful, sparking interest to further develop new PEGylated macromolecules. Since then, new methods for PEG conjugation have been introduced resulting in a wide array of macromolecules that can potentially be modified. To attach PEG to a molecule, it is important to functionalize one or both PEG termini with a functional group that is chosen based on the reactive group on the molecule being PEGylated. Amine conjugation, considered first-generation PEG chemistry, is the most common technique for the attachment of PEG molecules to proteins [71]. Amine reactive PEG derivatives form secondary amine linkages by substituting with multiple nucleophilic amino acid groups (lysine, serine, tyrosine, cysteine, and histidine) found in the protein, thus forming a heterogeneous mixture of PEG-protein conjugates [72]. First-generation PEG chemistry methods were first referred to as “gentle chemistry” due to the mild reaction conditions and use of simple, linear PEG molecules [73]. This simplicity, however, is important in maintaining the activity and three-dimensional structure of proteins. As previously mentioned, proteins are extremely sensitive to their environment, so it is of utmost importance to limit the amount of harsh chemical used during conjugation.

Second-generation PEGylation methods refer to any newly developed method of PEG conjugation that typically uses more complex PEG derivatives and harsher chemical

conditions than first-generation methods. These PEG derivatives, unlike the linear amine derivatives used in first-generation chemistry, contain multiple different functional groups such as aldehyde, carboxylic acid, and thiol groups [74, 75]. Depending on the desired product and degree of PEGylation, altering the chemical environment for most of the second-generation PEG derivatives creates a more site-specific PEGylation method. For instance, by changing the pH of the environment to acidic, mPEG-priopionaldehyde, a PEG derivative containing a reactive aldehyde group, selectively reacts with a protein's N-terminal α -amine, because nucleophilic substitution will only occur when the pH of the molecule is near the residue's pK_a [76, 77].

1.3.1. Complications with PEGylation

Since the 1970's, concrete evidence has proven PEGylation as a viable manipulation method to improve pharmacokinetic properties of biotherapeutic proteins. PEG-protein conjugates display the "stealth" properties that are desired for the optimal drug deliverance of biotherapeutic agents [78]. PEGylation allows for the therapeutic protein to "sneak" by the body's immune system by sterically shielding the protein's surface from degradative agents, while also maintaining the water solubility and protein activity needed to be a viable treatment option [79]. Although PEGylation seems to be an ideal manipulation method, several limitations arise during the characterization and purification processes for these newly formed conjugates. First-generation PEGylation relies on the coupling of PEG derivatives to different reactive amino acids on the protein. Most therapeutic proteins rely on non-specific PEGylation occurring through the between hydroxyl- or aldehyde- functionalized PEG monomers and amine groups found on lysine

side chains or the N-terminus of the protein [80]. This coupling method, although highly reactive at physiological conditions, occurs at random positions, ultimately producing a heterogeneous mixture of PEG-conjugates. Since lysine makes up nearly 10% of all protein amino acids, it is incredibly difficult to characterize exactly which and how many lysine residues on the protein were PEGylated. These heterogeneous PEG-protein conjugates, called “isomers”, differ in molecular weights, protein stabilities, and even in the level of activities [81]. In 2003, the PEGylation of INF- α 2a produced nine different isomers, each differing in the level of bioactivity. The difference in bioactivity of these isomers was theorized to directly affect the interferon receptor binding kinetics and stabilities [82]. The heterogeneity of PEG-protein conjugates lowers molecular activity of the therapeutic protein causing variations in treatment mechanisms and clinical side effects [83]. For FDA approval of non-site specific PEGylated protein drugs, the individual PEG-protein conjugates must be fully characterized, and biological analyses must be run on each conjugate to determine their pharmacodynamic properties. The more homogenous the product is, the better chance it has at getting approved by the FDA [63]. To achieve a homogeneous PEG-protein conjugate, site-specific coupling or effective purification methods must be incorporated. Purification methods are not only costly, but are inefficient and difficult as well. The purification will need to separate three molecules (PEG, PEG-protein, native protein), where the separation of PEG and native protein is simple, using filtration and size-exclusion methods [84]. Difficulties arise when trying to isolate the desired PEG-protein conjugate from the others due to similar characteristics between the conjugates. As a result, several different methods, such as Ion Exchange Chromatography and Hydrophobic Interaction Chromatography, are completed in succession to fully purify

the product [85]. With consideration to the high manufacturing costs associated with the current production of therapeutic proteins, the production and purification of PEGylated proteins is economically infeasible. The overall process to achieve a high purity product is at the direct expense of high yield. To make up for the loss in yield, protein production will need to be increased, ultimately driving up manufacturing costs exponentially. Therefore, the more homogeneous the PEG-protein conjugate product formed, the less purification needed, decreasing the amount of protein lost, while improving the cost of manufacturing.

To combat the heterogeneity issues with amine-coupling (first generation) researchers are developing second generation PEGylation methods that are focused on a more site-specific coupling of PEG derivatives to amino acids on the protein. The attachment of PEG to the thiol group of cysteine is considered site-specific because it accounts for only 1% of the total amino acid content in proteins. However, many of these cysteine groups will undergo disulfide bonding with each other, lowering the number of active thiol groups suitable for PEGylation. The undesired bonding between cysteine residues results in very few proteins possessing active cysteine groups capable of reacting with PEG monomers. To fix this dilemma, researchers are interested in introducing site-specific cysteines to the protein sequence through genetic engineering, but little is known regarding the effect this will have on protein activity [86]. Various other amino acids and functional groups have been of interest for second generation PEGylation, but most, if not all, have limitations that void them as suitable coupling agents. For instance, arginine is another amino acid that is less abundant than lysine, but has similar reaction chemistry involving the coupling of PEG to an amine group. Based on these two parameters, one could assume it to be a perfect option

for site-specific PEGylation producing an active, monodisperse PEG-protein conjugate. These assumptions are proven to be false, as the coupling requires long reaction times that drastically decrease protein stability and site specificity [87, 88]. Carboxyl groups can be PEGylated, but only when amines are not present, virtually eliminating the use with proteins and peptides [89]. Like carboxyl groups, hydroxyl groups are only suitable for PEGylation for uses in non-peptide moieties such as matrices for chromatography and biocompatible surfaces [90]. The second generation PEGylation methods have potential to be effective, site-specific coupling mechanisms, but each method is limited to its own specifications resulting in a narrow range of proteins that can be modified.

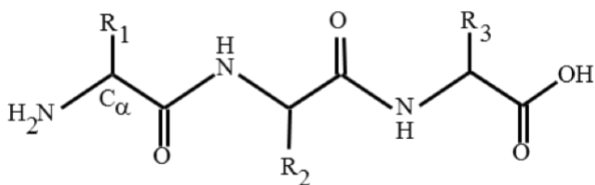
While PEGylation has a countless number of unique functionalities, it presents major drawbacks in modifying biotherapeutic proteins due to the heterogeneity of PEG resulting in characterization and purification limitations. Along with the characterization and purification, being able to maintain native protein activity, while improving *in-vivo* drug half-life is crucial in developing novel modifier of biotherapeutics.

1.4. Peptoids (Poly-N-Substituted Glycines)

Peptoids, or *N*-substituted glycines, are synthetic peptidomimetic oligomers that structurally resemble α -peptides, but have side chains attached to the amide groups on the backbone instead of the α -carbon as in peptides (Figure 1.1.) [91]. This structural modification generates an achiral backbone that eliminates the potential for hydrogen bonding, resulting in a protease-resistant polymer that exhibits good cell permeability and protein binding characteristics resembling that of more “drug-like” molecules [92]. In

peptides, hydrogen bonding occurs between the amide hydrogen and the carbonyl oxygen on the backbone, which, although critical for the formation of distinct secondary structures can result in the denaturation of the molecule. The backbone hydrogen bonds are weakened and susceptible to breakage when exposed to extreme conditions (heat, ultraviolet radiation, strong acids or bases, organic solvents, and enzymes) causing the denaturation of the peptide's secondary structure [93]. This is unfavorable because many peptides rely on the secondary structure to determine functionality and bioactivity; therefore, the high-sensitivity of peptides to denaturation presents a major drawback for the usage as biotherapeutics [94].

Peptide



Peptoid

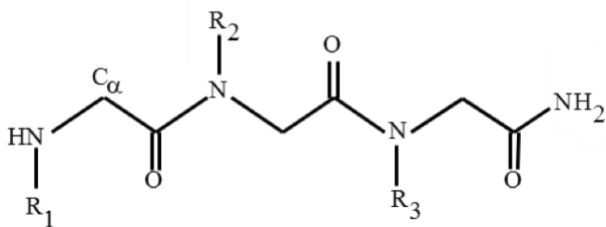


Figure 1.1. Peptide and peptoid structures.

1.4.1. Peptoid Synthesis

Peptoids can be produced via a sequence-specific, solid-phase synthesis method comparable to that of peptides. Unlike peptide synthesis, where submonomers must be protected prior to addition, peptoid synthesis allows for the precise addition of unprotected submonomers greatly simplifying the process. The submonomer method is a highly efficient, low cost synthesis technique that allows for the addition of a wide variety of side chains as primary amines. Using a solid-phase support (Ex: Rink Amide Resin), submonomers are added from carboxylic to amine termini via a submonomer “cycle” made up of two-steps: (1) acylation and (2) amination (nucleophilic substitution) (Figure 1.2.) [91]. The first reaction of the submonomer cycle, acylation, adds an activated carboxylic acid derivative onto a receptive amine generating a tertiary amide bond. In general, bromoacetic acid and diisopropylcarbodiimide (DIC) are used for acylation. The bromoacetic acid is activated by DIC separately, and then added to the solid-phase support [95]. The second step in the cycle, amination, involves the nucleophilic displacement of the halide (typically bromine) by a primary or N-terminal secondary amine (side-chain). As the halide group is removed from the haloacetamide, the primary nitrogen submonomer attacks the alpha-carbon forming an ammonium salt. The halide ion then removes hydrogen from the ammonium salt producing hydrogen bromide [95, 96]. The amination step creates the molecular diversity that is present in peptoids due to the thousands of commercially available amine side-chains.

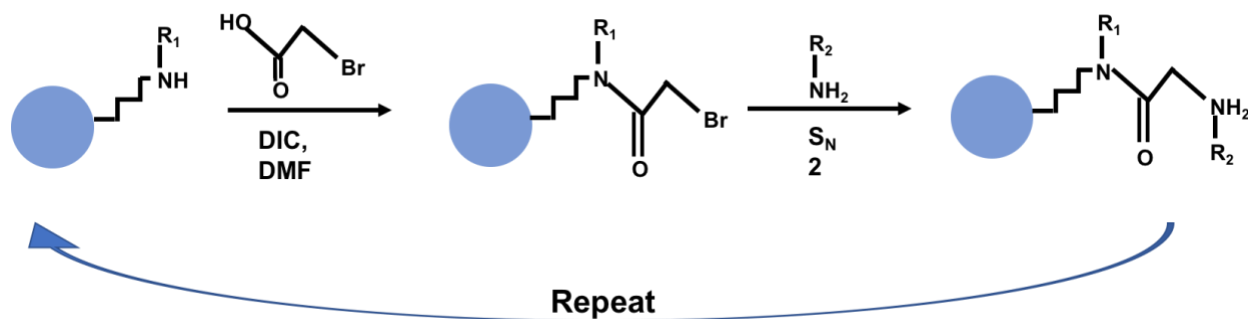


Figure 1.2. Submonomer cycle for peptoid synthesis.

1.4.2. NMEGylation

To overcome stability and half-life issues seen in most biotherapeutic proteins, researchers in the last decades have worked to develop new modification and delivery techniques.

Although many of these newly developed delivery methods, such as the use of nanoparticles and lysosomes, show great promise, their use is still hampered by an increase in protein aggregation, ultimately decreasing drug efficacy. The newly discovered modification methods commonly use well-known coupling chemistries for the incorporation of a wide range of molecules to increase resistance to enzymatic degradation. However, as seen in PEGylation, many of these modifications result in the formation of heterogeneous protein conjugates that have a decrease in native protein activity, and require inefficient, costly purification methods. Thus, there is a critical need to develop a site-specific protein modification method that can produce homogeneous products to enhance bioavailability and efficacy of therapeutic proteins.

Peptoids are attractive as novel biomimetic polymers that can potentially be used in therapeutic protein modifications and delivery systems because of their resistance to

degradative enzymes, great cell permeability, and low immunogenicity [97]. The peptoid sequence is fully customizable through the addition of side chains with various chemistries. By incorporating side chains with specific functional groups into the sequence, common coupling chemistries can be used to cross-link the peptoid to peptides, proteins, nanoparticles, and other molecules [98]. Protein modifications involving sequence specific peptoids offers a promising means for overcoming stability and absorption issues commonly displayed by current biotherapeutics.

Like PEGylation, the challenge still lies in forming homogenous products that maintain native protein bioactivity and conformation [99]. Since PEG groups have traditionally shown an increased stability to serum enzymes, but failed to produce homogeneous products, increased efforts have been to find a PEG alternative. *N*-methoxyethylglycine (NMEG) is a hydrophilic peptoid monomer that resembles the structure of PEG (Figure 1.3.). The NMEG monomer can be produced by incorporating 2-methoxyethylamine side chains onto the peptoid backbone via the solid-phase synthesis method. The NMEG peptoids can be covalently attached to a desired molecule by site-specific coupling reactions, called NMEGylation [100]. Previous research shows that, like PEG groups, oligoNMEGs are promising as antifouling agents. NMEGylated protein conjugates of varying lengths showed a decrease in serum protein adsorption and a resistance to cell attachment for an extended period *in vitro*, both desired characteristics for use as a biotherapeutic [101]. As predicted and proven in recent studies, the NMEGylation of polypeptides resulted in an increase in desirable properties such as increased water solubility, hydrophobicity, and serum stability, while also maintaining the original binding affinity of the peptide to its

target [100]. These studies for the NMEGylation of peptides/proteins are promising, however, the true efficacy of this method needs to be explored further.

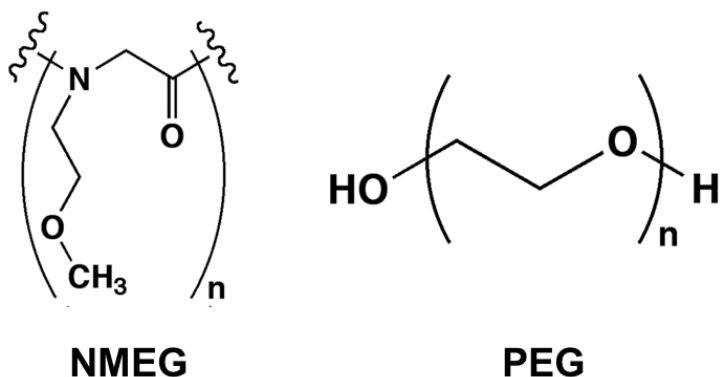


Figure 1.3. Structural comparison between NMEG and PEG.

1.5. Green Chemistry

Chemistry has played, and will continue to play an important role in nearly every aspect of modern life, from pharmaceutical manufacturing to polymer development. Over the last three decades, green chemistry has been an emerging field internationally, supported through the creation of hundreds of governmental initiatives working to achieve sustainability [102]. Green Chemistry is originally defined as the “design of chemical products and processes to reduce or eliminate the use and generation of hazardous substances” and can be characterized by the careful planning and molecular design of experiments [103]. In 1998, Paul Anastas and John Warner, often known as the “fathers” of the Green Chemistry field, developed The Twelve Principles of Green Chemistry as a framework for the design of new chemical processes [104]. This framework focuses to reduce potential hazards, including toxicity, physical hazards, and environmental hazards,

across all stages in chemical processes [105]. The first principle, waste prevention, involves the reduction in the amount of waste produced. Since the amount of waste produced is often directly correlated to many of the remaining principles, it is considered to be the most impactful in developing a “Green” chemical process. Chemical companies have started investing in waste management techniques, in part due to the increased public awareness in the environment, but mainly because of a governmental increase in the cost of waste removal, amounting to nearly 40% of the overall production costs [106]. When analyzing the waste management process, it is important to look at the efficiency of the chemical reaction taking place. Yield, chemical selectivity, atom efficiency, energy spent, solvent usage, and renewable raw materials are all impactful to the overall efficiency of the reaction and can be managed to decrease waste production [107, 108, 109, 110].

1.5.1. Green Chemistry Oxidation

Inspired by The Principles of Green Chemistry, the work of researchers has focused on developing “greener” chemical reactions that reduce or eliminate the use and generation of toxic chemicals. The original focus has been on waste elimination, or what is known as the E factor (environmental factor). The E factor is a metric used to quickly assess the environmental effect of manufacturing processes, and is typically measured in kg waste per kg product [111]. To determine the true E-factor value and amount of waste produced, it is important to look into the stoichiometric equation for the overall process [112]. Along with the E factor, in 1991, using what is known as “atom economy”, scientists began investigating the reaction efficiency and where in the process the waste content originates. By analyzing waste production of inefficient processes (high E factor values), they found an

increased amount of organic salts, metal (Na, Mg, Zn, Fe) and metal hydride (LiAlH_4 , NaBH_4) reducing agents and oxidants such as permanganate, manganese dioxide, and chromium (VI) reagents [113]. These results prove the important role that catalysis and solvent choice play in the development of green chemistry reactions [114].

Traditionally, catalytic oxidation of alcohols (primary, secondary, allylic, propargylic, etc) to aldehydes, ketones, and carboxylic acids require the incorporation of many harsh reagents. Chromium (VI) oxides are well-known oxidants for the conversion of primary and secondary alcohols to aldehydes and ketones; however, this method requires the use of harsh organic solvents such as pyridine, dimethylformamide (DMF) with sulfuric acid, and others [115, 116, 117, 118]. The handling of chromium (VI) compounds is crucial due to its chronic toxicity and contamination of product. A study of workers exposed to chromium (VI) compounds have reported the development of asthma and other signs of respiratory distress, accompanied by a 20% decrease in forced expiratory volume of the lungs [119].

The Swern oxidation, typically using dimethylsulfoxide (DMSO), oxalyl chloride, and trimethylamine in methylene chloride solvent does not produce heavy metal waste products, thus, making it a more environmentally friendly option [120]. However, drawbacks still exist in that oxalyl chloride is known to have toxic and corrosive properties, and methylene chloride is carcinogenic, hepatopathic, and neuropathic [121]. Other methods, such as the use of manganese dioxide (MnO_2), can oxidize allylic and benzylic alcohols, but are still faced with hazardous and toxic reagents, and the potential for residual metal contamination [122].

1.5.2. Bleach/TEMPO Oxidation

The oxidation of primary alcohols to aldehydes and carboxylic acids is a fundamental transformation in organic chemistry. To create a more green chemistry oxidation method researchers have been investigating the use of nitroxyl radicals and transition metal salts. Both nitroxyl radicals tetramethylpiperidine-N-oxyl (TEMPO) radical and phthalimide-N-oxyl (PINO) radical in the presence of small amounts of manganese (II) nitrate and cobalt (II) nitrate have displayed excellent results in the aerobic oxidation of benzyl alcohols to aldehydes and carboxylic acids [123]. However, the oxidation with these reagents is limited when dealing with less reactive aliphatic and allylic alcohols [124].

The development of Anelli's oxidation procedure has proven that aliphatic primary alcohols can be oxidized into aldehydes and carboxylic acids in a more efficient, green chemistry manner, by reacting the alcohol with a dichloromethane-water mixture containing bleach (sodium hypochlorite) in the presence of sodium bicarbonate, potassium bromide, and a catalytic amount of TEMPO free radical [125, 126]. TEMPO free radical serves as the primary oxidant for the transformation of alcohols into aldehydes by forming reactive oxoammonium salts. The secondary oxidant, sodium hypochlorite, is typically used to activate the TEMPO free radical by forming the oxoammonium salt, but subsequently plays a major role as the primary oxidant in the conversion of aldehydes to carboxylic acids. Typically, the reaction is done in an excess of sodium hypochlorite and a phase-transfer catalyst forming to form a high yield of carboxylic acid [127]. However, by eliminating the phase-transfer catalyst and lowering the amount of sodium hypochlorite used the oxidation can produce aldehydes with low reaction times [128]. This modified

version of Anelli's procedure allows for the fast, efficient, and low-cost oxidation of primary alcohols to aldehydes.

2. Research Rationale

While knowledge of different types of protein delivery systems and modification methods has grown in the last decades, the difficulty in characterization, susceptibility to enzymatic degradation, loss of stability and bioactivity, and economic costs continue to hinder them as viable treatment options. Thus, there is a critical need to develop a post-translational modification method that produces homogeneous conjugates that can be easily characterized and purified. This technique should increase protein resistance to enzymatic degradation and maintain native protein stability and bioactivity. The covalent attachment of NMEG-peptoids (NMEGylation) is a promising method to achieve this goal due to its favorable properties, such as an increase in water solubility and serum stability. It is believed that NMEGylation can fix the heterogeneity issues seen in PEGylation by modifying proteins in a site-specific manner. Unlike PEG-conjugates, NMEG peptoids allow for the precise positioning of specific chemical functional groups for attachment to reactive amino acids on the protein.

The overall goal of this project is to develop a protein modification method that improves the serum stability and efficacy of biotherapeutic proteins to be used as potential treatment options. We hypothesize that the NMEGylation of a target protein will result in the production of a homogenous protein-peptoid conjugate that withstands enzymatic degradation and displays native protein conformation and activity. The modification will

occur through the cross-linkage, via reductive amination, of an aldehyde functional group on the peptoid and primary amines, namely lysine, on the surface of the protein. Due to the expensive and instable nature of commercially available aldehyde side chains, we will implement a modified Anelli's oxidation method for the green chemistry oxidation of a primary alcohol peptoid side chain into the desired aldehyde functional group (Figure 2.1.). To minimize waste production and the use of toxic reagents, only sodium hypochlorite (bleach) and TEMPO free radical will be utilized for the oxidation reaction.

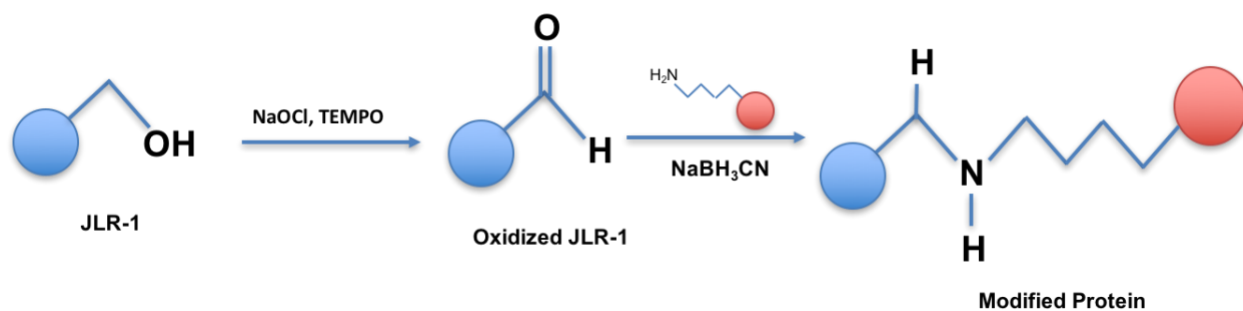


Figure 2.1. Schematic for overall protein modification with NMEG peptoid (JLR-1).

The hypothesis was supported by completing the following aims:

1. Investigate the Green Chemistry oxidation of peptoid.

We hypothesize that the oxidation using only bleach and TEMPO free radical will form the desired aldehyde side chain. The oxidation of primary alcohols occurs in two main steps, first to an aldehyde, then to a carboxylic acid. It is believed that by altering chemical kinetics, the oxidation can be stopped after the first step, forming a stable aldehyde group. It is believed that the concentration of bleach and reaction time plays a major role in the level of oxidation that occurs. LC-MS and MALDI-TOF mass

spectrometry will be used to determine the level of oxidation, and to confirm the formation of a stable aldehyde side chain.

2. Investigate the cross-linkage of the NMEG peptoid to a target protein.

We hypothesize that, in the presence of a reducing agent, the NMEG peptoid can be cross-linked to a target protein. The aldehyde group on the peptoid can be reacted, via reductive amination, with primary amines of lysines on the protein. Due to the abundance of lysine amino acids, it is predicted that multiple peptoid molecules will cross-link to the protein, drastically improving the ability of the protein to withstand protease degradation. By performing the reaction at physiological conditions, it is believed that the native activity and conformation of the protein will be maintained. Analytical HPLC and MALDI-TOS mass spectrometry will be used to confirm the cross-linkage of peptoid to protein. Tricine SDS-PAGE gel and trypsin degradation assays will be performed to assess enzymatic resistance and protein stability.

3. Materials

MBHA rink amide resin was purchased from NovaBiochem (Gibbstown, NJ). The amine sub-monomer *tert*-butyl N-(4-aminobutyl) carbamate was purchased from CNH Technologies Inc. (Woburn, MA), 2-methoxyethylamine and ethanolamine were purchased from Acros Organics (Pittsburgh, PA). Piperidine was purchased from Sigma-Aldrich (St. Louis, MO). Sodium hypochlorite came from original Clorox™ purchased at Walmart (Bentonville, AR). 2,2,6,6-Tetramethylpiperidinoxy (TEMPO, free radical) was purchased from BeanTown Chemical Corporation (Hudson, NH). Sodium cyanoborohydride was purchased from Alfa Aesar (Haverhill, MA). All other reagents and consumables were

purchased from VWR (Radnor, PA) and were used without further modification, unless otherwise specified.

4. Methods

4.1. Peptoid Synthesis

Peptoids were synthesized via the solid-phase submonomer method on MBHA rink amide resin [91]. Initially, the resin was swelled with dimethylformamide (DMF), then the Fmoc protection group was removed by two separate incubations in 20% piperidine solutions in DMF for 30 seconds and again for 12 minutes. The resin's secondary amine was acylated with a fresh solution of 0.4 M bromoacetic acid and N,N'-diisopropyl carbodiimide (DIC) at a ratio of 4.25:0.8, mixing for 1 minute, forming a tertiary amine bond. The amine side chains were attached via nucleophilic displacement at concentrations ranging from 0.5-1.0 M in DMF depending on the side chain. The two-step submonomer cycle was repeated until the desired sequence was obtained (Figure 5.1.). The peptoid was cleaved from the resin by mixing with 95% trifluoroacetic acid (TFA), 2.5% water, and 2.5% triisopropylsilane on an orbital shaker (Belly Dancer, Stovall Life Sciences, Greensboro, NC). The acid was removed using a Heidolph Laborota 4001 rotary evaporator (Elk Grove Village, IL), the peptoid was dissolved in a 50:50 acetonitrile:water solution, and dried to a powder using a Labconco lyophilizer (Kansas City, MO).

4.2. Peptoid Purification

The peptoid was reconstituted in a 25:75 acetonitrile:water solution and purified using a Waters Delta 600 preparative reversed-phase high performance liquid chromatography (HPLC) (Milford, MA) with a Duragel G C18 150 x 20 mm column (Peeke Scientific, Novato, CA). Due to the hydrophilic nature of the NMEG peptoid, equilibration times were extended from 20 minutes to 60 minutes, the injection volume was reduced to 1.2 mL per injection, and a 10-minute delay following injection was necessary prior to the linear gradient of 0-65% solvent B (94.9% acetonitrile, 5% water, 0.1% TFA) in A (99.9% water, 0.1% TFA) over 65 minutes. Peptoids were confirmed to be >98% pure via analytical HPLC (Waters 2695 Separation Module) with a Duragel G C18 150 x 2.1 mm column (Peeke Scientific, Novato, CA) using a gradient of 5-95% solvent D (99.9% acetonitrile, 0.1% TFA) in C (99.9% water, 0.1% TFA) over 30 minutes. Purified peptoid fractions were combined, lyophilized into a powder, and stored at -20 °C.

4.3. Peptoid Characterization

Synthesis and purification of the desired peptoid sequences were confirmed via matrix assisted laser desorption/ionization time of flight (MALDI-TOF; Bruker, Billerica, MA) mass spectrometry using 2,5-dihydroxybenzoic acid as a matrix substance. The oxidation and cross-linkage products were confirmed via a combination of liquid chromatography-mass spectrometry (LC-MS) and MALDI-TOF mass spectrometry.

4.4. Oxidation of Alcohol to Aldehyde

To form the desired aldehyde from the oxidation reaction, a stoichiometric equivalent amount (1:1) of sodium hypochlorite to peptoid was used. The reaction solution contained 0.1% bleach, 1% of 1.4 M TEMPO free radical, and 25% peptoid in phosphate buffered saline (PBS). The concentrations of bleach and peptoid were varied to optimize reaction yield. The reaction time was varied from 0 to 3 hours at 23 °C and a pH of 7.4.

4.5. Cross-linkage Reaction

Prior to the oxidation reaction, a 2 M amine reducing agent was prepared by dissolving sodium cyanoborohydride in a 5 M NaOH solution and allowing it to incubate for 1 hour before use. A 10% protein and water solution was prepared by a ten-fold dilution of 4 mg/mL protein solution. The 10% protein and sodium cyanoborohydride solutions were added to the oxidated peptoid reaction solution. The reaction time was varied from 0 to 4 hours at 33 °C and a pH of 7.4. At hour increments, the reaction was assessed by analytical HPLC using a gradient of 5 to 95% solvent D (99.9% acetonitrile, 0.1% TFA) in C (99.9% water, 0.1% TFA) over 30 minutes. The reaction product at each time point was spotted and the cross-linkage was confirmed by MALDI-TOF.

4.6. Trypsin Degradation

The protein was dissolved in a 50 mM ammonium bicarbonate solution. A 500 mM 1,4-Dithiothreitol (DTT) solution was added to the protein sample to a final concentration of 20 mM (1:25 dilution), then incubated at 60 °C for 1 hour. A fresh solution of 1 M 3-

indoleacetic acid (IAA) was prepared using 50 mM ammonium bicarbonate. The 1 M IAA solution was added to the reduced protein sample to a final concentration of 40 mM (1:25 dilution) and allowed to incubate at room temperature for 30 minutes protected from light. The alkylation reaction was quenched by adding 500 mM DTT to a final concentration of 10 mM (1:50 dilution). Trypsin solution was added to the sample to form a final protease to protein ratio of 1:30 to 1:100 (w/w). The final solution was incubated at 37 °C for 24 hours and stored at -20 °C to stop the digestion reactions. The digested fractions were analyzed using MALDI-TOF and a Tricine-SDS-PAGE gel.

5. Results and Discussion

5.1. Peptoid Sequence Rationale and Characterization

Previous studies have shown that NMEGylation is a viable post-translational modification method to improve the desirable characteristics of biotherapeutic proteins. The peptoid sequences, referred to as JLR-1 and BiCK-5 (Figure 5.1a & 5.1b), each include five N-methoxyethyl glycine (NMEG) side chains to improve the water solubility and serum stability of the target protein. Both, JLR-1, and BiCK-5, contain cysteine-like side chains on the C-terminus to enable covalent linkage to slide surfaces and other molecules. The hydroxyl-group on the ethanol side chain of JLR-1 can be oxidized into an aldehyde to enable the covalent linkage to amine groups on a target molecule. The lysine-like side chain of BiCK-5 provides a primary amine group that can be reacted with the aldehyde on JLR-1 to test the efficiency of and optimize the cross-linkage reaction. MALDI-TOF (Figure 5.2.) and analytical HPLC (Figure 5.3.) confirmed the desired sequences were synthesized.

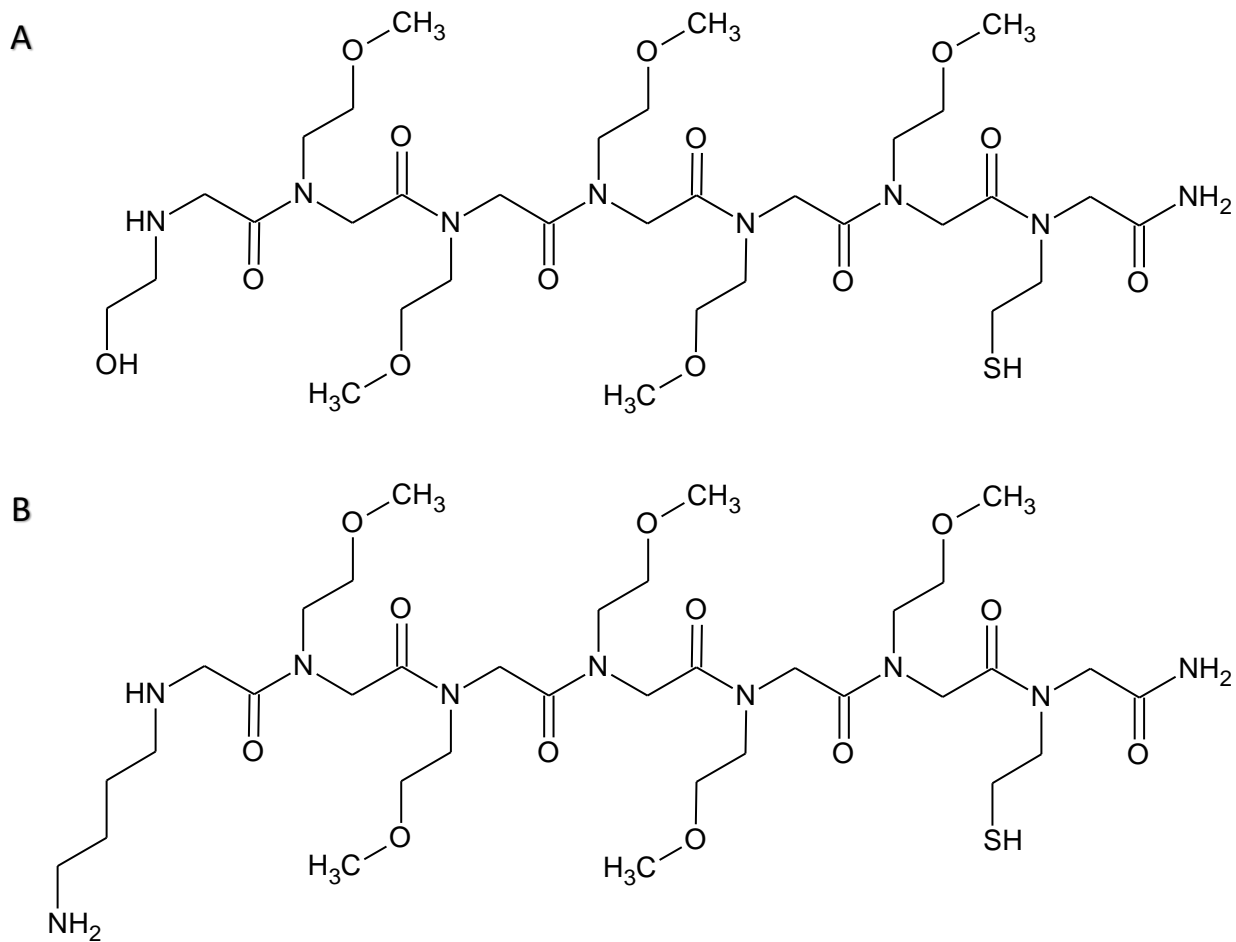


Figure 5.1. Molecular structures for (A) JLR-1 (B) BICK-5 peptides

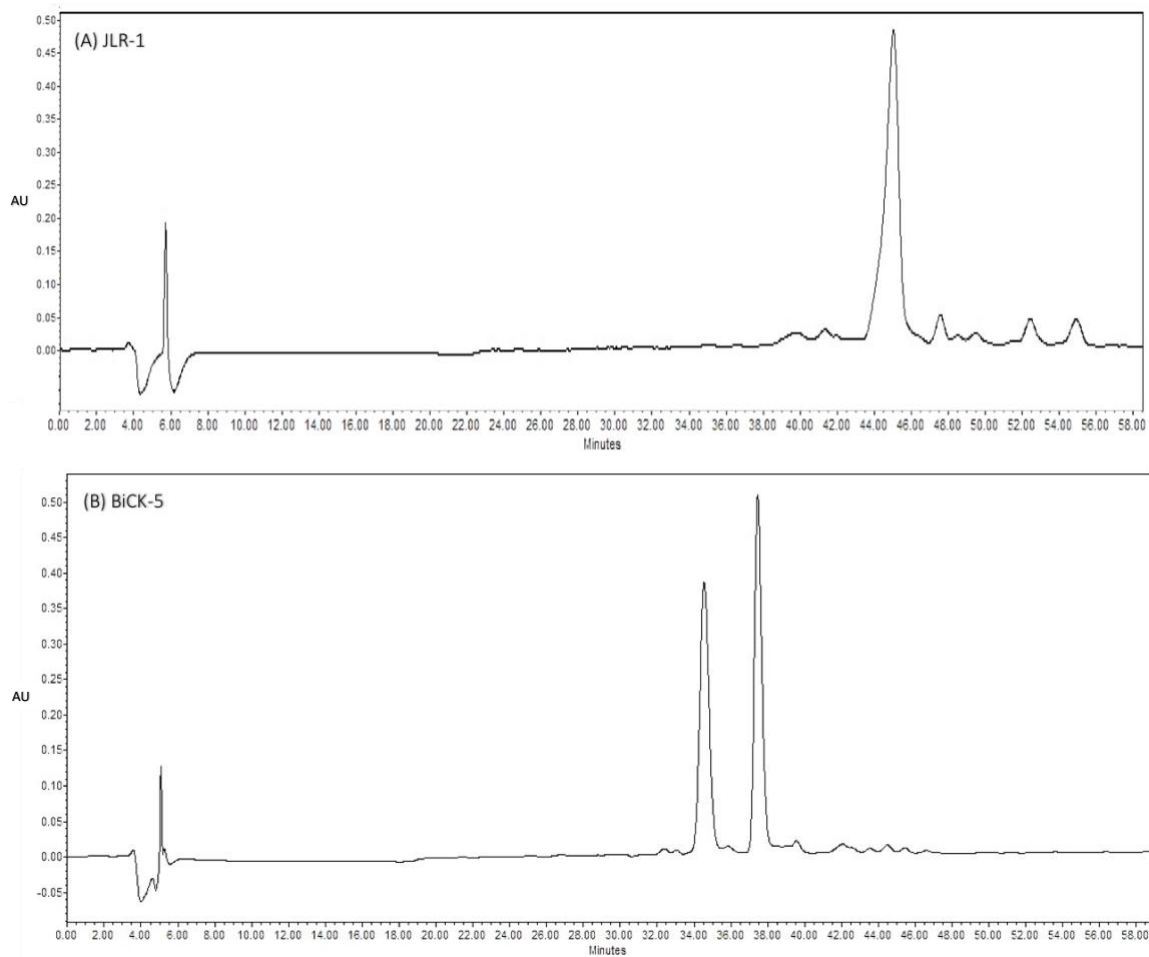


Figure 5.2. Preparative HPLC spectrum for (A) JLR-1 (B) BiCK-5

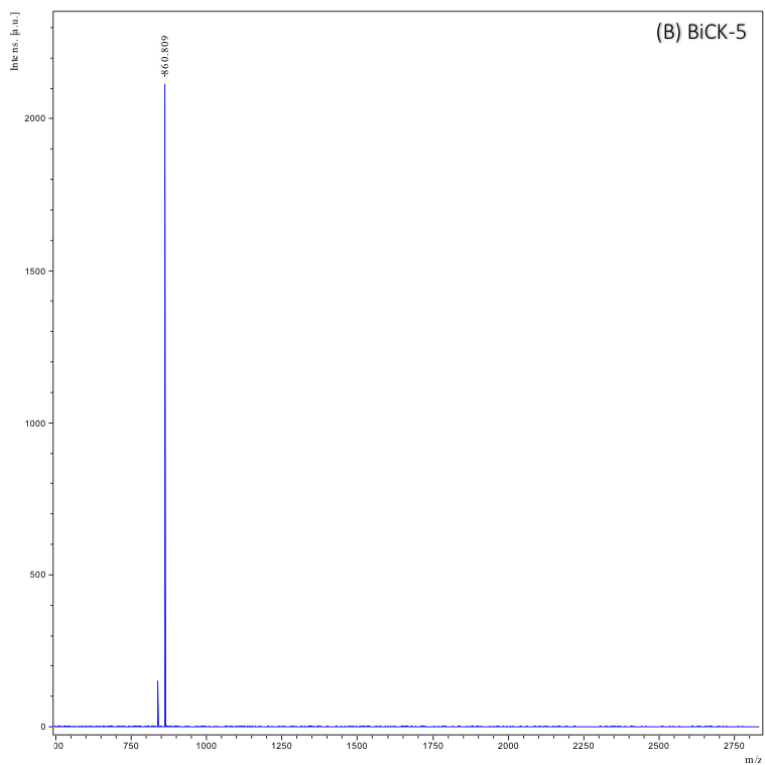
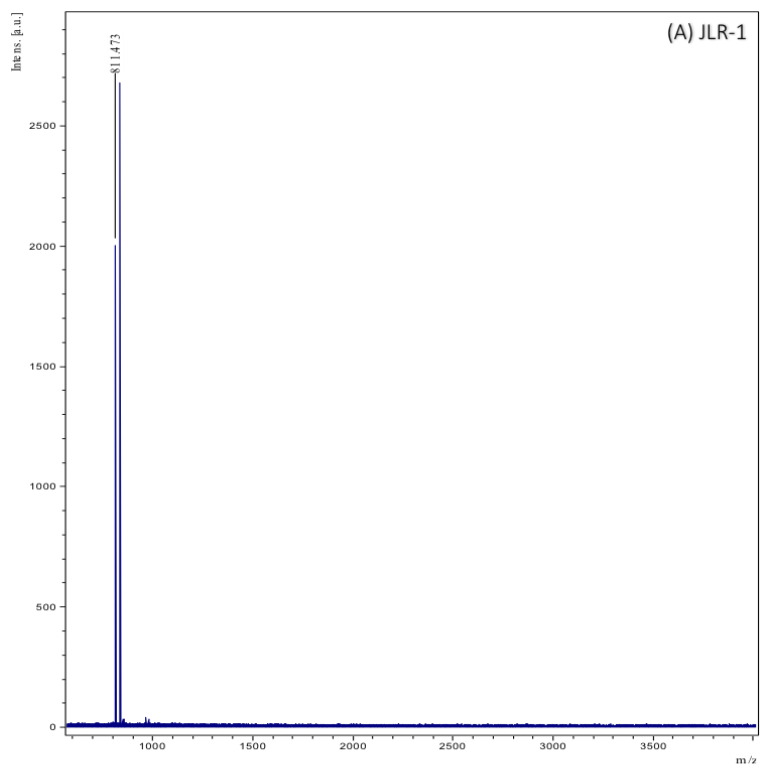


Figure 5.3. MALDI-TOF spectra for (A) JLR-1 and (B) BiCK-5 peptides.

5.2. Oxidation of Alcohol to Aldehyde

The product formed during the oxidation of primary alcohols is directly affected by the amounts of oxidizing agents used, the reaction time, and the reaction temperature. A common issue that arises in the formation of aldehydes is that the oxidation reaction progresses too far, forming an unwanted carboxylic acid group. Thus, to form the desired aldehyde it is important to find the optimal bleach and TEMPO free radical concentrations, as well as an optimal reaction time.

Initially, a solution containing 1% bleach and 1% TEMPO free radical was reacted with JLR-1 for 3 hours at room temperature. The amount of sodium hypochlorite in the reaction was nearly 10x higher than the amount of peptoid used. LC-MS (Figure 5.4.) and MALDI-TOF (Figure 5.5.) results of this oxidation demonstrated the formation of the undesired carboxylic acid group on JLR-1. The LC-MS spectrum showed two main peaks at 6 and 7 minutes, corresponding to the initial JLR-1 and JLR-1 with carboxylic acid, respectively. The mass spectrometry spectrum for the second peak confirmed the formation of the carboxylic acid group, shown by the peak at 825.5 m/z. Based on these results, we can conclude that the oxidation reaction of JLR-1, using 1% bleach, 1% TEMPO free radical, and a reaction time of 3 hours, fully progressed, passing the formation of an aldehyde, ultimately producing a carboxylic acid.

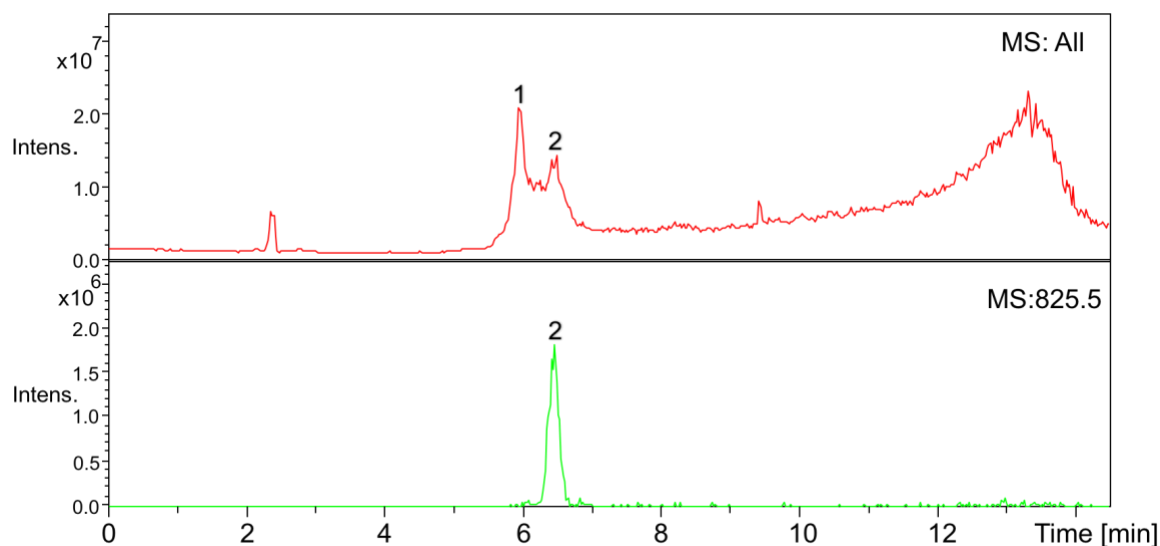


Figure 5.4. LC-MS spectrum for the oxidation of JLR-1 with 1% bleach at 3 hours. (Top) Overall LC spectrum (Bottom) LC peak for JLR-1 with carboxylic acid

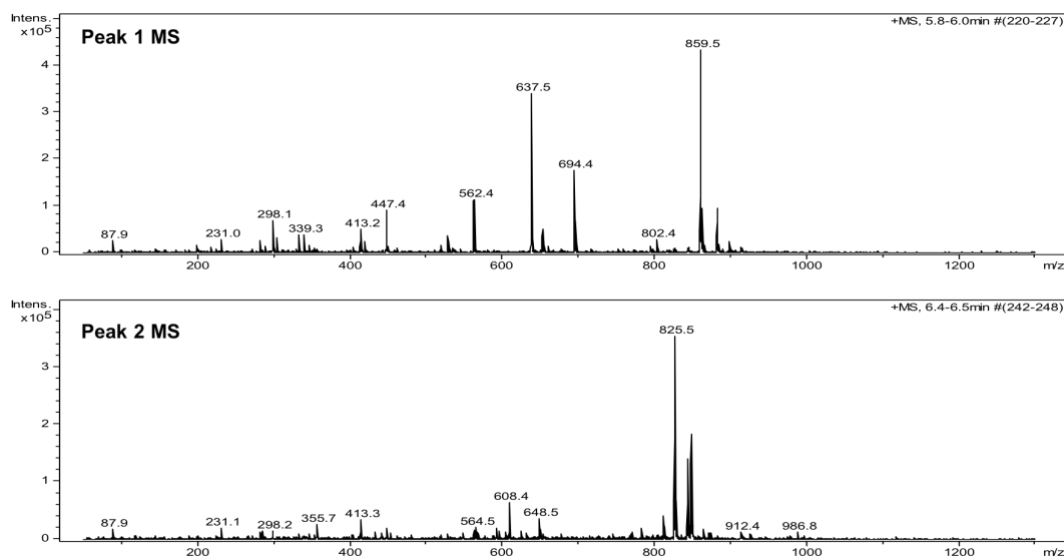


Figure 5.5. Mass spectrum for LC-MS of JLR-1 with 1% bleach at 3 hours.

The amount of bleach used in the oxidation reaction was decreased 10-fold, forming nearly a 1:1 ratio of sodium hypochlorite to peptoid. The reaction time was decreased from 3 hours to 15 minutes, while the TEMPO free radical concentration, pH, and temperature

were kept constant. To determine the time at which oxidation occurs, the reaction solution was spotted for MALDI-TOF at time points of 0, 1, 5, and 15 minutes.

As expected, no oxidation occurred at 0 minutes, shown by a single mass peak at 811 m/z (Figure 5.6.) representing an unoxidized peptoid. At 1 minute, the MALDI-TOF spectrum (Figure 5.7.) began showing a peak at 809-810 m/z indicating the formation of the desired aldehyde group on JLR-1. The intensity of this peak (809-810 m/z) and the sodiated version (831-832 m/z) increased at the 5-minute mark (Figure 5.8.). By 15 minutes, the MALDI-TOF results (Figure 5.9.) showed a drastic increase in the intensity of the mass peak for the newly formed aldehyde peptoid. The sodiated version of this peak displayed an intensity that was greater than that of the unmodified JLR-1 peak (833-834 m/z).

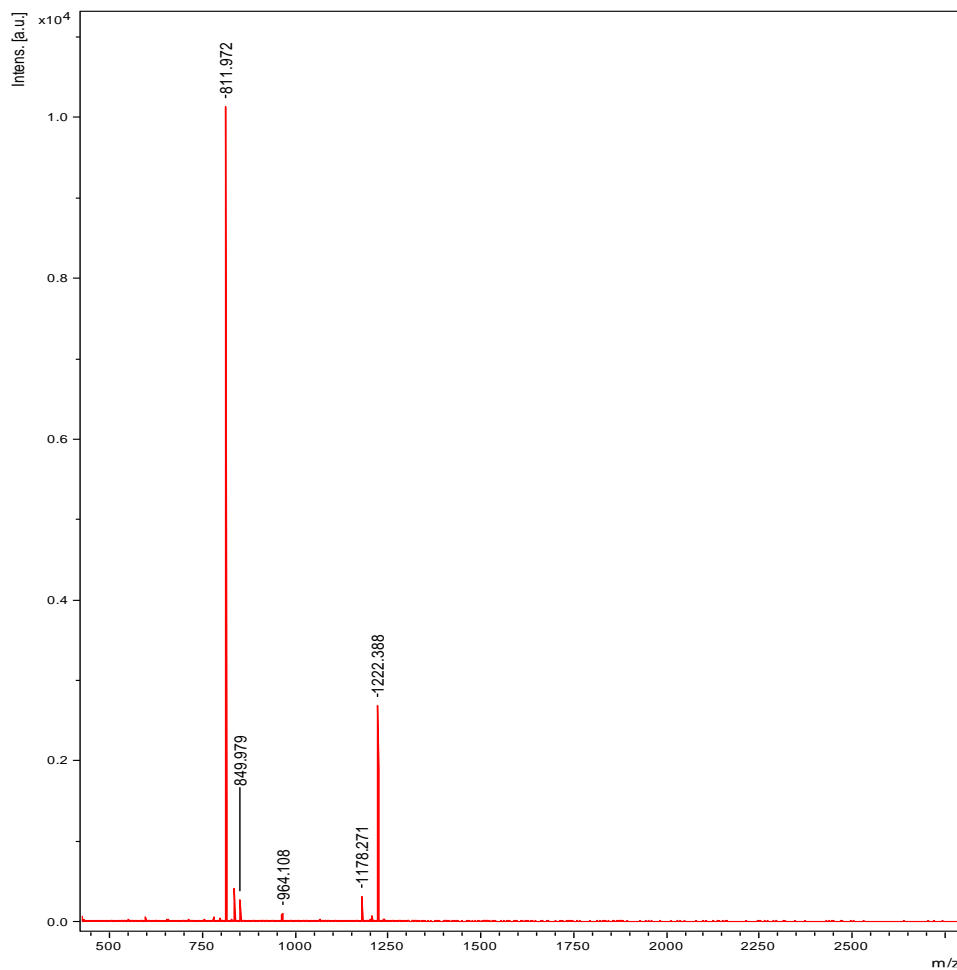


Figure 5.6. MALDI-TOF spectrum for JLR-1 peptoid oxidation with 1% bleach at 0 minutes.

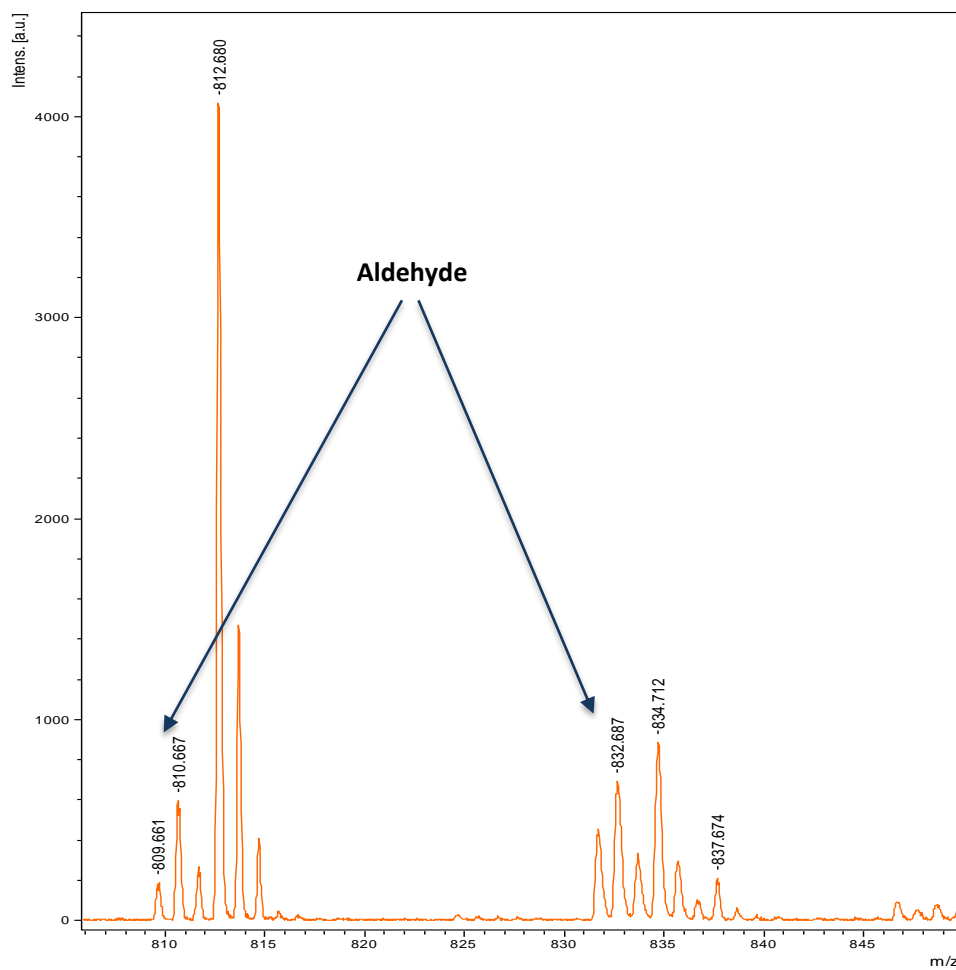


Figure 5.7. MALDI-TOF spectrum for JLR-1 with 0.1% bleach, 1% TEMPO after 1 minute.

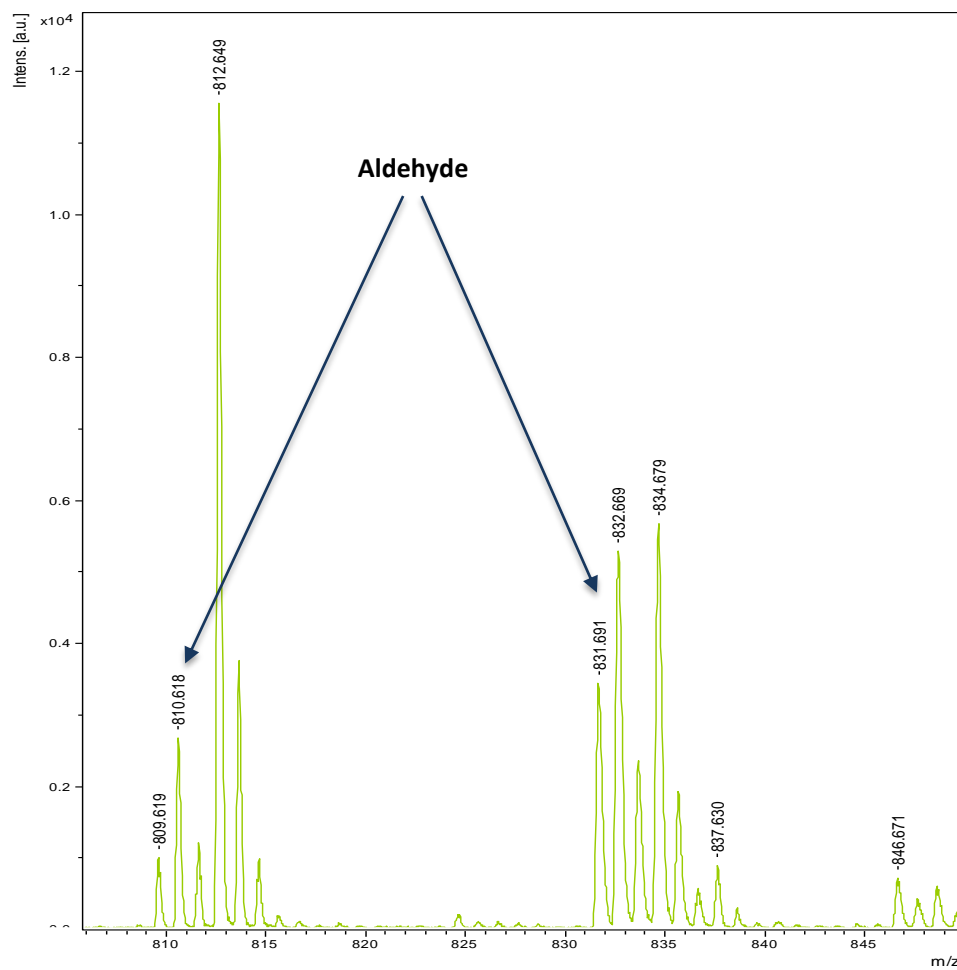


Figure 5.8. MALDI-TOF spectrum for JLR-1 with 0.1% bleach, 1% TEMPO after 5 minutes.

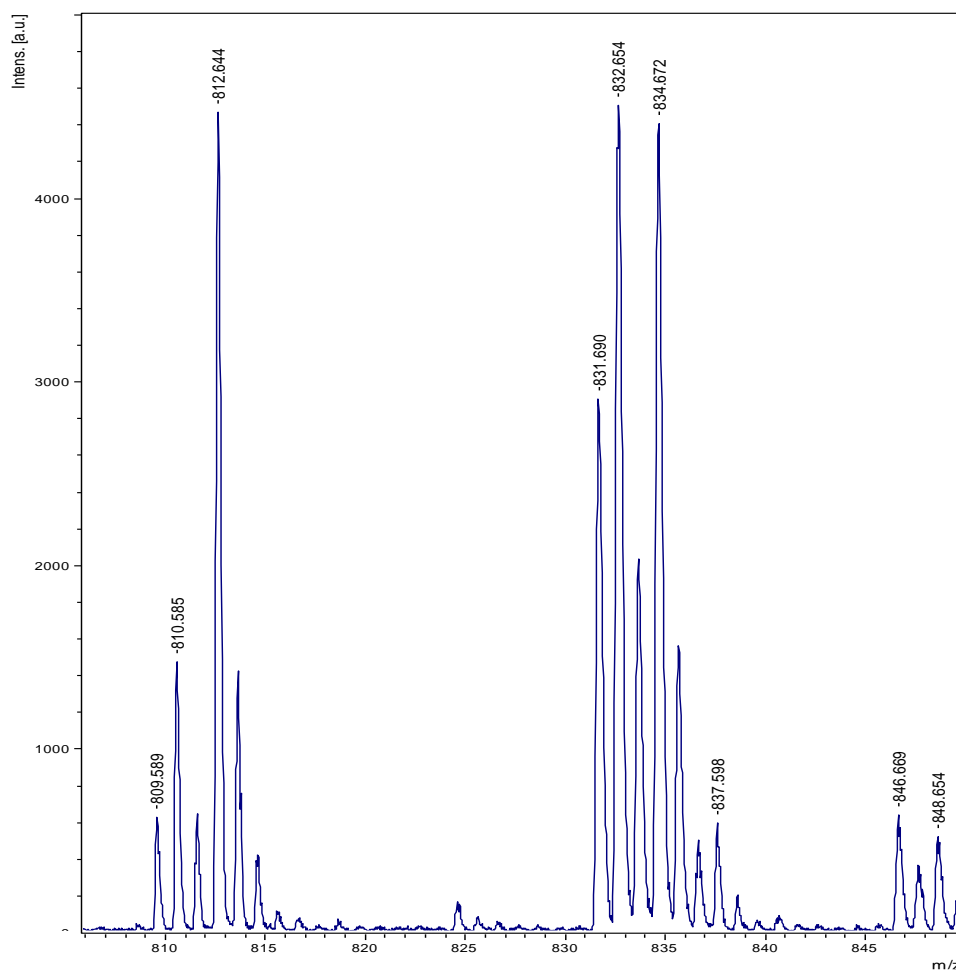


Figure 5.9. MALDI-TOF spectrum for JLR-1 with 0.1% bleach, 1% TEMPO after 15 minutes.

As hypothesized, the primary alcohol of JLR-1 was oxidized, using bleach and TEMPO free radical, and the desired aldehyde group was formed. The amount of bleach and reaction time was proven to be critical in controlling the reaction kinetics. By controlling these kinetics, the oxidation reaction was essentially 'stopped' at the aldehyde, instead of progressing to the formation of a carboxylic acid. Along with a reduction in reaction time, a stoichiometrically equivalent amount of sodium hypochlorite to peptoid is beneficial in forming stable aldehyde molecules from the oxidation of primary alcohols.

5.3. Cross-linkage (NMEGylation)

The reductive amination reaction was tested by cross-linking the JLR-1 and BiCK-5 peptoids. The aldehyde group on JLR-1 covalently attaches to the primary amine on BiCK-5 in the presence of a strong reducing agent (sodium cyanoborohydride). The reaction product was analyzed by MALDI-TOF, which shows a mass peak at 1670 m/z that could potentially indicate a successful cross-linkage (Figure 5.10.). The expected molecular weight of the JLR-1 and BiCK-5 cross-linkage is 1631 Da; however, the 1670 m/z peak could represent the cross-linked peptoid in the presence of a potassium ion (+39 m/z). Unfortunately, the 1670 m/z peak could also be due to the undesired reaction between thiol groups on the cysteines of both peptoids. This thiol linkage results in an expected peptoid conjugate mass of 1647-1648 Da, which if sodiated, could also be representative of the 1670 peak.

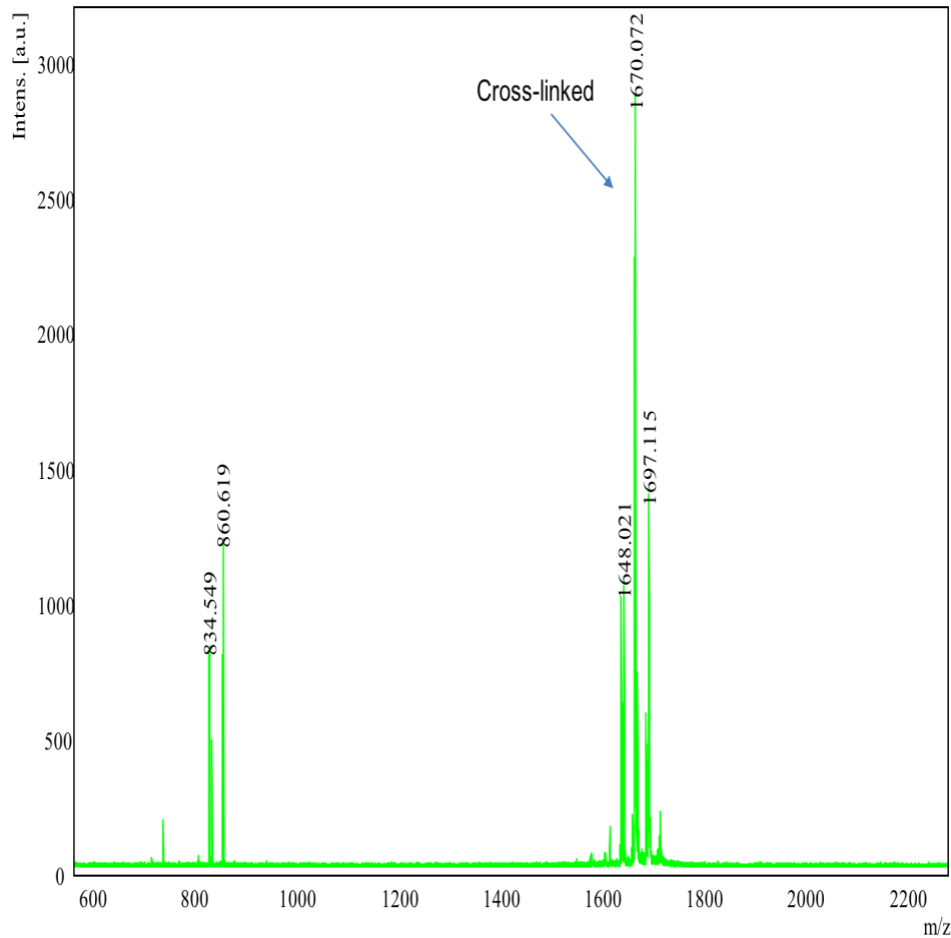


Figure 5.10. MALDI-TOF spectrum for the potential cross-linkage of JLR-1 and BiCK-5.

One issue observed in the cross-linkage between peptoids is the similarity in molecular weights between the two molecules. The molecular weight of the aldehyde version of JLR-1 (809 Da) was only 29 Da less than BiCK-5 (838 Da). Other issues arise due to the complexity in the cross-linkage reaction, the potential for unwanted reactions, and the presence of salts (sodium, potassium, etc), making the characterization, especially via MALDI-TOF, extremely difficult.

In order to overcome the issues observed with BiCK-5, the protein bovine cytochrome c was chosen for the cross-linking reaction with JLR-1. Cytochrome c has an abundance of

lysine amino acids, 18, that could be modified in the amination reaction, increasing the chances for successful NMEGylation. Immediately following the oxidation of JLR-1, bovine cytochrome c and sodium cyanoborohydride were added to the peptoid solution and allowed to react for 4 hours at physiological conditions (temperature of 33 °C and a pH of 7.4). The analytical HPLC results demonstrate a shift in the HPLC peaks that increases in intensity as time progresses (Figure 5.11.). The initial cytochrome c (Figure 5.11A) and JLR-1 (Figure 5.11B) peaks at 17 and 13.5 minutes, respectively, have shifted to the modified protein peak (Figure 5.11C) at ~10 minutes. We predict that this shift represents the formation of peptoid-protein conjugates through the successful cross-linkage of JLR-1 to cytochrome c. As reported in the literature, NMEGylation increases the water solubility of the protein; therefore, the modified protein would be expected to elute from the column faster than the original protein did.

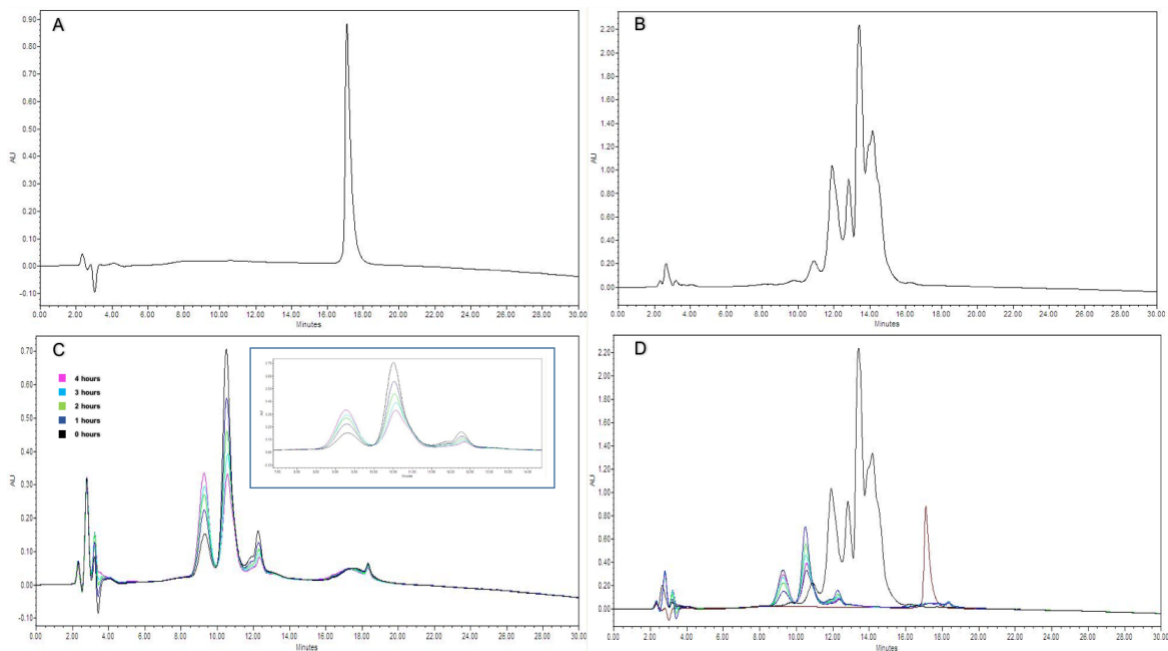


Figure 5.11. Analytical HPLC spectra for (A) cytochrome c protein (B) oxidized JLR-1 peptoid (C) cross-linkage of cytochrome c with JLR-1 at 0 (black), 1 (dark blue), 2 (green), 3 (light blue), & 4 (pink) hours (D) combination of A-C.

The MALDI-TOF results (Figure 5.12.) for the reaction also indicate that the protein was successfully modified; however, the extent of modification is still unclear. It is proposed that the modification occurred on several lysines, producing a mixture of conjugate polymers that reduce the intensity of the modified cytochrome c peak (12,232 m/z). The number of lysines that are accessible for modification is dependent on the three-dimensional structure of the protein. Another issue arises with the possibility of JLR-1 forming disulfide bonds with the heme prosthetic group of cytochrome c. The heme group serves to organize the protein structure of cytochrome c. It is possible that by binding with heme, JLR-1 interfered with the formation of native protein structure, resulting in an unexpected number of lysines to be modified.

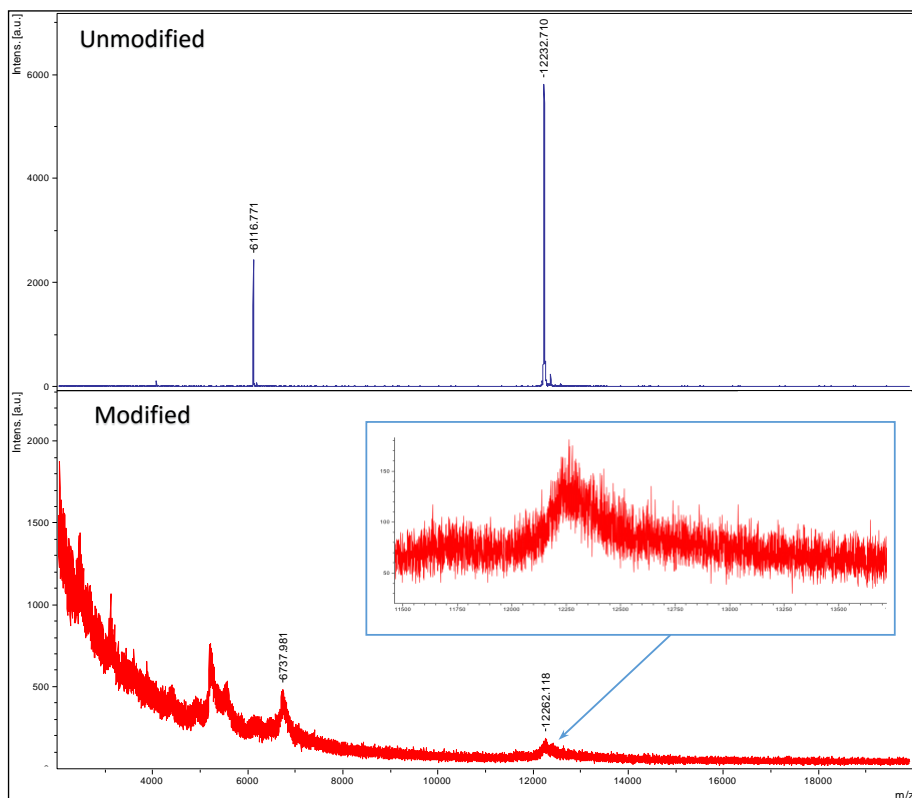


Figure 5.12. MALDI-TOF for the cross-linkage of JLR-1 to cytochrome c.

6. Conclusion and Future Work

The need for accurate, effective, and long-lasting biotherapeutic treatment options has led to the development of more intricate modification methods and drug delivery systems. Biotherapeutics require evolutionary advancements in serum stability and drug efficacy to be beneficial in future medicinal applications. The challenge lies in developing modification methods that form homogeneous products in a manner that native protein conformation and activity is maintained. We believe that the use of peptoids for the modifications of biotherapeutic proteins provides a propitious mode to achieve such goals.

We were able to successfully design a green chemistry oxidation method that modified a peptoid side chain to be used as a reactive functional group in the NMEGylation of a target protein. We found that by altering the reaction time and concentrations of reagents, the reaction rate can effectively be controlled and the desired oxidative product can be obtained. We demonstrated this ability by forming a stable aldehyde functional group from the oxidation of a primary alcohol using only bleach and TEMPO free radical as oxidative agents. Initially, higher bleach concentrations and longer reaction times caused the reaction to by-pass the aldehyde, and directly form a carboxylic acid. Through a ten-fold dilution in bleach concentration and a reduction in reaction time to 15 minutes, we were able to produce the desired aldehyde. Sodium hypochlorite activates the TEMPO free radical which serves as the main oxidant for the reaction. We believe that the reduction in sodium hypochlorite limited the amount of TEMPO free radical that was activated; ultimately stopping the reaction at the aldehyde. It is important to note that although the reaction was successful in producing the desired product, there is still room for

improvement in the efficiency of the reaction. It would be interesting to assess the effect of lowering the TEMPO concentration on aldehyde formation. The ratios of TEMPO and bleach can be varied to optimize the aldehyde production. If the amount of bleach and TEMPO can be reduced even further, then it would make it more environmentally friendly, improving the E-factor rating of the reaction. Also, instead of going straight into the cross-linkage it would be intriguing to investigate purification methods, and stability tests for the newly formed aldehyde peptoid.

We have studied the attachment of NMEG peptoid to a target protein to overcome stability and absorption issues displayed by current biotherapeutics. We proposed that an aldehyde group on the peptoid can be reductively aminated with primary amines on the target protein to form a homogeneous NMEG peptoid-protein conjugate. It is believed that this conjugate will carry some of the desirable properties seen in PEG and NMEG monomers, such as increased water solubility and resistance to enzymatic degradation. We have evaluated the use of NMEG peptoids for site-specific modification to biotherapeutic proteins; however, are still limited in characterization techniques. Initially, the amination reaction was tested between two peptoids, JLR-1 and BiCK-5, but due to similarities in molecular weights the product could not be fully characterized. It is believed that the cross-linked product shown on MALDI-TOF is an unwanted disulfide linkage formed between the cysteine groups on both peptoids. To eliminate this possibility, our lab will further investigate the synthesis and usage of peptoids without cysteine side chains. Also, the molecular weight disparity can be increased by synthesizing peptoids with various NMEG chain lengths ($n=5, 10, 15$).

We have demonstrated that the NMEGylation of target proteins results in many of the same limitations that currently hinder PEGylation as a viable modification method. When evaluating the cross-linkage of JLR-1 to cytochrome c, the mass spectrometry results indicated that a cross-linkage between the two molecules possibly occurred, but the degree of modification (number of lysines modified) could not be determined. From the MALDI-TOF results in Figure 3.10., we believe that the number of lysines modified with peptoid varied from molecule to molecule, forming a heterogeneous mixture of peptoid-protein conjugates. The covalent bonding between thiol functional group on the cysteine side chain of JLR-1 and the heme group of cytochrome c may play an important role in the characterization issues. Any factor, whether it's the binding to heme, or a change in reaction conditions, can cause the protein to unfold, revealing hidden amino acids that are now able to react. Depending on the reaction environment, each individual protein molecule may undergo a unique conformational change, resulting in a wide array of reactive groups. It is important to note that although heterogeneous conjugates were potentially formed, the viability of NMEGylation as a biotherapeutic protein modification method needs to be further investigated. It would be interesting to complete a more thorough study on the cross-linkage of various NMEG peptoids to different target proteins. To avoid potential heme binding, several target proteins, such as myoglobin, can be used in place of cytochrome c. Other peptoid sequences can be adopted to pursue different conjugation chemistries with the target protein. Trypsin assays and Tricine-SDS-PAGE gel electrophoresis can be used to further analyze the reaction products formed and to test resistance of the newly developed protein-peptoid conjugate to enzymatic degradation.

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