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Disulfide bridge based PEGylation of proteins ☆

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Abstract

PEGylation is a clinically proven strategy for increasing the therapeutic efficacy of protein-based medicines. Our approach to site-specific PEGylation exploits the thiol selective chemistry of the two cysteine sulfur atoms from an accessible disulfide. It involves two key steps: (1) disulfide reduction to release the two cystine thiols, and (2) bis-alkylation to give a three-carbon bridge to which PEG is covalently attached. During this process, irreversible denaturation of the protein does not occur. Mechanistically, the conjugation is conducted by a sequential, interactive bis-alkylation using α,β-unsaturated-β′-mono-sulfone functionalized PEG reagents. The combination of: — (a) maintaining the protein's tertiary structure after reduction of a disulfide, (b) bis-thiol selectivity of the PEG reagent, and (c) PEG associated steric shielding ensure that only one PEG molecule is conjugated at each disulfide. Our studies have shown that peptides, proteins, enzymes and antibody fragments can be site-specifically PEGylated using a native and accessible disulfide without destroying the molecules' tertiary structure or abolishing its biological activity. As the stoichiometric efficiency of our approach also enables recycling of any unreacted protein, it offers the potential to make PEGylated biopharmaceuticals as cost-effective medicines.

Keywords: Site-specific; PEGylation; Protein disulfide bonds; Therapeutic proteins

Contents

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1. Introduction

Several clinically important milestones have unequivocally demonstrated the importance of covalently conjugating poly (ethylene glycol) (PEG) to proteins. This has led to PEGylated proteins becoming approved therapeutic medicines [1]. As the field grows and starts to mature, there is a need for new and improved PEG reagents. It is in this context that we have designed and developed thiol-specific bis-alkylation PEGylation reagents that can increase the site specificity and the efficiency of PEGylation [2,3]. These reagents are capable of undergoing addition reactions with the two sulfur atoms that are liberated by the mild reduction of an accessible disulfide. This results in PEG conjugation via a three-carbon bridge that links the two original sulfur atoms. In particular, we can now prepare homogeneous PEG-protein conjugates in high yield using conditions that are reproducible, scalable and affordable.

The main purpose of PEGylating proteins is to increase their half-life in the vascular circulation while also maintaining a large therapeutic index [4,5]. Recent clinical studies have suggested however that PEGylation provides significant unexpected, and as yet unexplained, therapeutic benefits for protein-based medicines [6–8]. The most dramatic of these has been the eradication of Hepatitis C virus in more than 50% of the patients who have been treated with PEGylated interferon α-2 and ribavirin for 6 months [6].

PEGylation is usually performed with electrophilic PEGylation reagents that undergo alkylation or acylation reactions with protein nucleophiles; i.e., typically the amino group on lysine [9–12]. This has led to several clinically useful medicines. There is now considerable effort focused on trying to make PEGylated products with increased purity [13–23]. In addition, there is a need to ensure the PEGylation process is efficient so that yield and purification processes are economically viable [24]. To this end, we have developed bis-thiol specific PEGylation reagents because most therapeutic proteins have accessible disulfide bonds, and the nucleophilic addition chemistry of sulfur is more selective and thereby more efficient than the conjugation chemistry of amines.

1.1. Disulfide bridging PEGylation

Most therapeutically relevant proteins do not have free unpaired cysteines that can be used for site-specific conjugation [25,26]. Rather most therapeutic proteins have an even number of cysteines that pair up to form disulfides [27–29]. Small proteins tend to have more disulfides than large proteins because the former need to compensate for their relatively low number of hydrophobic interactions. As a solvent accessible disulfide is usually present in most proteins, we hypothesised that it should be possible to chemically reduce a solvent accessible disulfide to its two free cysteine sulfur atoms, and still maintain the protein's tertiary structure [29–31]. PEGylation could then be accomplished by bis-alkylation to reconnect the two cysteine sulfur atoms via a three-carbon bridge (Fig. 1). The advantage of our approach is that the selective and efficient addition chemistry of thiols can be exploited without the need to recombinantly engineer the protein to introduce a free cysteine [32]. More specifically, our methodological approach exploits the chemical reactivity of *both* of the sulfur atoms in a naturally occurring disulfide.

Our PEGylation reagents have a substituted propenyl group as the conjugating moiety on the end of the PEG reagent **1** (Scheme 1). This conjugation moiety comprises an electron-withdrawing group (e.g., carbonyl), an α,β-unsaturated double bond, and α, β′ sulfonyl group that is prone to elimination as sulfinic acid. The electron-withdrawing group is required to promote thiol addition and to lower the p*K*a of the α-proton so that the elimination reaction can proceed. This juxtaposition of chemical functionality results in a latently cross-conjugated system. The conjugated double bond in the PEG mono-sulfone **1** is required to initiate a sequence of interactive and sequential addition–elimination reactions (Scheme 1). The addition of the first thiolate allows the elimination of a sulfinic acid derivative. This generates another conjugated double bond at the α,β′-position for the addition of a second thiolate. If the two thiols are derived from a protein disulfide bond, a three-carbon bridge

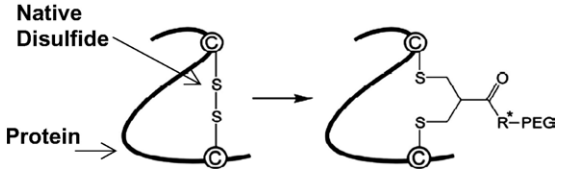
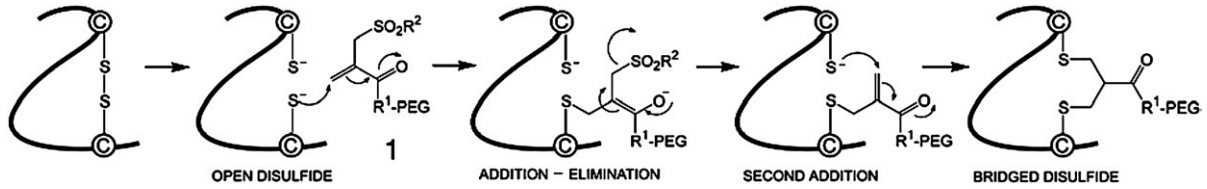


Fig. 1. Illustration of disulfide bridging PEGylation with a three-carbon bridge spanning the original disulfide bond.



Scheme 1. Site-specific, disulfide bridging PEGylation of an accessible protein disulfide is achieved by disulfide reduction followed by reaction with the functionalized PEG 1. These latently crossed functionalised PEGylation reagents are capable of sequential and interactive addition–elimination reactions leading to bis-thiol alkylation. Mechanistically, PEGylation involves (i) a first thiol addition to the PEG mono-sulfone, (ii) sulfinic acid elimination to generate a second double bond, and (iii) a second thiol addition.

is formed between the cysteine sulfur atoms of the original disulfide.

2. Extracellular proteins have disulfide bonds

Naturally occurring proteins that are synthesized for secretion into the extracellular environment usually have disulfides [33,34]. A naturally occurring unpaired cysteine is invariably buried within the protein where it is usually required for the protein's function and is therefore protected from oxidation [33]. This location also helps to prevent aggregation of the protein. Most, but not all [35] therapeutic proteins have disulfides with only the occasional accessible unpaired cysteine [25,27–29].

It is generally accepted that the native conformation of a protein is a result of non-covalent interactions between its primary amino acids [36]. While there are many protein folding pathways [37] that have been studied *ex vivo*, protein folding is also directed by non-covalent interactions [38,39]. As folding proceeds, there can be an increase in these cooperative interactions. Disulfide bonds stabilize these folded but intermediate structures [40–43]. In the context of the fully folded protein, disulfide bonds influence its physicochemical and biological properties in subtle and complex ways [34,44].

The controlled reduction of disulfides to study the unfolding of proteins has been useful to examine the mechanisms underlying the correct folding of a protein and to study those non-covalent interactions that stabilize its tertiary structure [29–31,45–50]. These studies have led to an understanding of how disulfide bonds function to stabilize a protein's structure. There are several examples where these disulfides are derived from cysteines that are well separated along the protein's main chain. For example the Cys6–Cys120 bond in α-lactalbumin is important for the protein's stability [51]. Other examples [52] provide support for the concept that solvent accessible disulfides contribute primarily to the stability of the protein rather than to its biological function [27].

2.1. Reduction of disulfides and their chemical modification

Accessible disulfides can be selectively reduced in the presence of buried disulfides. While there are examples of small disulfide rich proteins of less than 10 kDa that can easily undergo reduction of all of their disulfides [53], selective reduction is also possible in many proteins using mild conditions and without the use of denaturants. We typically use dithiothreitol (DTT) or tris (2-carboxyethyl) phosphine (TCEP–HCl). Because the disulfides of interest are accessible we do not typically use denaturants. Avoiding denaturants ensures that inaccessible disulfides are not reduced. Buried disulfides [27,54] usually contribute to a protein's tight packing and are therefore more difficult to reduce chemically. As a result, denaturants are often required to disrupt the protein's tertiary structure and thereby allow access of the reductant to the buried disulfide. The use of mild conditions enables the reduction of an accessible disulfide bond whilst still maintaining its tertiary structure. For reductions at neutral pH values we tend to use DTT. Excess DDT is then removed by eluting the reaction mixture over a PD10 column while maintaining deoxygenated conditions [2,3]. If a protein is more readily reduced in slightly acidic conditions (e.g., somatostatin) or if upon reduction it is not efficient to elute it over a PD10 column, then a slight excess of TCEP can be used. This is then followed by addition of the PEGylation reagent [2,3]. Once an accessible disulfide is reduced, the two free cysteine sulfur atoms become available for reaction.

There are examples where modification of a disulfide bond results in a major loss of tertiary structure and biological activity [48,55,56]. There are also examples where chemical modification, especially of accessible disulfides that have been reduced, does not lead to a loss of either structure or activity [57–59]. The chemical modification of an accessible disulfide with our bis-alkylation PEG reagent **1** (Scheme 1) differs from other forms of chemical modification. Instead of chemically functionalising each cysteine sulfur atom with a separate molecule to block the reformation of the disulfide bond – as many modifications do – a covalent connectivity with our PEGylation reagent is always maintained between the two cysteines of the original disulfide bond through the three-carbon bridge.

Our disulfide bridging PEGylation reagents are fundamentally different to other PEGylation reagents. The other di-thiol reactive PEGylation reagents that have been described have two separate thiol accepting moieties; e.g., two maleimides [60,61] or two vinyl sulfones [62]. As these other reagents are chemically independent, they are not capable of controlled bis-alkylation by the sequential addition–elimination reactions exemplified by our PEGylation reagents (Scheme 1). The sequential nature of the addition reactions is important to ensure that efficient rebridging of the original disulfide bond can occur. Since the distance between the reactive sites in these other reagents is much greater

than the distance between the reactive sites for our three-carbon bridge, there is little chance that a bridge can form between the sulfur atoms of a native disulfide.

2.2. Computationally modelling modified disulfides

Molecular modeling studies can aid the process of selecting the target protein by determining the potential effects of inserting a three-carbon bridge into the accessible disulfides of a protein. Our search of protein databases (e.g., Protein Data Bank, PDB — www.pdb.org) suggests that most therapeutically relevant proteins have at least one disulfide bond that is close to the surface, and that can be chemically modified without resulting in a loss of the protein's structure or function. Using these databases, we have developed a method for computationally determining which disulfides are solvent accessible, and therefore amenable to PEGylation using a three-carbon bridge [63]. We can also computationally predict whether the insertion of a three-carbon bridge will lead to the loss of the protein's tertiary structure [63–65]. Using this approach [63] (which does not require specialist expertise), we can also evaluate the effect on a protein's active surface using information from its crystal structure, from protein–receptor/ligand complexes, or from biological data if these complexes have not yet been crystallized. This computationally generated modeling information can be invaluable in defining the biological consequences of modifying a protein. It can also be applied to proteins that have to be recombinantly engineered to incorporate an optimized disulfide that will be amenable to PEGylation using a three-carbon bridge [66,67].

These molecular modeling studies are undertaken using the integrated molecular modeling packages Maestro v6.5 and Macromodel v9.1 [64,65,68,69]. For example, our modeling studies have described the insertion of a three-carbon bridge into each of the two disulfides in interferon-α2 (IFN). They demonstrated that only minimal disturbance of the overall tertiary structure of the protein would occur [64,65]. This conclusion was subsequently and experimentally confirmed by (1) circular dichromism — which indicated that the disulfide reduced IFN and the bridged PEG-IFN maintained their native structure, and (2) biological studies — which showed that the monoPEG-IFN had antiviral activity that was similar to amine PEGylated versions of IFN [2].

2.3. Modeling PEG in the presence of protein

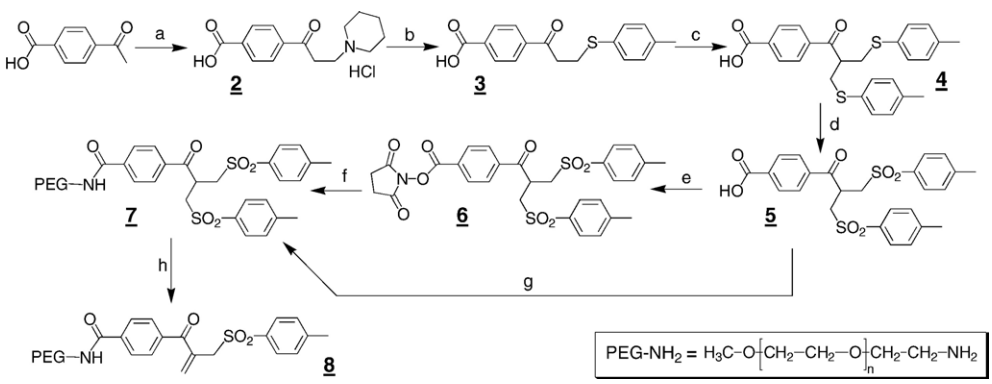
Understanding the consequences of having a PEG in the immediate vicinity of a protein remains a subject of active research. For example, having high molecular weight PEGs in solution can stabilize the native and compact structure of human albumin even though the interaction between PEG and albumin is not thermodynamically favourable [70]. Therefore, it can be inferred that PEG does not obscure the protein's active surface. We also have found that PEG folds independently of the protein; even when the tripeptide glutathione is considered, the small tripeptide does not become enveloped by the much larger PEG [64].

3. Reagent synthesis

We have prepared several versions of the PEG reagent **1** (Scheme 1). Most of our effort to date has been focused on the PEG mono-and bis-sulfones **7** and **8** (Scheme 2). While the reaction pathway for PEGylation (Scheme 1) requires the PEG mono-sulfone **8** to be present for the first addition reaction, the precursor PEG bis-sulfone **7** has also been found to have utility for PEGylation. This is because the bis-sulfone **7** can be used to generate the mono-sulfone **8** *in situ* using mild conditions while in the presence of the protein. This provides a means by which the rate of the first addition reaction can be controlled.

3.1. PEG mono-and bis-sulfones

The PEG mono-sulfone **8** is typically prepared from the known carboxylic acid bis-sulfone acid **5** [71–73] (Scheme 2). Coupling of mono-amine terminated PEG is routinely accomplished using the active ester bis-sulfone **6** or by direct carbodiimide mediated coupling with the carboxylic acid bis-sulfone **5**. Analogous coupling reactions can also be conducted with the carboxylic acid bis-sulfide **5** (or its NHS ester) to give the corresponding PEG bis-sulfides. These can then be gently



Scheme 2. Preparation of PEG mono-sulfone **8**: (a) paraformaldehyde, piperdine hydrochloride, ethanol, reflux; (b) toluene thiol, ethanol, reflux; (c) formalin, ethanol, toluene thiol, reflux; (d) OXONE; (e) DIPC, NHS; (f) methoxy-PEG-NH_2; (g) DCC, CH_3O-PEG-NH_2; (h) phosphate buffer, pH 7.8.

oxidized to give the corresponding PEG bis-sulfone **7** without any changes to the structural characteristics of the PEG.

The synthesis of the PEG mono-sulfone **8** can be accomplished by incubation of the PEG bis-sulfone **7** (25 mg/mL; Scheme 3) in 50 mM phosphate buffer (pH 7.8) and purification by reversed-phase (RP) HPLC. PEG bis-sulfone **7** undergoes elimination of toluene sulfinic acid **10**, probably through the enolate intermediate **9** to give the PEG mono-sulfone **8** at pH values of 6.0 or higher. We have monitored this elimination reaction by ^{1}H-NMR and RP-HPLC to characterize it for the different PEG bis-sulfones. The rate at which this elimination reaction occurs is dependent upon the structure of the PEGylation reagent, pH, concentration and temperature. Several *para*-and *meta*-substituted [74] reagents using different PEG precursors have been prepared and the preliminary computational studies of the elimination reaction published [64].

The PEG bis-sulfone **7** is important because the rate at which the PEG mono-sulfone **8** is generated *in situ* can be matched to the thiol reactivity of the protein after the reduction of its disulfide(s). As the reduced protein must retain its tertiary structure, there could be varied thiolate reactivities for different proteins. This means that the conjugation conditions have to be tailored to avoid the multiple conjugation of PEG to a single protein molecule.

When it is necessary to conduct PEGylation at an acidic pH value because of, for example, issues related to the protein's solubility or its stability, we have found it best to isolate and use the PEG mono-sulfone **8** directly in the conjugation reaction [3,75]. In this context, we have found that some peptides and proteins are more soluble when reduction of the disulfide is conducted in slightly acidic conditions; e.g., reduction of somatostatin at pH 6.2 followed by conjugation with the PEG mono-sulfone **8** [3]. However, for the majority of PEGylation reactions, it is more practical to use the PEG bis-sulfone **7** to generate the PEG mono-sulfone **8** *in situ*. We have prepared the PEG-bis-sulfone reagent **7** using linear PEG's of 5, 10, 20 and 30 kDa with nominal differences in the efficiency of the conjugation reaction. The reagent is easily prepared on a multigram scale. We have also prepared and used reagents derived from other water-soluble polymers [76].

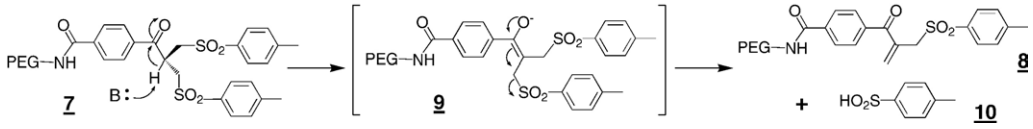
4. Characteristics of disulfide bridging PEGylation

There are two steps involved in our PEGylation strategy: — (1) disulfide reduction, and (2) bis-alkylation PEG conjugation to rebridge the original disulfide. This is followed by purification, which is usually straightforward, because we have found that typically only one equivalent of the PEG reagent is required for each disulfide that needs to be PEGylated. There are three central physicochemical features that underpin the success of our approach to site-specific PEGylation: — (1) maintenance of the protein's tertiary structure for cysteine propinquity in the folded protein after the reduction of a disulfide bond, (2) steric shielding by the PEG after the first addition reaction has occurred, and (3) selectivity and reversibility of thiol addition reactions to conjugated double bonds.

4.1. The need to preserve the protein's tertiary structure

Throughout the disulfide reduction and PEGylation steps, it is important to preserve the protein's tertiary structure after the reduction of the disulfide. This is in order to: — (i) keep the free thiols close to each other, (ii) minimize any potential for the irreversible denaturation and aggregation of the protein, and (iii) prevent disulfide scrambling reactions if more than one disulfide has been reduced. Maintenance of the protein's tertiary structure after the reduction of the disulfide is also necessary to ensure that competitive reactions are inhibited during the conjugation reaction. This ensures that the second cysteine sulfur atom is nearby so that its closeness in time and in space favours the second addition reaction that is required to connect the three-carbon bridge.

Mechanistically, protein thiol addition reactions with our reagents are sequential (note Scheme 1) with only one double bond being available for thiol addition at any given moment in time. The β, β′ bis-sulfone groups undergo elimination more readily than nucleophilic displacement reactions [71–73]. Lawton et al. [71–73,77] first demonstrated that β,β′-bis-sulfone functionality could undergo bis-thiol alkylation via the alkenyl β-mono-sulfone to crosslink proteins by the mechanism shown in Scheme 1. He studied this functionality with ribonuclease [77] and with antibodies [71,72]. Wilbur then examined this functionality with antibody fragments [78], and others have used this functional motif in organic synthesis (e.g., [73,79–83]). These propenyl compounds are not water-soluble. Reaction of a disulfide reduced protein in the presence of an organic solvent usually leads to protein denaturation. This will cause multiple intermolecular and competitive reactions with other amino acid residues. Hence, to utilize the latently functionalized, cross-functionalised propenyl system for bis-alkylation of thiols, there is a crucial need for PEG (or another water-soluble polymer) because this enables the use of experimental conditions that allow the protein to maintain its tertiary structure throughout the conjugation process. Maintenance of the tertiary structure also helps to ensure that the desired three-carbon bridging reaction occurs between the two sulfur atoms from the original disulfide bond.



Scheme 3. Elimination of toluene sulfinic acid **10** from PEG bis-sulfone **7** generates PEG mono-sulfone **8**. It is required for the first thiol addition reaction to occur.

4.2. The presence of PEG

After the first addition reaction (Scheme 1), the steric shielding of the PEG will inhibit the approach of a second protein molecule with a free thiol. The second addition reaction (note Scheme 1) will also be favoured by the fact that the bis-alkylating propenyl group is on the end of a much larger PEG molecule. This will prevent the competitive intermolecular reactions that could result in two protein molecules being conjugated to one PEG molecule. Likewise, steric shielding will delay the approach of a second molecule of the bridging PEGylation reagent, and thereby prevent one molecule of PEG becoming conjugated to each of the cysteine sulfur atoms from a disulfide. If such a situation were to arise, it could result in the formation of a complex mixture of products.

4.3. Thermodynamically driven PEGylation

Another characteristic of our approach to site-specific PEGylation is the reversible nature of thiol addition reactions to conjugated double bonds [84]. Coupled to this is the general inefficiency of amine alkylation and acylation reactions because they often require basic conditions and involve competitive reactions with water. Thiols are nucleophilic at neutral pH and therefore most amine residues will be protonated. If a protein's tertiary structure is maintained, disulfide bridging PEGylation will be driven to its thermodynamic product; i.e. thiol selectivity will lead to the formation of a three-carbon bridge between the two cysteine sulfur atoms. Thiol selectivity leads to the formation of a three-carbon bridge between the two cysteine sulfur atoms that are derived from a disulfide. In contrast, most amine-specific PEGylations are kinetically driven reactions that lead to the generation of a heterogenous product.

4.4. Control reactions

A range of proteins have been examined to determine the scope for disulfide bridging PEGylation. Digestion, NMR and MALDI studies are all consistent with PEGylation having occurred by disulfide bridging [2,3]. In all cases, control reactions were conducted where the unreduced protein was allowed to incubate with the PEG reagent. A typical example is shown in Fig. 2 for asparaginase [3]. This tetrameric protein has one disulfide per monomeric unit while also displaying 22 lysines per monomeric unit. In this example the reactive PEG mono-sulfone **8** (1.3 Eq.) was incubated overnight at ambient temperature. There was no observed reaction when the protein was incubated with 1.3 equivalents of the PEG-mono-sulfone 8 at pH values ranging from 6.0–8.6. Once the disulfide was reduced with DTT (lane 9), quantitative PEGylation was observed (lane 10) using 1.3 equivalents of the PEG mono-sulfone **8**. This example also illustrates the efficiency of this approach to PEGylating proteins.

4.5. Yield

For proteins with one accessible disulfide (e.g., leptin, asparaginase), we have found that quantitative PEGylation

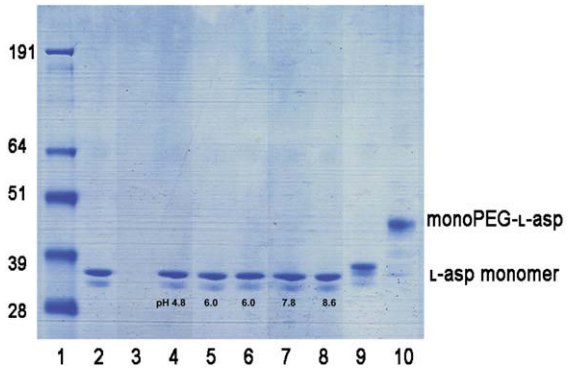


Fig. 2. Example gel of control reactions. Lane 1 — protein molecular weight standards, lane 2 — native L-asparaginase monomer, lane 3 — PEG mono-sulfone reagent **8**, lane 4 — Native L-asparaginase incubated with 1.3 Eq. of PEG_{5K} mono-sulfone reagent at pH 6.0, lane 5 — Native L-asparaginase incubated with 1.3 Eq. of PEG_{5K} mono-sulfone reagent at pH 7.0, lane 6 — Native L-asparaginase incubated with 1.3 Eq. of PEG_{5K} mono-sulfone reagent at pH 7.8, lane 7 — Native L-asparaginase incubated with 1.3 Eq. of PEG_{5K} mono-sulfone reagent at pH 8.6, lane 8 — Native L-asparaginase, lane 9 — 100 mM DTT reduced L-asparaginase, lane 10 — Reduced L-asparaginase incubated with 1.3 Eq. of PEG_{5K} mono-sulfone reagent at pH 7.8.

occurs with one equivalent of the PEG bis-sulfone **7**. If a protein has two accessible disulfides and both are reduced (e.g., interferon-α2) and then one equivalent of the PEG bis-sulfone is added, we observed yields of 60–70% of the mono-PEGylated IFN. The remainder of the product mixture was unPEGylated protein and diPEGylated IFN (~5%). Typically these PEGylation reactions [3] are conducted by incubating the protein (in this case IFN) in phosphate buffer at pH 7.8 containing EDTA. After the addition of DDT, the solution is allowed to incubate for 30 min followed by elution on a PD-10 column. One equivalent of the PEG bis-sulfone reagent **7** is then added and the reaction mixture incubated for time periods up to 16 h at 4 °C. If two equivalents of the PEG bis-sulfone are added, then diPEGylated IFN was the exclusive product. If it is found that the initial conjugation reaction is slow or that the conjugation is better conducted in acidic conditions, then the PEG mono-sulfone **8** can be used.

4.6. Recycling unPEGylated protein

It is important to ensure that the any disulfides that have been reduced, but not PEGylated are then reoxidised. This will ensure the retention of the protein's biological activity. For example, when both of the disulfides of IFN were reduced, and one equivalent of the PEG bis-sulfone **7** was added, the unPEGylated and reoxidised protein retained its biological activity. Therefore any reduced disulfides that have not been PEGylated, can easily be reoxidized [3]. This enables efficient recycling of any unPEGylated protein for another disulfide bridging PEGylation reaction.

5. Illustrative examples

The therapeutically relevant proteins that have been examined with disulfide bridging PEGylation include cytokines

and antibody fragments (Fabs) [3]. Examples of cytokines in the clinic include PEGylated interferon α2 [85,86] and granulocyte colony-stimulating factor (GCSF) [87]. In the case of Fabs the greatest interest has been in blocking tumour necrosis factor-α [88].

5.1. Interferon α-2

The insertion of a single three-carbon bridge (without PEG attached) had a negligible effect on the biological activity of IFN. However, when a single PEG was attached to IFN, the biological activity of the PEG-IFN was reduced to a level that was similar to the in-vitro activity of the amine PEGylated IFNs in clinical use [89–94]. This is approximately 7% of the antiviral activity of IFN. This result illustrates the minimal effect of the three-carbon bridge compared to the PEG on the in-vitro biological effect of IFN. Interestingly, the biological activity of our PEG-IFNs was independent of the molecular weight of the PEG attached. This is in contrast to the experience with amine PEGylation where biological activity is inversely correlated with the size of the PEG [94]. We have also observed this independence of biological activity as a function of the size of the PEG with asparaginase. We believe that this is due to the site-specific nature of the disulfide site-specific PEGylation. As expected, the *in vivo* half-life of the PEG-IFN was directly correlated with the size of the PEG [2,3].

5.2. Leptin

Computational studies showed that the disulfide bond in leptin was accessible and stochastic modeling indicated that leptin would maintain its tertiary structure if the disulfide was modified with a three-carbon bridge to which PEG was attached [95]. When leptin was compared with IFN, the disulfide bond in leptin (Fig. 3) was found to be located in a very flexible loop of the protein. It was therefore possible that reduction of the disulfide in leptin could lead to the separation of the free cysteine sulfur atoms making rebridging of the disulfide difficult. However, PEGylation using the PEG bis-sulfone **7** (1.3 equivalents) in phosphate buffer at pH 7.8 with 2 M L-arginine gave a high yield of the mono-PEGylated leptin (~67% by SEC).

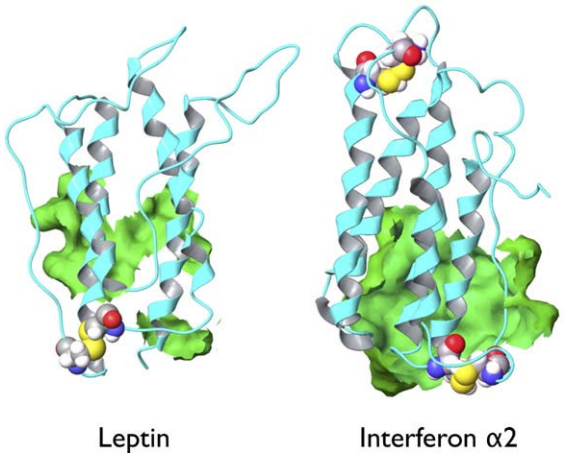


Fig. 3. The structures of interferon α2 and leptin highlighting the accessible disulfides.

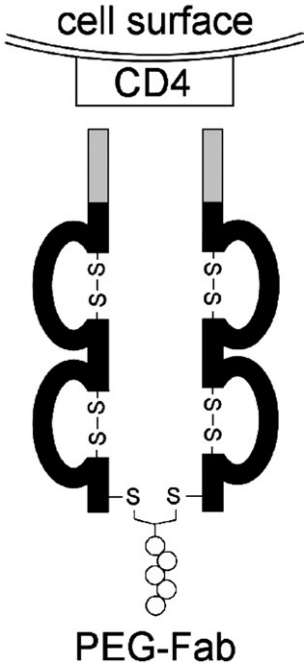


Fig. 4. A representation of anti-CD4 PEG-Fab with PEG conjugated at the hinge disulfide.

5.3. Antibody fragments

In the case of antibody fragments (Fabs), there is an accessible disulfide in the vicinity of the hinge region. It is distal to the Fab's receptor binding site (Fig. 4). There are also multiple non-covalent interactions between the light and heavy chains that maintain the Fab's tertiary structure when this hinge region disulfide is reduced. The conditions required to reduce the accessible disulfide are much milder than those required to reduce the inaccessible disulfides located within a Fab. This enables the site-specific PEGylation of the accessible disulfide in the Fab [2].

Using this approach, we have shown that the anti-HIV-1 activity of an anti-CD4 Fab is retained but its affinity for the CD4 cell surface receptor on lymphocytes is reduced (Fig. 5). These

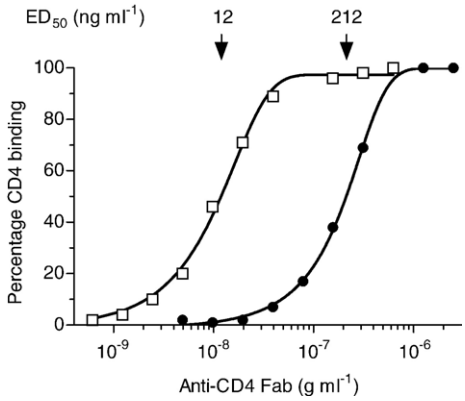


Fig. 5. Binding of Fab (□) and PEG-Fab (●) to cell surface CD4 on the T lymphocyte C8166: concentration is that of Fab in PEG-Fab ($n=3$).

results are consistent with the observations of Kubetzko et al. [96] where the attachment of a 20 kDa PEG near the hinge region of an antibody fragment resulted in a 5-fold reduction in the apparent in-vitro affinity of a PEGylated antibody fragment to its receptor. It was due to a reduction in the association rate of the PEGylated molecule for its receptor with no change in the dissociation rate. Their modeling studies also suggested that PEGylation results in <7% of a PEGylated molecule being available for binding to its receptor at any moment in time [96]. The remainder has intra-molecularly blocked binding interfaces and is in rapid equilibrium with the functional molecule. Although this reduces the observed association rate, all of the PEGylated molecules can eventually bind to the receptor. Similar and related observations have been made for PEG-IFN [93,94]. In addition, the PEGylated molecules inter-molecularly block the attachment of neighbouring molecules causing an apparent reduction in the number of binding sites, minimizing protein aggregation and increasing protein stability [97].

6. Efficacy, metabolism and toxicology studies

Although efficacy studies of PEGylated proteins are performed using laboratory based biological model systems, these are not adequately representative of the biological activity for PEGylated biopharmaceuticals in animals or man. The lack of clinically validated animal models for many of the diseases in which PEGylated biopharmaceuticals are likely to have an exciting new therapeutic role , complicates their evaluation and development. For example, there is no animal model for chronic Hepatitis C infection. In addition, metabolism studies of the biological fate of PEGylated proteins are very difficult to perform. Therefore, during the clinical development of a PEGylated biopharmaceuticals, toxicology studies will have to be performed in a relevant species (e.g., cynomolgus monkeys for PEG-IFN) prior to conducting clinical trials in humans. The kidney and the liver play a role in the excretion of PEG with urinary excretion being the major route of elimination for PEGs with a MWt of <190 kDa [4].

It is also reassuring that the consensus from the published literature is for PEG associated biologicals to have a low exposure-toxicity relationship in animals and humans. This is coupled to a large therapeutic index [4]. Therefore, if the toxicological evaluation of the biological molecule is complete, and a therapeutic window has been identified, the association of PEG with a protein should not represent an additional or unexpected risk in human studies. This means that the acute or chronic administration of a PEGylated protein by a range of routes should be safe. However, as for any therapeutic protein that is being developed for use in humans, a full toxicology package in relevant species will be required prior to starting clinical trials.

7. Conclusions

PEGylation is a clinically proven strategy for increasing the therapeutic efficacy of protein-based medicines. Our approach to site-specific PEGylation exploits the thiol selective chemistry of the two cysteine sulfur atoms from an accessible disulfide. The PEGylation reagents are designed to undergo bis-thiol alkylation by an interactive mechanism that involves addition–elimination reactions. The result is that PEG is attached to a three-carbon bridge and is then linked to the two sulfur atoms from an accessible protein disulfide. Our site-selective approach to protein PEGylation is chemically efficient. UnPEGylated protein can be recovered and recycled. Disulfide bridging PEGylation is therefore a cost-effective process for making a homogenous product. It also offers the potential to make PEGylated biopharmaceuticals as cost-effective medicines.

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