ABSTRACT

An Expansion on Protein PEGylation for cancer therapeutics: from Bench to Bedside

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Director: James A. Marcum, PhD

PEGylation is a biochemical modification of biomolecules using polyethylene glycol (PEG) that confers several desirable properties to the modified biomolecule. It has been shown to improve stability, solubility, increase half-life and reduce immunogenicity of biological molecules like proteins, enzymes and nanomolecules. PEGylation then is a good means for possibly improving drug therapeutics. The process of PEGylation is complex and has undergone many changes and improvements. The first chapter of this thesis is a review article I published with a cancer research lab. It explored the first- and second-generation methods of PEGylation including the chemistry, synthesis, benefits, and pitfalls of PEGylation. It also listed some of the marketed PEGylated therapeutics in use at the time. The second chapter is an expansion of that review article almost three years later. It explores the third generation of PEGylation, the efficacy and current use of the therapeutics listed in the first chapter and explores the possible future of PEGylation.

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AN EXPANSION ON PROTEIN PEGYLATION FOR CANCER THERAPY: FROM BENCH TO BEDSIDE

A Thesis Submitted to the Faculty of

Baylor University

In Partial Fulfillment of the Requirements for the

Honors Program

By

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Waco, Texas

May 2020

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ACKNOWLEDGEMENTS

I want to acknowledge all the people who have helped me get where I am today because this success is as much theirs as it is mine. First I would like to thank Dr. Marcum for believing in me and giving me the freedom to take my time and explore my ideas with this thesis. I would also like to thank Dr. Brown and Dr. Davidson for taking time out of their schedules to review my thesis and be on my committee.

Second, I would like to thank Dr. Banerjee, Viju and the entire Cancer Research Unit at the Kansas City VA. They welcomed me with open arms and not only introduced me to the world of research but pushed me to dive in headfirst. Because of them, I was able to publish my own research review, something I never imagined I would be able to do in college. They are the reason I was able to write this thesis.

Third, I would like to thank my parents for everything they have done for me. In turn, everything I do is for them. I want to thank my friends for always keeping me in high spirits. They are the best pick-me-ups. Thank you Swetha for keeping me accountable and staying by my side while I wrote this.

I would also like to thank my dad, Dr. Beck and Robin for encouraging me to stay in the Honors program and finish the thesis. I really appreciate their support whenever I doubted myself. This is just the beginning.

To the essential workers and scientists.

Thank you for your bravery during this pandemic.

Research is how we advance forward

PROLOGUE

In 2018, along with the cancer research lab at the Kansas City VA Hospital, I published a review article titled Protein PEGylation for cancer therapeutics: from bench to bedside. The article discusses PEGylation, what it is, it's purpose, benefits, pitfalls, synthesis, chemistry and its current uses in therapeutics. With PEGylation's primary benefit being reducing the immunogenicity and increasing retention time of PEGylated biomolecules, its probable use in cancer therapeutics is valid and relevant. That being said, I decided to expand on this article. I have learned a lot since publishing that article; medicine and healthcare is more complex now than it ever has been. Comprising about 17 percent of the nation's gross domestic product (GDP), healthcare is a multi-trillion dollar industry that is growing at an ever-increasing rate. I wanted to see how PEGylation is doing now-- in current therapeutics. Does it work and what does its prevalence look like in future areas of medicine? Precision medicine is a recently growing sector of cancer therapeutics. It uses each patient's unique genetic pattern to curate treatment. I'm wondering if PEGylation can fit into that.

This thesis aims to explore these areas of PEGylation as a mechanism for improved therapeutics, with a focus on cancer treatments. Chapter 1 will consist of the published review article titled "Protein PEGylation for cancer therapy: bench to bedside" and includes a detailed explanation of PEGylation, specifically, the creation of PEGylated therapeutics. It is untouched, the work of multiple authors and a product of the Kansas City VA Medical Center Cancer Research Unit. Chapter 2 will be an update: including a look into the current research in PEGylation, from 2017 forward, as well as a literature review

of current PEGylated drugs and their efficacy to treat specific diseases. This section focuses more on the application and efficacy of the PEGylated therapeutics.

CHAPTER ONE

Protein PEGylation: Bench to Bedside[1]

Introduction

Polyethylene glycol (PEG) is a non-immunogenic biological compound made of repeating ethylene glycol units (Figure 1a) (2). Covalent and non-covalent attachment of PEG to biological molecules including proteins and enzymes, called PEGylation, has had a proven effect of increased half-life, reduced immunogenicity, and improved drug solubility and stability (3). Each PEG molecule can combine with two or three water molecules, making the overall compound larger and more hydrophilic (4). PEG molecules can be branched or linear, each having their own advantages when reacting with certain proteins. Branched PEG molecules tend to increase certain "stealth" properties of a conjugated biomolecule, thereby also increasing "in vivo" half-life. PEG conjugation has the ability to modify physiochemical properties and increase the retention of the therapeutic in the body, which is why it is useful for newer drug therapies (1).

Early work on PEGylated proteins and enzymes was conducted in Frank Davis's laboratories during the late 1970's. They laid down the founding materials for protein PEGylation as a targeting drug delivery system for researchers today. Davis's work included linking a methoxy-PEG group to the amino acids present in proteins using cyanuric chloride as the coupling agent (Figure 1b). Their work demonstrated that

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PEGylated proteins had longer half-lives in the bloodstream and decreased immunogenicity (5). Because of the newfound use of proteins in therapeutics, PEGylation has emerged as a well-established method in the field of targeted drug delivery systems.

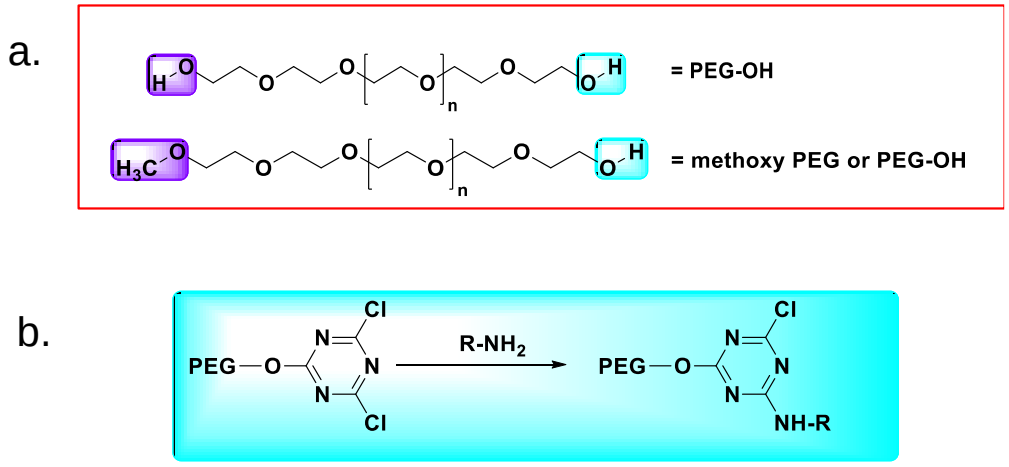

Figure 1. (a) Poly(ethylene glycol) with the most frequently used endgroups, diol on top, and methoxy PEG at the bottom panel. (b) First generation PEGylation using cyanuryl chloride as coupling agent.

The advantage of PEGylation is that it leads to retention and enhancement of favorable properties of protein therapeutics without loss of function. Chemical, molecular, and structural properties of PEGs, as well as their conformational behavior in aqueous solutions govern the pharmacological disposition of PEGs and PEGylated products in physiological compartments (6, 7). Key chemical properties that direct the capacity of PEG to modulate pharmacokinetic and pharmacodynamic profiles of small molecular drugs, proteins or nanoparticles are: (1) molecular weight (2) polydispersity (3) conformation, and (4) end group functionality. PEG molecules can vary in size and shape, thereby providing options to modulate pharmacological output for different drug therapies. This is especially

useful when proteins are needed for an extended period of time with non-significant degradation or loss of function (8). PEG molecules are an important part for other site-specific targeted therapeutics such as with the use of nanoparticles (NP). Surface modification of the nanoparticles with PEGs of differing chain length, shape, density, and molecular weight allow for a more advanced drug delivery system for anti-cancer therapy with superior targeting capacity and biocompatibility (9). PEG molecules render NPs more biocompatible and efficacious by masking undesirable properties, such as surface cationic charge, or by enhancing water solubility. The high stability and low immunogenicity of PEGylated proteins result in sustained clinical response to drugs and allows for minimal dose and less frequent administration. PEGylation of liposomes improves the stability, circulation time, and improves the targeting ability known as enhanced permeation retention effect. These effects improve the therapeutic outcome and reduces the toxicity of the encapsulated drug (10).

PEGylation is considered to be the first successful technology to improve the pharmacokinetic profiles of therapeutic agents and has been applied in the clinic for more than 25 years. It is also the most established half-life extension technology in the clinic which has proven to be safe and FDA approved (11). This review examines the functionalities of protein PEGylation, its application and significance in the area of targeted drug delivery, and potential drawbacks that might inhibit their widespread use in designing site-specific drug delivery systems.

Chemistry of PEG

The process of PEGylation starts by obtaining the PEG molecules from a monomeric ethylene oxide, using ring-opening polymerization (1). Polymerization with water can result in bifunctional or monofunctional PEG chains. Monofunctional PEG molecules or mono-methoxylated PEGs (mPEGs), obtained by methanol initiation, are typically preferred as the starting material for protein PEGylation (1). The results from functionalizing PEG molecules is a mixture of molecules that are mono-dispersed with a range of molecular weights and therefore, typically requires additional purification steps (1). Crude results from first-generation, non-specific binding are a combination of isomers and polymers of different sizes and molecular weights. There is usually a combination of mono-PEGylated, di-PEGylated and fully PEGylated conjugates, each of which have slightly different properties, especially in terms of hydrophilicity and charge distribution (1). Since PEGylation is typically an additional step post-synthetic to protein, many methods have been tried to achieve the best results along the most economically viable route (1).

Chemically describing, with a general structure of $HO\text{-}(CH_2CH_2O)_n\text{-}CH_2CH_2\text{-}OH$, preparation of polyethylene glycol theoretically can be achieved through the reaction of ethylene oxide with water, ethylene glycol or ethylene glycol oligomers. The reaction could follow either anionic or cationic ring opening polymerization route, the former being preferred to realize a low polydispersity product (Figure 1b). As the most frequently used route, poly (ethylene glycol) is synthesized through an anionic ring-opening polymerization of ethylene oxide initiated by nucleophilic attack of a hydroxide ion on the epoxide ring (12). Polydispersity (the ratio of weight average to number average molecular

weight of a polymer, M_w/M_n) of commercially available PEG is usually < 1.1 (13). Monomethoxy PEG (mPEG) (CH_3O-$(CH_2CH_2O)_n$-CH_2CH_2-OH, which is heavily used for polypeptide modification, is synthesized by anionic ring opening polymerization initiated by methoxy ions. PEGs with different end groups can be realized by using different initiator/terminating reagents. Both homo (same functional groups on both ends of PEG chain) and hetero (different functional groups on both ends of PEG) forms have been synthesized. Different functional groups at the end of the PEG chain can also be introduced by the post-polymerization approach. The common functional groups that have already been synthesized and commercially available are listed in Figure 2.

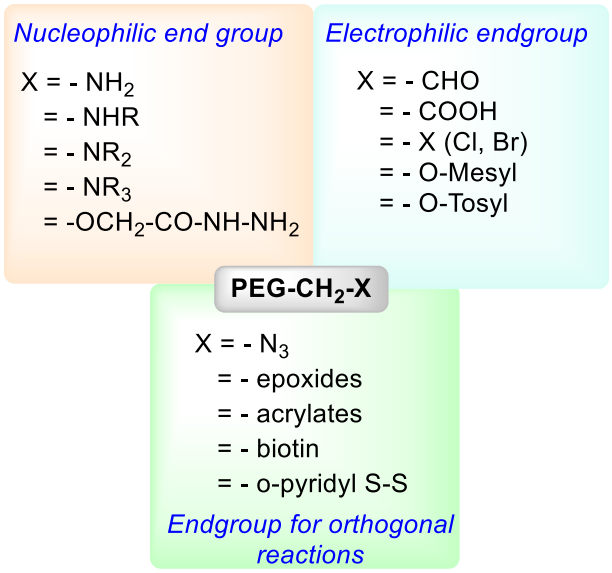

Figure 2: Common end groups that are available for PEGylation of proteins and different substrates.

PEG's unchallenged success in therapeutic formulation can be attributed to the following properties of this macromolecule: (a) broad-spectrum of solubility in both organic and aqueous media (b) minimum toxicity and immunogenicity compared to

polymers of equivalent molecular weight (c) non-biodegradability (d) hydrophilicity, and (e) fast clearance. In addition, the stealth behavior of PEGs to physiological surveillance mechanisms such as immune or reticuloendothelial system (RES) has also triggered the use of PEG in variety of systemic and non-systemic preparations. Historically, and in most of the cases until now, attachment of small or macromolecular therapeutics are generally realized through covalent bonding with terminal primary OH groups of PEG. The presence of a finite number of functional end groups on linear PEGs suppresses the tendencies of the molecule to crosslink, thereby improving dispersity and homogeneity of the intended conjugate, particularly if the target substrate has multiple numbers of complementary functional groups.

Molar mass of PEG controls the bio-distribution of PEG-conjugates. As any ring-opening polymerization, molecular weight of PEG is determined by controlling the ratio of monomer to initiator. Molar mass is one of the most critical factors that governs biocompatibility, stealth behavior and ability of PEG to modulate pharmacokinetic profiles of other conjugated protein molecules. Typically, PEG of molar mass 400 Da to 50 kDa has been used in different biomedical applications, with 1-5 kDa PEGs are often being used for conjugating antibodies and nanoparticles while 20-50 kDa PEGs are intended for use in conjugation with low molecular weight drugs or highly unstable products such as oligonucleotides and siRNA (Ref/US). With larger assemblies, addition of PEG decreases the opsonization and elimination by the reticuloendothelial system, while for smaller and unstable targets, PEGylation improves systemic half-life through minimization of renal clearance. Most consumer products available for healthcare, cosmetics and households contain PEGs of different molecular weights, reflective of the broad-spectrum

biocompatibility of this product. For example, PEGs of 3-5 kDa have been approved by the FDA for use in laxative preparations (Ref/US) and has been considered as a GRAS (generally recognized as safe) product for use in pharmaceutical preparations. Molar mass of a molecule, along with the molecular architecture, determines the hydrodynamic volume it's going to occupy in aqueous environment, which controls the rate of excretion of the polymer from circulation.

Synthesis of PEG is accompanied by low polydispersity. Homogeneity of a synthesized polymer is indicated by a polydispersity index (PDI), which in turn is defined by the ratio of number average and weight average molecular weights of the product. A PDI value less than 1.1 indicates a homogenous distribution of the polymer in terms of the efficiency and reproducibility of the macromolecule synthesis (16, 17). Obtained through anionic polymerization of ethylene oxide, PEG exhibits PDI around 1.01, thus providing excellent uniformity in terms of pharmacokinetic properties (6).

Solubility of PEG. PEG exhibits moderate to high solubility in most of the common organic solvents, as well as in water. Hence, chemical modification of PEG at its end group is facile, as well as cleaning-up of the product through an aqueous purification method (such as dialysis of gel filtration chromatography) is relatively easy. Aqueous solubility is also key to the usage of PEG in biological applications. PEG is generally attached to biologically active molecules through its terminal primary hydroxyl (OH) groups. Hence, most of the OH-group chemistry, such as esterification, etherification, and carbonate linkage formation are employed for the preparation of PEG-conjugates. Although some of these pathways yield cleaner products, in many instances, it is difficult to separate the targeted, transformed product from the reaction mixture containing the unreacted polymer

and the polymer that underwent a side reaction. Excellent differential solubility of PEG in various organic solvents and water enables easy separation of products to yield a clean, final conjugate. It is also notable that, while for small molecular drug conjugates, purification of the final product is extremely critical, protein-conjugated PEGs can be separated from the unreacted reaction components based on molecular weight, charge or hydrophobicity. Hydrophilicity of PEG is also critical for maintaining and enhancing water solubility of non-polar drug molecules. Such solubility enhancement is essential not only for in vivo stability of drugs, but also for increasing storage stability. Such enhancement of physical and thermal stability is connected to so-termed 'conformational cloud' originated from the molecular flexibility of PEG (6).

Chemical diversification of PEG structure. A vast array of chemical diversification strategy of PEG has been tested, reported and commercialized. The main objective of such diversification is to render the polymer amenable to conjugation to small and macromolecular therapeutic agents, including drugs, oligo- and polypeptides, proteins (enzymes and antibody), oligonucleotides, and biomaterials surfaces. PEG has also been used as a cross-linker in case of which bifunctional derivatives of PEG has been synthesized. The functional end group requirement of PEG is largely directed by the complementary functional group of the substrate that will be coupled to PEG. For example, in order to conjugate with lysine residue that is present in most of the proteins, carboxylic acid terminated PEG is most frequently used. Other reactive amino acids present in proteins, which are typically conjugated with PEG include lysine, cysteine, histidine, arginine, aspartic acid, glutamic acid, serine, threonine and tyrosine (12).

For lysine, alpha and epsilon amino groups are most often conjugated with PEG. Harsh chemical conjugation techniques are generally avoided. PEG derivatives that has been synthesized earlier for conjugating to amine groups of lysine (or other amine groups) are summarized in Figure 1. These first generation PEG modification strategies for amine groups of amino acids, as compiled by Harris et al., include: a) PEG dichlorotriazine b) PEG-tresylate c) PEG-succinimidyl carbonate d) PEG-benzotriazole carbonate e) PEG-p-nitrophenyl carbonate f) PEG-trichorophenyl carbonate g) PEG-carbonylimidazole and h) PEG-succinimidyl succinate. These PEG chemistries use acylation mechanisms to modify amine groups of proteins. In many cases, these reactions showed limitations in terms of impurities, low molecular weight products, unstable linkages, and lack of selectivity. Acylation of amino groups by PEG, mediated by urethane linkages has also been explored, where p-nitrophenyl carbonate, trichlorophenyl carbonate, and carbonylimidazole PEG has been used for polypeptide modification. PEG succinimidyl succinate (PEG-SS) is another first-generation PEG-reagent that has been used for protein PEGylation, but exhibited issues of hydrolysis and immunogenicity.

Reductive amination has found application in protein PEGylation with the advent of more targeted PEGylation reagents, such as PEG-aldehyde. However, this molecule has later been replaced by acetal derivatives of PEG-propionaldehyde or PEG-acetaldehyde (18), which is in-situ converted to aldehyde hydrate by acid hydrolysis. Acetal derivatives have longer storage stability than PEG-propionaldehyde or PEG-acetaldehyde.

Active esters of PEG carboxylic acid have been the most frequently utilized reagents for PEGylation chemistry (Figure 3). Active ester covalently conjugates with amines to form amide bonds which are stable at physiological conditions.

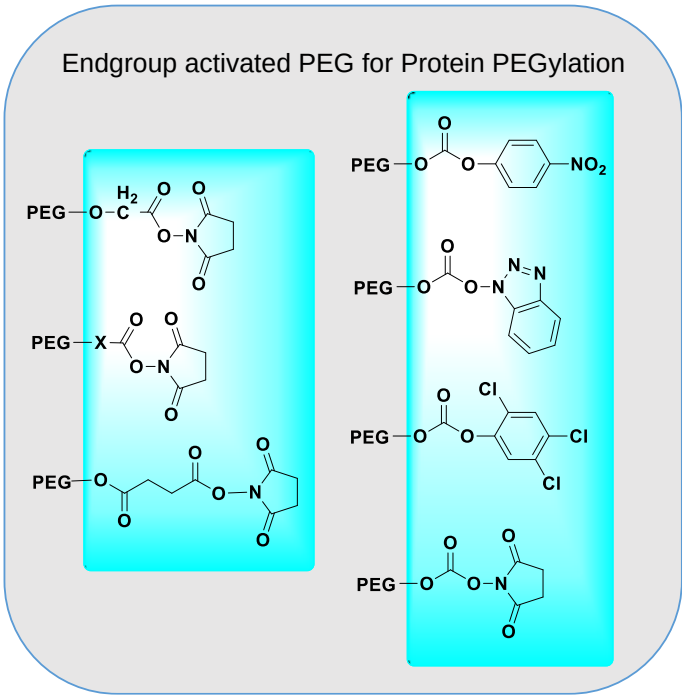

Figure 3: Activation of carboxylic end groups of PEG for coupling to N-terminal of peptides.
These coupling chemistry belong to the so-termed ‘first generation’ PEGylation strategy.

Active esters of PEG can also be purified easily from unsubstituted or over-substituted compounds by ion-exchange chromatography (19). Most frequently encounters active esters of PEG carboxylic acids are N-hydroxysuccinimide (NHS) and carbodiimides. Carboxymethylated PEGs (CM-PEG)(20) have been used as a substitute for SS-PEG to avoid premature dePEGylation of the PEGylated compounds. Succinimidyl ester of carboxylmethylated PEG is a very reactive intermediate (t1/2 of 0.75 minute at pH 8 and at 25C). Distance between carboxylic acid group and PEG-backbone has a significant effect of hydrolysis of the PEGylated compound. Harris et al synthesized propionic and butanoic acid derivatives of PEG. It was found out that, SBA-PEG with two additional methylene units shows a longer hydrolysis half-life than SPA PEG. (23 min at pH 8 and 25C for PEG-SBA compared to 16.5 min for PEG-SPA under similar conditions). PEG-

maleimides, vinyl sufone, iodoacetamide and orthopyridyl disulfide are representative PEG derivatives that can be used for the PEGylation of proteins via modifying the cysteine residue. Where PEG-vinyl sulfone reacts with thiol to form a stable thioether linkage, the reaction rate is slow in acidic conditions, but fast in slightly basic conditions (pH 7-8). On the other hand, PEG-maleimide (PEG-Mal) is reactive to thiol under acidic conditions. PEG-MA, as reported by Harris, is not stable in water and can undergo ring opening or addition of water across the double bond. Orthopyridyl disulfide PEG (PEG-OPSS) and PEG-iodoacetamide (PEG-IA) are also two reagents which found application in site-specific conjugation of PEGs to cysteine residue in protein (12). While PEG-OPSS generates a disulfide bond in the product, which is susceptible to reduction, PEG-IA conjugation results in generation of iodine which may react with other amino acids. For carbohydrates, PEGylation chemistry essentially involves oxidation of the carbohydrate residue to aldehydes first. Hydrazide or amine derivatives of PEG are than reacted with these aldehyde groups to immobilize PEG on carbohydrates through hydrazone or Schiff base type linkers.

Reversible PEGylation of proteins is also possible by using a degradable linkage between the target protein and PEG. Intron® has been synthesized by Enzon to improve pharmacokinetic half-life of interferon alpha-2b (21). PEG was coupled to Nδ1 position of imidazole ring in histidine to form a carbamate linkage. Such carbamate linkages degrade over time, releasing free interferon. Pegasys®, which is the branched PEG40 kDa-interferon alpha-2a conjugate, however showed a better pharmacokinetic profile in vivo than the PEG-intron. Tag-free, cleavable PEGylation strategies have been reported on by different investigators. Greenwald et al. utilized 1,6-elimination pathway (22), Bently et

al used mPEG-phenyl ether succinimidyl carbonates and mPEG benzamide succinimidyl carbonates (23) and Zalipsky et al. used p- or o-disulfide of a benzyl urethane (24) to design cleavable PEGylated proteins to recapitulate full bioactivity of the native protein upon PEG-cleavage. Heterobifunctional PEG has also been synthesized which has found applications in drug conjugation, synthesizing targeted nanoparticles and in surface immobilization with bioactive substances.

Chemistry of PEGylation

PEGylation has evolved significantly since its first development from non-specific conjugations known as the 'first generation PEGylation'. The variations of PEG conjugations including whether the PEG molecule is branched, the site of PEG attachment as well as the mass of the PEG molecule called for a "trial and error" method of PEGylation that brought to light many problems. Apart from achieving a solution of different sized PEG and protein conjugates, the resulting conjugates are also not uniform, resulting in many positional isomers (1,8). This, however, isn't completely ineffective, as multiple drug therapies such as Pegasys, used to treat Hepatitis C, utilizes non-specific PEGylation (1). Non-specific PEGylation typically uses amine conjugation. A subset of most frequently used PEGylation chemistry is presented in Figure 4.

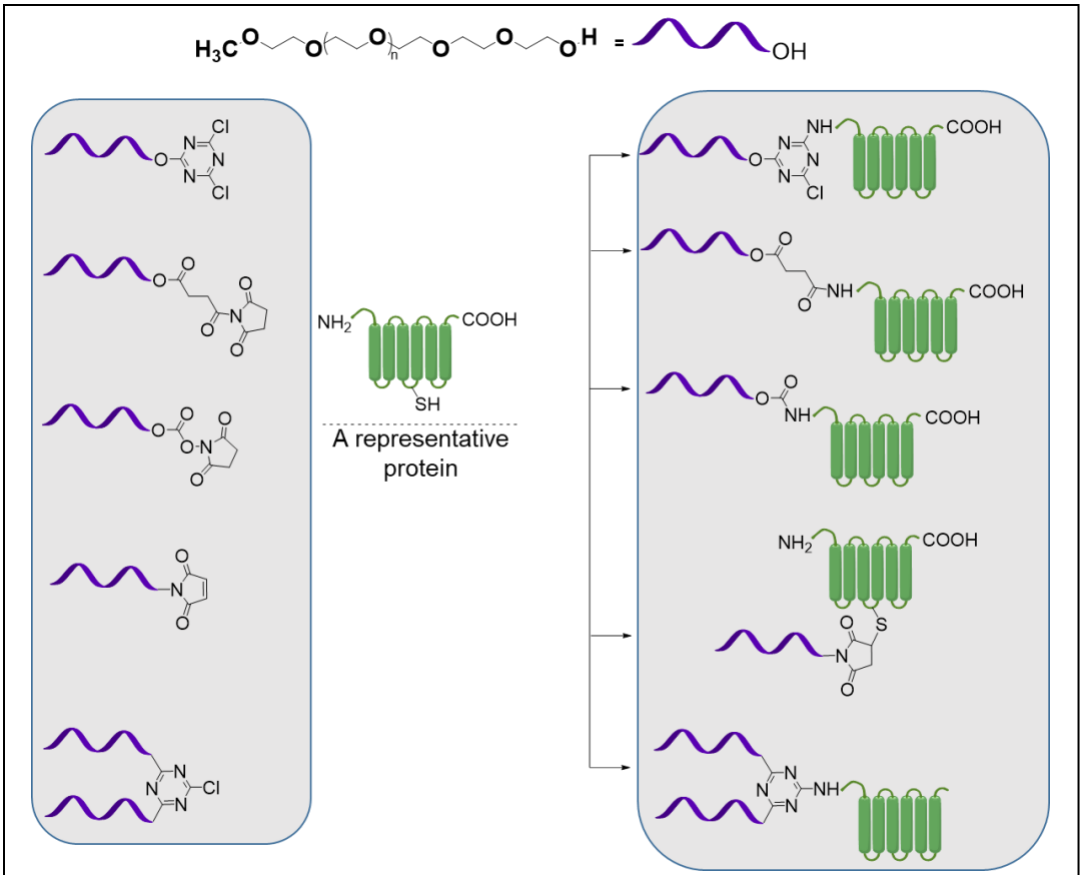

Figure 4. Common PEGylation of proteins using canonical amino acids.

It has since evolved into a site-specific conjugation method known as ‘second generation PEGylation. The availability of more specific functions of PEG molecules, having the capability of reacting to particular moieties in the protein, should be accredited for the increase in PEGylation specificity (25). There are multiple pathways for site-specific conjugation. N-terminal PEGylation, thiol and bridging PEGylation, histidine tags and enzymatic PEGylation (Figure 5a) are a few of methods currently being used, based on where on the amino acid sequences the PEG molecules will attach (1). The objective of site-specific PEGylation and main pathway is through reversible conjugation, or releasable prodrugs (9). Reversible conjugation is even less inhibiting on drug activity than irreversible conjugation, used in first-generation PEGylation. Second-generation

PEGylation looks to temporarily attach PEG molecules via cleavable linkages (Figure 5b)(9). This way, drugs can be released according to a specified time schedule, in vivo via hydrolytic cleavage.

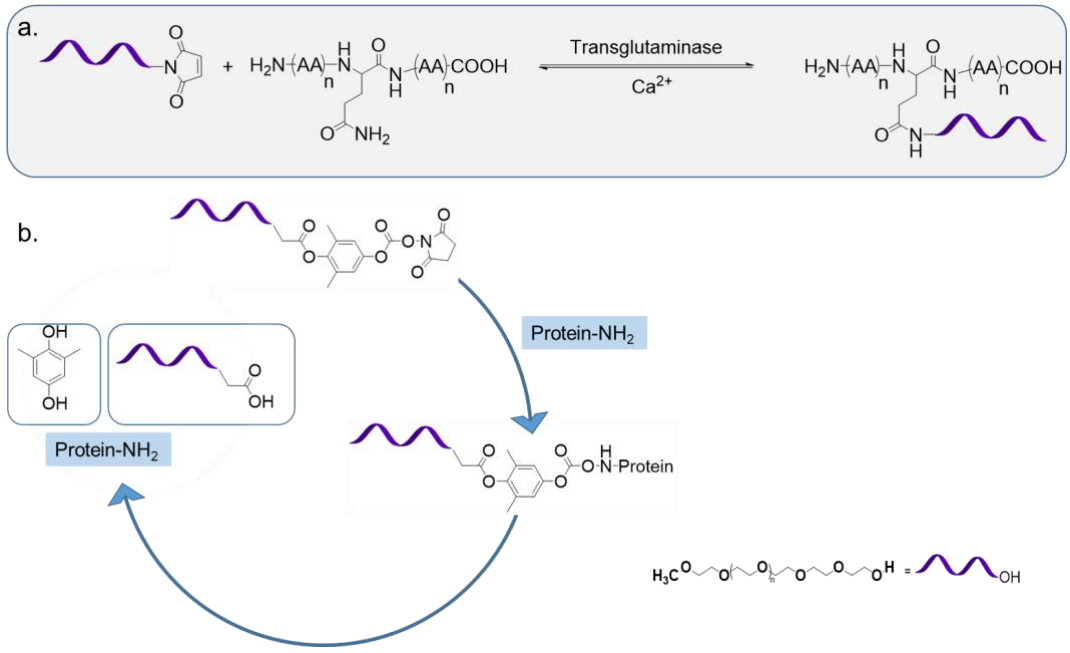

Figure 5. (a) Enzymatic ligation of PEG to glutamine residue of a protein (b) Attachment of cleavable PEG to a protein which is capable of releasing the active protein upon hydrolysis.

The future of PEGylation looks toward third-generation PEGylation, which aims to achieve the highest potency and circulation half-life without compromising fast-acting, site specificity and lower dosages (11). This is through noncovalent PEGylation based on electrostatic linkages (1). One method that is being explored is through pre-targeting of PEG engagers. Used with effective results in targeting Epidermal Growth Factor Receptors (EGFRs) in Triple Negative Breast Cancer, PEG engager pre-targeting was used to minimize the regulatory challenges of lowered shielding and reduced uptake (26). This could be very useful for cancer therapeutics because it utilizes a targeting molecule and

separate linker molecule. Based on the linker molecule, the pro-drug can enter tumor cells via endocytosis which can be receptor-mediated or not, based on the type of cancer cell and pro-drug (9). Site-specific ligation of PEG to amino acids is an exclusive strategy (27) that can result in PEGylation of proteins bearing either cysteine, or non-canonical amino acids (28, 29). Michael type thiol-maleimide reactions (Figure 6a) and azide-alkyne click cycloaddition (30) (Figure 6b, top panel) have gained extensive traction in realizing such PEGylation strategies. To avoid introduction and removal challenges of copper ions, metal free chemoselective reactions have been introduced for site-specific ligation of PEGs to azide containing proteins. Known as 'Strain promoted azide alkyne cycloaddition (SPAAC), developed in the laboratories of Bertozzi, Boons and in van Delfts laboratories separately, this process relies on the ring strain of cyclooctyne derivative of PEG (Figure 6b, bottom panel).

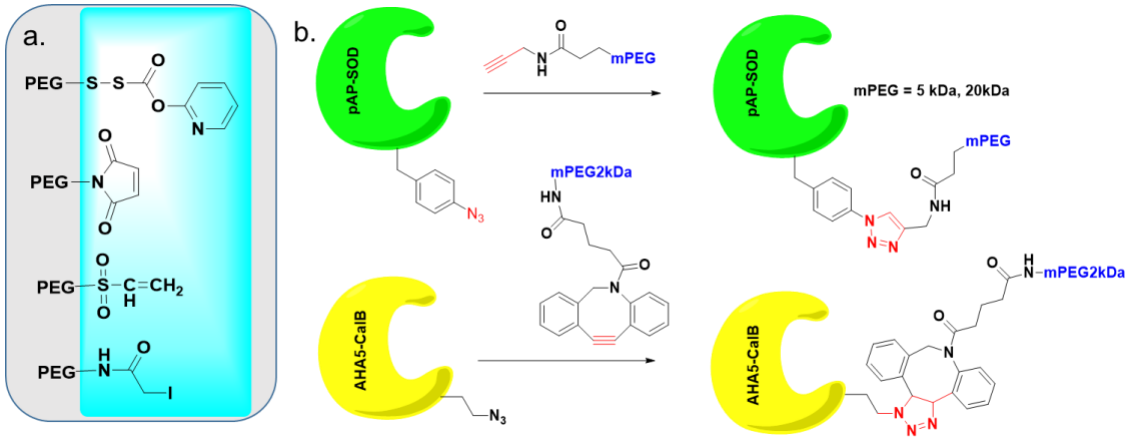

Figure 6. (a)Thiol reactive PEGs for site-specific PEGylation of proteins containing cysteine residue. (b) CuAAC PEGylation of human superoxide dismutase1, and (B) SPAAC PEGylation of lipase CalB.

As an illustrative example of such site-specific ligations, *Candida antartica* lipase B (CalB) was expressed in an auxotrophic strain of *E.coli*. The enzyme contained five (05) azido homo alanines, one of which was selectively exposed by solvent. A dibenzocyclooctyne PEG derivative of 2kDa molecular weight, was able to connect itself to the azido-CalB in only 3h in PBS buffer at room temperature (31-34). This type of chemoselective reaction will open an exclusive opportunity to PEGylate wide variety of therapeutic proteins which contains the non-canonical amino acids located in non-functional domain.

Additionally, the ability of PEG to reduce the cytotoxicity of a therapy has helped prove its potency in treating diseases. For example, mitochondrial oxidative damage can be treated by the shielding of the mitochondria with mitochondriotropic antioxidants, but this therapy has yielded questions about its cytotoxicity (35). As a result of this, PEGylation was brought in to modulate the cytotoxicity of AntiOxCIN6, a mitochondriotropic antioxidant which is cytotoxic to hepatocarcinoma (HepG2) (35). The modulation was done by the conjugation of PEG with Caffeic acid (CAF) and Triphenylphosphine (TPP+), which acted as antioxidant and targeting arms respectively. The results from the process revealed that with PEG conjugation, the antioxidant abilities related to CAF were maintained in the CAF - PEG - TPP conjugate (known as CPTPP) and that the PEGylation reverted the loss of the ability to chelate iron associated with just AntiOxCIN6 (35). In addition, it was observed that CPTPP was not toxic to human HepG2 cells whereas both AntiOxCIN6 and CAF, when alone, acted in a harmful way to the same cell lines. The successful modulation of the AntiOxCIN6 cytotoxicity along with the maintained oxygen consumption of mitochondria and efficacy of the antioxidant during the PEGylation

demonstrated the ability of PEG to act as a method that not only increases the half-life of a drug and retains its efficacy, but also one that can reduce the cytotoxicity of a treatment.

Functional Significance of PEGylation

Protein therapeutics and protein engineering are one of the newest and most promising biologic drug therapy routes. Due to their high specificity and rapid onset, they are preferred to synthetic therapeutics (1,4). Currently, these biologic drugs cannot be used to their full potential because of short half-lives, protein degradation and other interfering pharmacokinetic (PK) properties of these therapies (3, 11). With protein PEGylation, these biomolecules can have an increased half-life in the body and be protected from rapid renal filtration via the kidneys (4). PEGylation can bestow a number of noteworthy and distinct pharmacological advantages over the unmodified form of the biomolecule such as, improved drug solubility, reduced dosage frequency, toxicity and rate of kidney clearance, an extended circulating life, increased drug stability, enhanced protection from proteolytic degradation, decreased immunogenicity and antigenicity, and minimal loss of biological activity (36). This can all be done without a loss of function, making it an attractive for further drug therapies, and especially cancer therapies.

CCN5, also known as Wnt1-inducing signaling pathway protein 2 (WISP-2) is the fifth member of the CCN family protein. Structurally, CCN proteins are composed of four functional domains, namely IGF binding protein domain, von Willebrand factor C (VWF-C) repeat, thrombospondin type 1 repeat and a cysteine rich C terminal domain. CCN5 does not contain the C-terminal domain and is hence distinct from its siblings. Over the years, CCN5 has emerged as one of the key regulators of cancer and other diseases. We

have carried out extensive investigation on the functional role of CCN5 in breast cancer and have found that CCN5 plays an important role in proliferation of mammary tumor cells (37). Further, CCN5 is over-expressed in several breast cancer cell lines (38) and is crucial for the proliferation of ER-alpha positive breast cancer cells, like MCF-7 (39). Interestingly, CCN5 activation is the mechanism via which EGF (epidermal growth factor) induced proliferation of non-invasive MCF-7 breast cancer cells (Banerjee et al, 2005). This pro-proliferative effect of CCN5 was accelerated under the influence of IGF-1 (40). We further showed that mutant p53 silences CCN5, thereby converting non-invasive breast cancer cells into invasive type (41), thus CCN5 exerts anti-invasive role in breast cancer. Convincing evidence for the potential therapeutic role of CCN5 necessitates ways to make this protein "druggable". CCN5 is a small protein of ~29 kDa, thus it is very likely that the retention time in the body will be too short to exert its beneficial effect. PEGylation of CCN5 using mPEG propionaldehyde through reductive amination pathway (Figure 7) was found to increase circulation half-life of the protein without compromising its biological efficiency.

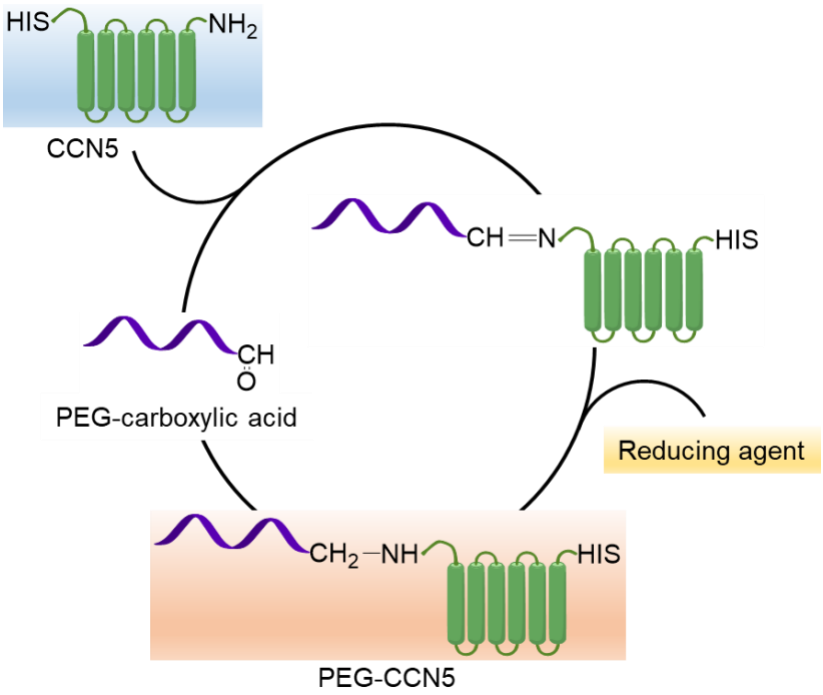

Figure 7. Reductive amination of CCN5 using mPEG propionaldehyde of different molecular weight resulted in PEGylated CCN5, which extended the biological half-life of the protein.

One of the most common anti-cancer therapeutics is the use of monoclonal antibodies (9). These antibodies attach to specific sites on tumor cells that can activate apoptosis, or cell death, or block cell growth pathways. However, since they don't typically have the Fc region that binds to cell surface receptors and is linked to antibody solubility and stability, their in vivo half life is short (11). Through PEGylation, antibody glomerular filtration and immunogenicity has been reduced, all the while, maintaining binding affinity for the receptors (9). The attachment of PEG to angiogenesis inhibitor (CDP791) increased its efficacy and has been shown in clinical studies of colorectal, ovarian and renal cancers (9).

PEGylation has been utilized in drug therapies that have been FDA- approved and had a proven track record of success. The first marketed drug that used PEGylation appeared in 1990, called Adagen, used to treat adenosine deaminase (ADA) deficiencies

in severe combined immunodeficiency disease (SCID) (1). Since then, over 10 PEGylated drug therapies have been FDA-approved and on the market, while more than 20 are currently undergoing clinical trials (11). These longer-acting solutions have made for a less frequent need to apply the drug and a lower dosage as well.

Why PEGylation is Important for Protein Therapy

Over the last thirty years, protein therapy has emerged as a major pharmaceutical method for treating diseases. When conjugated with PEG (resulting in PEGylation), proteins are granted new capabilities that improve their clinical potential. For example, PEGylation delays proteolytic degradation and glomerular filtration through the kidneys in vivo (4). In addition, the most successful protein conjugates have been with PEG (42). This is due in part to the increased half-life of the serum as a result of increased hydrodynamic volume. An obstacle that is occasionally faced when dealing with protein therapy is the immunogenicity, or the ability to provoke an immune response, of the protein. The immunogenicity of a protein can make a drug less effective, so PEGylation overcomes this problem by reducing the immunogenicity of proteins (although not all PEGylated proteins do this) (43, 44). PEGylation has the capability of altering the physicochemical properties in the parent protein, including electrostatic and hydrophobic properties (25). PEGylation allows for prolonged circulation time of conjugated therapeutics in the body through a decreased rate of kidney clearance and reduction of proteolysis and opsonization. The high hydration capacity in PEG molecules helps increase the hydrodynamic radius of the conjugate approximately 5-10 folds higher than if it would be from the molecular weight alone. The increased radius then allows for

lesser chances of glomerular filtration and aids its solubility (45). PEGylation has been chosen as the method of choice in extending protein half-life after many years of intense studies. It has been selected due to its extraordinary flexibility, hydrophilicity, variably size, and low toxicity. As of now, there are over 10 different kinds of PEGylated products approved by the FDA, with many others in developmental stages (5).

Table.1 List of PEGylated compounds currently in use or approved for use

Currently Approved Drug	**Purpose and/or Effect**	**Reference**
Krystexxa	Lowers uric acid levels in order to aid in removing gout crystals.	Hershfield, M. Arthritis Research & Therapy, 2014
PEGASYS (peginterferon alpha 2b)	Used with other hepatitis C medicines to treat adults with chronic hepatitis C and certain liver problems. Can be used with ribavirin to treat both adults and children with chronic hepatitis C.	Wang, Y.S. Biochemistry, 2000
Adagen (pegademase)	Modified enzyme used for Enzyme Replacement Therapy (ERT).	Ellis, KM British Journal of Pharmacology, 2003
Oncaspar (pegaspargase)	Given to patients with acute lymphoblastic leukemia as part of a group of chemotherapy treatments.	Abuchowski, A. Cancer Biochemistry Biophysics, 1984
Somavert (pegvisomant)	A prescription medicine for acromegaly, a disease caused by the surplus of growth hormones in the body. The goal is to have a normal IGF-1 level in the blood.	Thorner, MO The Journal of Clinical Endocrinology and Metabolism, 1999
Neulasta (pegfilgrastim)	Administered to reduce the risk of infection after strong chemotherapy.	Johnston, E. Journal of Clinical Oncology, 2000

Mircera (CERA; PEG-EPO)	Used to treat symptomatic anaemia associated with chronic kidney disease (CKD).	Macdougall, IC Current Hematology Reports, 2005
Cimzia (certolizumab)	An injected prescription medication that works to prevent inflammation that may result from an overactive immune system.	Choy, EH Rheumatology, 2002
Macugen (pegaptanib)	Utilized for the treatment of neovascular age-related macular degeneration.	Bayés, M. Methods and Findings in Experimental and Clinical Pharmacology, 2002
Plegridy (peginterferon beta-1a)	Indicated for the treatment of patients with relapsing forms of multiple sclerosis.	Pepinsky, RB The Journal of Pharmacology and Experimental Therapeutics, 2001

Drug Nomenclature adapted from table from (11). Purpose and references adapted from approved drug websites.

Pitfalls of PEGylation

PEGylation has come a long way since it first arrived in the market 30 years ago. It has undergone procedural changes from first-generation PEGylation to second-generation PEGylation and now, there are attempts to increase the efficacy with third-generation PEGylation. Although becoming one of the most widely used drug delivery technologies, PEGylation has had structural challenges. The size and position of PEG molecules on the conjugates strongly affects its properties. PEG dispersity index, degree of PEGylation, and PEGylation site specificity are some of the key problems with PEGylation that need attention.

Cyclic dimer of ethylene oxide, 1,4-dioxane, is the major side product associated with the synthesis of PEG. International Agency for Research on Cancer (IARC) categorizes dioxane as being possibly carcinogenic in humans based on data generated from animal models. 1,4-dioxane is generally stripped off from the final product under low pressure. Side products also include residual ethylene oxide and formaldehyde, both of which are classified by IARC in group 1 (carcinogen in humans). Hence, using pharmaceutical grade PEG for biomedical applications is always recommended (6). In earlier synthetic processes, PEG synthesis was also accompanied by the presence of PEG diols, which in some cases exceeded 15% of the composition of mPEG. Presence of PEG diols has been addressed by Harris et al, by using benzyloxy-PEG with diol impurity, followed by exhaustive methylation and deprotection of benzyl group. In this technique, inert dimethyl ether derivative of PEGs can be removed after polypeptide attachment. Ion-exchange chromatography has also been employed to remove PEG-diols after converting PEG to PEG carboxylic acid (12).

There have been multiple studies to confront these issues. One study reported that PEGylation sites could be found through the comparison of native and PEGylated proteins, but the method was not sufficient in locating PEGylation sites to a certain residue (26). In addition, although the PEG chain can be identified in smaller peptides, the PEG site may be impossible to detect with larger peptides. The problem lies in the difficulty of isolating and purifying parts of the enzyme digestion and also in the obstruction of the specific cleavage by proteolytic enzymes (3). Another obstacle researchers have faced is the polydispersity of PEG, which ranges in value from 1.01 for low molecular weight (3-5

kDa) up to 1.2 for high molecular weight (20 kDa) (3). This is an undesirable characteristic due to its similarity in dispersity to the PEG conjugates.

In addition to these problems, it has also been found that the same process that prevents proteolytic enzymes from advancing towards the PEGylated protein can also refuse a substrate from the active site of the protein (3). This scenario is primarily observed in enzymes with greater molecular weight (polysaccharides, peptides, proteins) and greatly reduces the advantages of PEG conjugation. In order to counter this complication, researchers have developed a series of methods such as using an active-site protecting agent or an inhibitor. Although the problem was reduced through the use of an active-site protecting agent, the possibility of PEGylation still occurring in the area around the protected site renders the results insufficient. In response to this result, a process, based on the use of an inhibitor linked with an insoluble resin (agarose) was devised (3). This method, when held at proper pH and ionic fortitude, protects both the active site and the area surrounding it. The enzyme, after the removal of the inhibitor, continued to reflect biological activity towards substrates such as albumin and blood clots (with urokinase) (46, 47).

PEGylated therapies have also exhibited multiple side-effects on patients. PEGylated drugs, being small, can enter the vasculature easily, not only of the tumor, but also normal body tissues. HFS (Hand and foot syndrome), mucositis, and rash were common side-effects observed.

Conclusions

PEGylation, as a process, has evolved rapidly through research over the past three decades. Its diverse functionality grants it a prestigious standing in the world of site-specific targeting therapeutics. Currently, there are around 10 FDA approved PEGylated drugs on the market that treat a variety of diseases ranging from Hepatitis C to Multiple Sclerosis. The enhancements that these drugs grant proteins, such as extended half-life, reduced/modulated protein cytotoxicity, maintained protein efficacy, and binding affinity to cell receptors are just the current advantages, and future research looks to delve deeper into the benefits PEGylation can provide. The third generation of PEGylation will attempt to provide the greatest potency and circulating half-life without compromising site-specificity and low dosage. If completed successfully, with the help of further research PEGylation has the potential to become the primary method for protein therapy.

CHAPTER TWO

Two Years Later: Drug Review

Introduction

PEGylation has been studied as a possible pharmacological method for over thirty years, yet the exact pharmacokinetic behaviors of PEG are still unknown in vivo. PEG's non-toxic, non-immunogenic and non-antigenic properties combined with its capability to prolong in-vivo circulation time of biomolecules make it an ideal candidate for pharmaceutical application. Due to the lack of clarity surrounding its pharmacokinetic behaviors however, many PEGylated pharmaceuticals have failed in pre-clinical and clinical trials (49). In order to combat this high failure rate and improve the clinical application of PEG, an accurate in-vivo analysis of PEGylated molecules and their fragments is needed.

The current challenge in PEGylation technology surrounds the in vivo transformation of PEGylated molecules in order to free the drug for its attachment and accumulation at the target site. Only then, can the effects of the drug be exerted. This poses the issue of the then-free PEG particles that accumulate in the body, especially with long-term exposure. Previously, it was thought that there were no biological effects of PEG because of its inert nature, however, recent studies have exemplified its in vivo metabolization into carboxylic-acid PEG (PEG-acid). An accumulation of PEG-acid can then lead to dangerous cases of acidosis and hypercalcemia (49). The complex and intrusive nature of current in vivo analytical methods raise difficulties for the in vivo evaluation of PEGylated molecules' pharmacokinetic properties.

First-generation and second-generation PEGylation methods have resolved issues of drug-targeting and steric specificity. Third-generation PEGylation methodology looks to resolve the in vivo issues of PEG accumulation and detachment. The current problems and viable possibility of solutions are justified reasons for the push toward refining the a new, third-generation method of PEGylation.

Research into PEGylation has helped diversify its therapeutic uses. PEG molecules have been attached to various macromolecules such as proteins, carbohydrates and oligonucleotides, but also more recently, nanoparticles and antibodies, the latter of which has been explored as mechanisms of more potent therapeutics, especially in the field of cancer research. Current literature recognizes about twenty marketed PEGylated drugs. This chapter will discuss the ten listed in Table 1 of Chapter 1.

Third Generation of Protein PEGylation

As discussed in the review article, the technique of PEGylation has undergone many changes since its first discovery as a targeting drug delivery system. First generation PEGylation refers to the method of randomly attaching PEG molecules to proteins. The non-specific products result in isoforms which can have differing properties, making it difficult to be used as a specific, targeting therapeutic. Only two drugs from Table 1 were created using this method: Adagen and Oncaspar. Second-generation PEGylation uses various methods of site-specific PEG attachment as well as various forms of PEG, including different sizes and shapes, like branched or bifunctional PEG which has multiple functional groups that can act as active sites. This method allows for an increased diversity of specific PEGylated molecules (11). Most PEGylated pharmaceuticals on the market

today were created using second-generation PEGylation. Third-generation PEGylation is still in the process of being refined as a viable method of drug creation. The main goal is to preserve the PEGylated molecules bioactivity (50).

Current issues of PEGylated therapeutics include in vivo transformation, steric hindrance, anti-PEG antibodies and tradeoffs between increased half-life and potency. How PEG interacts with the active drug as well as the active site affects the drug's potency. Attachment of a larger molecule to a drug often sterically hinders it, affecting its ability to bind to the target and impeding the active site, which then affects drug potency and half-life (11). Methods of drug activation once the biomolecule is at the target site and not travelling through the body could combat this issue. Additionally, studies have shown that treating patients with PEGylated drugs has led to the creation of anti-PEG antibodies, unfavorably invoking the patient's immune response and leading to the clearance of the PEGylated biomolecule. The common response to these issues has been to change dosage intervals or increase drug dosage to patients but, since the effects of PEG accumulation are still unclear, third-generation PEGylation looks to prevent some of these problems from happening in the first place. Research into third-generation techniques has been largely in the use of linkers and PEG engagers (11, 51, 53). Since these methods are more specific and more expensive than the former generations, most PEGylation methodology studies have been conducted in regard to a certain therapeutic or disease.

Linkers are peptide-chains that link the PEG molecule to the active drug in order to maintain physical space between the two. Linkers are often permanent, but the idea of releasable linkers has been proposed to help transform the drug into its active form in vivo at the target site as well as reduce unwanted interactions between PEG and either the active

site or the drug itself (52). It has been shown that the length and conjugation method of the linker as well as it's protein makeup largely affect the stability of PEGylated proteins (54). One study used disulfide bridges to create a forked shape of the PEGylated protein which showed considerable efficacy in active site targeting (52). While linkers are the main alternative to direct conjugation of PEG, it is still an actively researched area, undergoing much trial-and-error. Like second-generation PEGylation, customized linkers increase the diversity of potential therapeutics. Different linkers are tested with various PEGylated molecules in a trial-and-error fashion.

PEG engagers are bi-specific PEG-binding antibodies that help with targeted drug delivery. This area of research is largely focused around cancer since this method helps target the tumor site directly. In a study of PEGylated nanoparticles as a treatment for triple-negative breast cancer, bi-specific, pre-targeted PEG engagers induced endocytosis of the PEGylated nanomedicine into tumor cells by binding onto both the PEG molecule and a receptor on the tumor cell. This engager enhanced drug delivery and targeting by binding on both active sites (51). PEG engagers can also be used with any PEGylated nanomedicine since PEGylated nanoparticles are coated with PEG (51). While it was thought that anti-PEG antibodies generated by patients may impede the drug delivery by binding with the PEGylated nanomedicine, the study showed that even patients with high levels of anti-PEG antibody saw the increased therapeutic effects of the enhancers, largely due to the enhancers increased affinity over the anti-PEG antibodies for the PEGylated nanomedicine. While this is not a modification to the PEGylated molecule itself, it is a more generic approach to improve efficacy of PEGylated nanoparticle therapeutics.

While it is unlikely that PEGylation as a targeting drug-delivery method will disappear, it's creation methodology may not see any more drastic improvements. Instead, there will be a movement to enhance its bioactivity through supplemental therapeutics.

PEGylated Drug Review

There are a couple dozen PEGylated drugs on the market that include of a wide range of therapeutics. Even of the ten listed in Table 1, no two are used for a similar purpose. The first PEGylated drug to be on the market was Adagen in 1990. Since then, various PEGylated drugs have been on the market with varying degrees of success. Some are widely used as a common treatment while others are used as a last resort option or have been phased out.

Of the ten drugs listed in table 1, one of the most widely used is Krystexxa, generically referred to as pegloticase. As previously been stated in table 1, pegloticase is used to lower uric acid levels in order to aid in removing tophi gout crystals (48). Gout is the most frequent cause of inflammatory arthritis which can cause severe joint pain due to high levels of urate or uric acid in blood serum. One of the ways to treat it and reduce serum urate is with the enzyme uricase (55). Recombinant uricase is characterized by poor solubility, rapid clearance and immunogenicity, making it a good candidate for PEGylation and resulting in the marketed drug pegloticase (48). Pegloticase is a recombinant, PEGylated uricase that is typically used when conventional methods of serum urate reduction aren't effective.

In a 2018 review of clinical trials and observational studies evaluating outcomes of pegloticase treatment, pegloticase was shown to effectively and quickly reduce serum

urate levels and tophi. However, a large proportion of patients developed anti-pegloticase, anti-PEG and anti-uricase antibodies after a few infusions, therefore reducing the efficacy of the treatment and eventually leading to discontinuation of treatment. The review stated many patient safety and cost concerns with pegloticase, including an increased risk of cardiovascular events in patients and frequent gout flares in the first three months of treatment. The review concluded with the prediction that pegloticase would eventually be a last resource option for gout treatment and tophi reduction (48, 55). This is not to say that pegloticase is not effective, just that the costs and risk may outweigh the benefit for a typical gout patient. There have been other treatments for gout, such as febuxostat and lesinurad that have been shown to be cheaper and more effective than pegloticase. In conjunction with other treatments, patients with more severe cases of gout or patients who have not been receptive to other treatments may find pegloticase to be their best option (55). As a general treatment for gout, however, its widespread use will probably continue to lower; the main issue being the creation of various antibodies leading to its diminishing efficacy with prolonged use.

PEGASYS, or peginterferon alpha-2a is a PEGylated interferon typically used with other medicines to treat chronic hepatitis and certain liver problems. Interferons are signaling proteins released by host cells in the presence of viruses; they can trigger immune responses or halt virus replication. Peginterferon alpha-2a has had proven efficacy in the treatment of viral diseases and was even explored as a possible treatment for COVID-19 to help stimulate antiviral responses (56). Though it is created using non-specific first-generation PEGylation, it is still widely used as an accepted treatment for chronic hepatitis B with clinical efficacy and lasting results. These results have been shown with solitary

use of peginterferon alpha-2a as well as its joint use with other therapeutics in a 2018 study publishing the consensus and guidelines of peginterferon use. The guidelines include dosage and stopping guidelines and serum levels to check the efficacy of treatment since it does diminish over time (57). Peginterferon alpha-2a has been used in treatments of other diseases like cancer as well. Because of its relative effectiveness and widespread use, it is likely to continue to be used as a viable treatment for hepatitis as well as continue to be researched as a treatment for other diseases.

Adagen, or pegademase is a PEGylated adenosine deaminase (ADA) enzyme used in enzyme replacement therapy (ERT) for patients that are immunodeficient (59). Adagen was the first approved PEGylated drug in 1990 created with non-specific first generation PEGylation (58). Since ERT is only used for rare immunosuppressive diseases, there are not many options for patients needing this type of therapy. Hence, there is not much research into different therapies and pegademase is used as a generally accepted treatment for ADA-deficiency. Studies showed no change in clinical outcomes due to a change in the pharmacokinetics of Adagen or development of anti-ADA antibodies, though development of anti-ADA antibodies was observed. Interestingly enough, bovine tissue has been the source of purified ADA and has recently posed challenges with consistent production and safety. Therefore, the same manufacturer that manufactured Adagen has developed a recombinant version of PEGylated ADA, which is currently in the last stages of clinical testing and awaiting approval as of April 2020. If this new drug gets approved, it is likely that the use of Adagen for ERT will phase out and the new drug will replace it (63).

Oncaspar, or pegaspargase, is a PEGylated asparaginase used for the treatment of acute lymphoblastic leukemia (ALL). It has been clinically proven to prolong elimination

half-life and reduce immunogenicity as compared to other modified asparaginase products. Pegaspargase has had many favorable clinical outcomes, such as a manageable tolerability profile, especially in conjunction with other treatments. It has been labeled as an effective first-line treatment for ALL and is likely to continue to be used in ALL treatment regimens (64).

Somavert, or pegvisomant, is a treatment for acromegaly, a disease caused by the surplus of growth hormones in the body. It has high morbidity and mortality rates and can result in tumors, often requiring surgery of the pituitary glands. Pegvisomant is a growth hormone receptor antagonist, usually used after surgery. Currently, somatostatin receptor ligands (LA-SRLs) are used as a first-line treatment after surgery but has shown increased efficacy when used in conjunction with pegvisomant (65). Multiple studies and cases have shown increased efficacy of combination therapy that includes pegvisomant so it is likely to continue to be used in clinical settings. However, it's solitary use as a therapeutic is low due to the quick buildup of antibodies (66).

Neulasta, or pegfilgrastim, is a drug used to reduce the risk of infection after strong chemotherapy. It is a recombinant granulocyte-colony stimulating factor (G-CSF) and one of the most effective of its type. However, there have been many clinical trials showing new similar drugs like B12019 with the same pharmacokinetic and pharmacodynamic profiles. These other drugs are also more cost effective, meaning there is likely to be a movement to phase out the use of pegfilgrastim and incorporate the use of more cost-effective therapeutics. This is not to say that pegfilgrastim is not effective, it is still used as a marker for other drugs' efficacy. However, due to its high cost and similar efficacy levels to cheaper, similar drugs, it is likely to stop being used as much (67, 68).

Mircera, or PEG-EPO is a recombinant erythropoietin used to treat anemia associated with chronic kidney disease. While it has shown clinical efficacy, longer half-life and higher bioactivity, its high cost has been a clinical burden. One study even showed the PEGylated therapeutic to block the binding of the EPO to its receptor more than the non-PEGylated version of the drug. However, since the overall pharmacokinetic benefits outweigh the poor binding profile, it is still used as the top therapeutic. Production of cheaper biosimilars to the PEGylated-EPO are undergoing clinical trials (69).

Cimzia, or certolizumab is an immunosuppressive medication that works to prevent inflammation from an overactive immune system. It is approved in the United States as a treatment for psoriatic arthritis, plaque psoriasis, Crohn's disease and many other ailments. Certolizumab was also looked at as a therapeutic to counteract the severity of respiratory disease that characterizes COVID-19 (70). Since it is used to treat so many diseases, its efficacy profile is different for each treatment. It does, however, continue to be used often in the clinic. In fact, a new electromechanical auto-injection device for self-injection of certolizumab is undergoing clinical trials, to make the therapy even more accessible to patients. It is likely that certolizumab will continue to be used widely for treatment of autoimmune diseases and continue to be explored as a possible therapeutic for other diseases (71).

Macugen, or pegaptanib is used for the treatment of vascular age-related macular degeneration. It is a PEGylated aptamer, which is a short, single-stranded oligonucleotide that binds to specific target molecule. Aptamers' high affinity and specificity make it useful as antibody analogs. Pegatanib is the first and only aptamer-based therapeutic, though some are currently undergoing clinical trials. Studies showed its high efficacy but also pointed

out high cost concerns and development of anti-PEG antibodies. Though pegaptanib paved the way for aptamer-based therapeutics, it has been largely replaced by the antibody-based therapy ranibizumab that recognizes more of the growth factors that characterize macular degeneration (72, 73).

Plegridy, or peginterferon beta-1a is used as a treatment for patients with relapsing forms of multiple sclerosis (MS). Interferon-beta treatments have been used to treat MS for over 20 years. This PEGylated form has shown prolonged half-life, increased systemic drug exposure and reduced dosing compared to other interferon-based therapeutics. Peginterfron beta-1a is one of the newer PEGylated drugs, approved in 2014. It was created to optimize interferon beta-1a by requiring less frequent dosing but maintaining the safety profile and has successfully yielded better results than the non-PEGylated interferon. The major side-effects have only been those associated with the non-PEGylated interferon and therefore, due to boosted therapeutic efficacy of peginterferon beta-1a, it is likely to continue to be used in a clinical setting (74).

While all of these PEGylated drugs are still on the market, it is clear that there are still some widespread issues with PEGylated therapeutics. Due to their complicated synthesis, the cost of creating them is relatively high compared to other classes of therapeutics. This translates into clinical cost for the patient and therefore, cost-effectiveness of these therapeutics is a major concern. Additionally, nearly all clinical studies of the PEGylated drugs have mentioned the presence or development of antibodies to PEG and/or the drug itself. The creation of anti-PEG antibodies depends not only on the PEG molecule but also the biomolecule it is attached to, meaning it is different for each drug (11). In one study, 38% of patients administered with Krystexxa once tested positive

for anti-PEG antibodies and even with extended periods of time in between dosages, generated anti-PEG antibodies rapidly. In another study, anti-PEG antibodies were detected in every patient treated with Onscapar (53). This is an issue because the development of antibodies prevents long-term efficacy of treatment. Though not all PEGylated drugs are needed for long-term treatment, it is a major barrier in this class of therapeutics.

PEG Alternatives

While PEG has undeniably been a useful tool for drug modification and drug delivery, there are some challenges with PEG that merit exploring other alternatives for biomolecular modification. One of the major issues that arises with almost any PEGylated therapeutic is the patients' immune response that creates anti-PEG antibodies, thereby reducing the efficacy of the drug after the first dose. Because of this reason as well as the confusion surrounding its pharmacokinetic properties, some researchers have suggested various polymer alternatives for PEG.

The three main categories of polymers that have been explored are synthetic polymers, natural polymers and zwitterionic polymers. Synthetic polymers can be designed to be hydrophilic and hyperbranched to mimic the immunogenicity and stealth behaviors of PEG. They may also be better suited to reduce antigenicity. Synthetic polymers that have been researched include polyoxazolines, poly(N-vinylpyrrolidone), polyglycerols and polyacrylmides (53). Some of these alternatives have shown equal, if not better empirical results than their PEG alternatives. In a recent study, interferon alpha-2b was modified with polysarcosine, a synthetic polypeptide and showed equal improvement in circulation half-

life, significantly reduced levels of anti-interferon antibodies, more rapid receptor association and overall improved efficacy more than its PEGylated counterpart, peginterferon, otherwise known as the drug PEGASYS. Another study showed significantly decreased antigenicity in a drug conjugated with POEGMA, a synthetic polymer, when compared to Krystexxa. However, these polymers also have drawbacks, such as their increased presence in everyday items inducing antibodies to the synthetic polymers after repeated exposure (59).

Natural alternatives include lipids, carbohydrates, proteins and polyaminoacids. Heparins and glycosaminoglycans (GAGs) in particular have bene explored since they have similar shielding, targeting and physical properties as PEG but are endogenous and therefore don't trigger an immune response. However, the research into natural alternatives is elementary and has not been studied enough to even undergo clinical testing. Since natural alternatives are already in the body for other purposes, their targeting ability is specific and difficult to manipulate for active-site targeting (59).

Synthetic zwitterionic polymers have been proposed as a PEG alternative because of their low immunogenicity and low clearance rates. Some zwitterionic polymers include poly(carboxybetaine)(pCB) and phosphobetaine-base polymers. They are good options due to their abundance of functional groups and chemical stability, however, some are difficult to synthesize and conjugate. In a study that compared uricase modified with high zwitterionic-dense polypeptides (PepCB) and PEGylated uricase, uricase-modified PepCB showed sustained activity and a longer half-life than PEGylated uricase, which is in the drug Krystexxa. The stronger hydration ability of carboxybetaine groups as compared to

poly-ethylene glycol provides an inherent advantage of zwitterionic polymers over PEG (59).

There are a variety of alternatives that could be used to enhance drug delivery instead of PEG, however, they each come with their own limitations. It is difficult to introduce completely non-antigenic drugs into the body, meaning that the creation of antibodies may always be an issue. That being said, since the creation of antibodies depends on both the biomolecule itself as well as the attachment (whether it be PEG, a natural polymer or a synthetic polymer), ideally, each biomolecule has an optimal modification to create the most effective drugs. However, this extent of specificity starts to become complicated and is yet to be holistically researched. The Precision Medicine Initiative aims to tackle problems like this by categorizing therapeutics based on patient differences.

Relation to Precision Medicine

According to the Precision Medicine Initiative, precision medicine is an "emerging approach for disease treatment and prevention that takes into account individual variability in genes, environment and lifestyle for each person" (61). It was a research effort started by former president Barack Obama in 2015 as a way to revolutionize disease treatment. The goal was to determine which treatment approaches are better suited for patients based on genetic, environmental and lifestyle factors.

While precision medicine seems like the ideal treatment method, it is a very broad and expensive endeavor. The National Institutes of Health (NIH) and most research surrounding precision medicine has focused on genomic research and finding trends

through data. In order to do this, the NIH launched the All of Us Research Program to recruit at least one million volunteers to provide genetic data, biological information, lifestyle information and personal history. This large data bank would be available for researchers to use, with a goal of creating better diagnosis and treatment techniques (61).

However, this database is not enough to kick start the large shift in healthcare that would need to take place in order to fully implement the practices of precision medicine. If precision medicine practices are to be used in a clinical setting, all healthcare providers would need access to the data and research and all patients would need to be routinely tested to determine what factor groups they would be in. These challenges make precision medicine difficult to implement, especially on a large scale.

Additional challenges include that the technology systems for data storage and collection and informatics tools to interpret molecular and clinical data have yet to be developed. Moreover, incorporating them into current healthcare systems would be a large challenge in itself. Current Electronic Health Record (EHR) systems aren't even universal or compatible among hospitals now. There would be a large learning curve to incorporate and use some sort of universally compatible database and to effectively understand gene alterations and genomic testing results. Another challenge is cost of research, DNA sequencing, technology and drug development. Obtaining funding from Congress or a private source may be difficult because of this initiative's novelty and large scale. Other issues include patient consent, legal issues and ethical problems, specifically, racial and ethnic bias and stereotyping.

However, not every part of precision medicine is so large-scale and daunting a task. One part of precision medicine is pharmacogenomics, the study of how variations in a

patient's genes affect their response to certain drugs (61). Pharmacogenomics explores many strategies of targeted drug delivery and is already being used in cancer treatments. Cancer classification has gotten more specific than being based on tissue of origin or appearance. Doctors can classify cancers based on the tumor's molecular signature, meaning a patient's genomic makeup can tell a lot about the disease (62). Cancer is caused by uncontrollable cell growth, typically due to a somatic mutation only present in the cancer cells. By distinguishing the mutations and identifying the cancer drivers, whether it be deactivation of tumor suppressors or activation of oncogenes, treatment can become much more specific. For example, melanoma is now recognized as BRAF-positive or BRAF-negative, a meaningful distinction for treatment (62). Tumor profiling is also particularly useful in breast cancer. Breast cancer is classified by the amplification of certain genes, making targeted drug delivery and individualized treatment possible. Based on the molecular classification of breast cancer, doctors can also know more about the probability of relapse. One study with routine clinical tumor profiling, 13% of patients carried a germline mutation in a cancer-susceptible gene (62). Clinically, this type of information can direct treatment decisions to create the most viable treatment strategy.

PEGylated drugs can be used as active targeting agents in precision medicine. Oncaspar is only one of the cancer drugs that PEGylation has been used for. PEGylation and other biomolecule modification techniques will continue to be used in the world of therapeutics, not only for cancer but for other diseases as well. However, through the realm of precision medicine, PEGylation has the potential to go even further. If doctors could pre-determine which patients already had anti-PEG antibodies or were prone to synthesize them quickly based on genomic data, they may be able to alter treatment plans or use an

alternately modified therapeutic. This would reduce a trial-and-error treatment style and ensure efficacy earlier in treatment.

Conclusion

Due to the immunogenicity and structural complications of PEG, researchers have been looking into PEG alternatives that can offer similar benefits of increased half-life, solubility and stability without the current complications PEGylated therapeutics face today. After reviewing the current marketed PEGylated therapeutics, it seems as though many still have issues with immunogenicity and the creation of anti-PEG antibodies, thereby reducing the therapeutics' efficacy after some use, bringing us back to the original problem. It is difficult to determine when modifications to PEGylation should stop and when research into a new drug-modification or delivery methods should start. PEGylation has undeniable benefits however, the current therapeutics may have exacerbated them. It has been difficult and costly to attempt to improve PEGylated drugs' downstream bioactivity, which is currently the biggest issue with regards to PEGylated therapeutics. However, there is still new research being done to improve the methods of PEGylation and some researchers still believe PEG to be the best system of drug delivery. It seems most likely that if there is a future of PEGylated therapeutics, it may be drug-specific through a precision medicine approach rather than general enough to apply as a third-generation method of PEGylation.

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