Bioconjugate Chemistry

Review

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Releasable Conjugation of Polymers to Proteins

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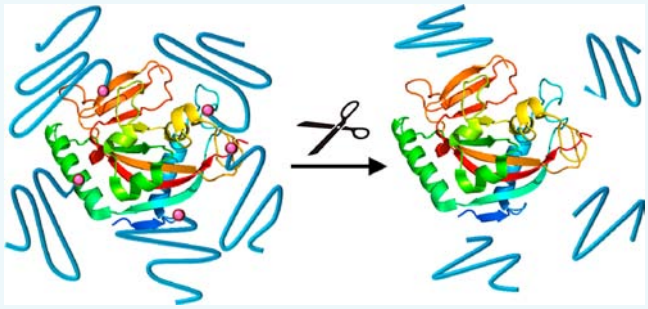
ABSTRACT: Many synthetic strategies are available for preparing well-defined conjugates of peptides/proteins and polymers. Most reports on this topic involve coupling methoxy poly(ethylene glycol) to therapeutic proteins, a process referred to as PEGylation, to increase their circulation lifetime and reduce their immunogenicity. Unfortunately, the major dissuading dogma of PEGylation is that, in many cases, polymer modification leads to significant (or total) loss of activity/function. One approach that is gaining momentum to address this challenge is to release the native protein from the polymer with time in the body (releasable PEGylation). This contribution will present the state-of-the-art of this rapidly evolving field, with emphasis on the chemistry behind the release of the peptide/protein and the means for altering the rate of release in biological fluids. Linkers discussed include those based on the following: substituted maleic anhydride and succinates, disulfides, 1,6-benzyl-elimination, host−guest interactions, bicin, β-elimination, biodegradable polymers, E1cb elimination, β-alanine, photoimmolation, coordination chemistry, zymogen activation, proteolysis, and thioesters.



1. INTRODUCTION

For roughly four decades, grafting methoxy poly(ethylene glycol) (mPEG) to proteins, a process referred to as PEGylation, has been exploited to prevent their renal clearance and their recognition by the immune system.[1−3] Currently, several PEGylated therapeutics are used in the clinic, including PegIntron (interferon-α2b), Pegasys (interferon-α2a), Neulasta (granulocyte colony stimulating factor), Mircera (epoietin-β), Somavert (growth hormone receptor antagonist), and Krystexxa (porcine-like uricase).[4−6] From a developmental context, several synthetic strategies are available for preparing well-defined conjugates of peptides/proteins with polymers. Owing to its simplicity, one attractive grafting approach is to employ residue-specific reactions, which permit the selective modification of all solvent exposed amino acid residues of a given sort. There currently exist residue-specific reactions for permanently coupling polymers to at least 10 out of the 20 canonical amino acids found in proteins.[7−10] However, in many cases, polymer-modification can lead to significant (or total) loss of peptide/protein activity. A classic example is lysozyme, whose ability to hydrolyze bacterial cell-wall polysaccharides is completely lost upon PEGylation with even a single 5 kDa mPEG chain.[11] One avenue for overcoming this challenge is to site-selectively PEGylate the protein in a region not involved in activity, based on an analysis of its 3D structure. For instance, Heredia et al.[12] have shown that the site-specific PEGylation of a V131C mutant of T4 lysozyme has no effect on activity, a dramatically different result from that achieved by random PEGylation. Nevertheless, protein engineering can be laborious, and introducing unpaired cysteine residues complicates oxidative refolding of proteins. Alternatively, the site-specific modification of naturally existing particularities on proteins, such as the solvent-exposed disulfide bonds on L-asparaginase, can be used to a similar effect. In this example, full catalytic activity of the enzyme was preserved though, unfortunately, full immunogenicity was also maintained.[13,14] Overall, even the best designed site-specifically PEGylated bioconjugates can demonstrate unacceptable biological properties. In addition, maintaining activity after site-specific PEGylation is especially challenging in the case of peptides/small proteins, proteins with macromolecular substrates, or those involved in binding events involving multiple cooperative interactions over a large area of its solvent-exposed surface. For instance, the site-specific coupling of mPEG to interferon-α2b at one of its native disulfide bonds imparts a 92% reduction of its antiviral activity, which is comparable to the 93% observed for random PEGylation with a single mPEG chain to a lysine residue.[15] Considering the ever-increasing number of therapeutic entities falling into the categories of proteins listed above, finding a solution for optimizing their pharmacological properties without compromising activity is of great interest, but remains a formidable task.

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One approach that is gaining momentum to address these challenges is to release the native protein from the polymer with time in the body.[16−21] The PEGylated protein is thus considered as a pro-drug, whose reconversion releases the fully active and unmodified native peptide/protein. This approach, termed releasable PEGylation (rPEGylation), incorporates an element of controlled release and is particularly useful for slowly regenerating the activity of proteins adversely affected by polymer modification. The two main challenges of rPEGylation are developing coupling chemistry that enable control of the rate of release of the protein from the polymer and, ideally, ensuring that the release mechanism leaves the protein completely unmodified (traceless) or minimally modified (nontraceless). Several excellent review articles exist which present how rPEGylation can be exploited to optimize the pharmacokinetics and pharmacodynamics of therapeutic peptides/proteins.[16−21] The scope of this contribution is to present the state-of-the-art linker chemistry available for the traceless reversible coupling of polymers to proteins, with emphasis on methods for tuning the rate of de-PEGylation of the bioconjugate. This review is limited to the conjugation of polymers to peptides/proteins, and will not discuss the conjugation of polymers to drugs, lipids, nucleic acids, other polymers, surfaces, and so forth, nor will it discuss the reversible conjugation of drugs to proteins. Review of these topics can be found elsewhere.[22,23]

2. SUBSTITUTED MALEIC ANHYDRIDE AND SUCCINATE LINKERS

One of the first rPEGylation strategies investigated was inspired by anhydride reagents that have previously been used to reversibly mask amino groups on proteins, for temporary selective protection from tryptic digestion during peptide mapping.[24] Unfortunately, while presenting an original concept, these seminal articles generally did not provide sufficient analytical data for a detailed description of the rPEGylation process using this type of linker. Garman and Kalindjian have prepared a 5 kDa mPEG derivative of a substituted maleic anhydride, which they coupled to tissue-type plasminogen activator.[25] However, the degree of PEGylation of the protein was not assessed, and the rate of polymer removal could not be calculated accurately from the supplied data. Roberts and Harris examined a similar approach in which lysozyme was modified with a 5 kDa mPEG bearing a substituted succinate linker.[26] Hydrolysis of the ester between mPEG and the linker in turn triggers the hydrolysis of succinate from the protein, a process which is accelerated by intramolecular catalysis at acidic pH. Recovery of lysozyme activity was observed with a half-life of ~2.5 h at 25 °C in a pH 8 buffer. However, a detailed analysis of the de-PEGylation process, including the analysis of full removal of the succinate from the protein, was not provided. It is interesting to note that removal of maleic anhydride itself from protein amino groups is typically achieved at acidic pH, and is generally not observed above a pH of 6. Thus, the long half-life at pH 6 (>10^3 hours) observed elsewhere,[27] suggests that at neutral pH elimination of this group from a protein at physiological pH will be very slow. rPEGylation using this chemistry is thus likely to be non-traceless, though evidence for this does not exist. Possibly due to limitations associated with linker immolation kinetics, and the requirement for acidic pH conditions to accelerate this process, rPEGylation based on this approach has, to the extent of our knowledge, not been further pursued.

3. THIOL−DISULFIDE EXCHANGE

Another early rPEGylation strategy, examined in the early 1990s, relied on the formation of a disulfide bond between the PEGylation agent and a reduced thiol on the protein. To our knowledge, the first reported example of this strategy was reported by Woghiren et al., who modified the active-site cysteine residue of papain with a 4-pyridyl disulfide activated mPEG.[28] When the activated mPEG was added dropwise to papain during the conjugation step, the recovered product did not contain any detectable conjugate. The authors rationalize this to dimerization of papain caused by nucleophilic attack of the thiol on papain on the papain−mPEG bioconjugate. This observation supports evidence of the potential for available free thiols to initiate de-PEGylation. Papain dimerization was not observed when the protein was added to excess activated mPEG. Pomroy et al. employed a comparable strategy for promoting the solubility of artificial hydrophobic peptides bearing a cysteine residue.[29] In the presence of triscarboxyethylphosphine, PEGylation was fully reversible. In another application, rPEGylation of the active site of the cysteine proteases chymopapain and ficin has been employed for their purification in fully active forms.[30,31] Purification of these enzymes is complicated by the existence of (iso)forms, of which some are catalytically inactive due to the absence of a thiol in the catalytic site. PEGylation of the active form, which possesses a free thiol group, allows for its selective isolation as a bioconjugate. De-PEGylation releases the native and fully active protein. More recently, Bontempo et al.[32] have reported the synthesis of thiol-reactive polymers (**1** in Figure 1) produced by atom transfer radical polymerization (ATRP), and their reversible conjugation to bovine serum albumin. More specifically, a pyridyl disulfide-functionalized initiator was prepared and used for ATRP of 2-hydroxyethyl methacrylate (HEMA) mediated by CuBr and 2,2′-bipyridine. Conjugation was achieved in methanol/phosphate buffer solution at basic pH, and release of the protein could be achieved with dithiothreitol. More recently, Wang et al. modified inorganic

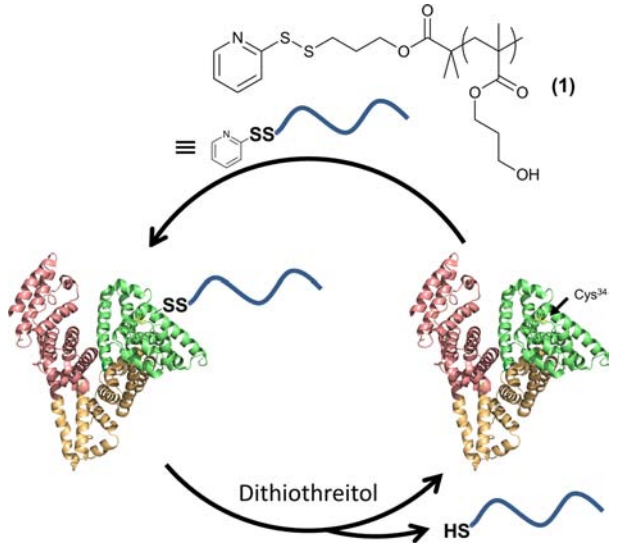

Figure 1. rPEGylation via thiol−disulfide exchange. Bontempo et al.[32] have synthesized a thiol-reactive pHEMA from a functional initiator for ATRP. This polymer was conjugated to cysteine-34 of bovine serum albumin, and could be removed by exposure to reducing agents, such as dithiothreitol.

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pyrophosphatase near its active site with this same polymer.[33] Enzymatic activity was fully inhibited, yet restored in the presence of a reducing agent. Considering the versatility of this polymerization approach, substantial opportunities exist for preparing a variety of thiol-reactive polymers for bioconjugation purposes.

Adjusting the kinetics of thiol−disulfide exchange has been an extensively examined topic, which could be exploited for controlling the rate of de-PEGylation. Steric hindrance imparted by addition of chemical groups adjacent to the disulfide has a predictable and very large impact on the rate of exchange. For example, Kellogg et al. have systematically altered the number and position of methyl groups adjacent a disulfide bond in an antibody−drug conjugate and have evaluated its stability in a redox buffer.[34] While the presence of 1−2 methyl groups increased stability of the disulfide bond by 7- to 22-fold in comparison to the unhindered bond, further modification led to an increased stability of 170- (2 methyl), 980- (3 methyl), and >22 000-fold (4 methyl) relative to the unhindered disulfide. Furthermore, a quantitative relationship between the local charge around the disulfide and its rate of reduction has also been established using model peptide disulfides.[35,36] The authors report the possibility of adjusting the rate of thiol−disulfide exchange with model endogenous thiols over several orders of magnitude, with fine and predictable tunability over the entire range. Substantial opportunities thus exist for exploiting thiol−disulfide exchange for adjusting the rate of de-PEGylation. One caveat to this approach for traceless rPEGylation, however, is the necessity that the protein naturally possess a solvent-accessible cysteine residue. This limits the applicability of this approach to a relatively small subset of proteins. Nevertheless, such a residue could be introduced by protein engineering, though the released protein will inevitably be a mutant of the wild-type protein.

4. THIOL−THIOESTER EXCHANGE

Recently, Chen et al. prepared a thioester derivative of mPEG containing an activated acid for conjugation to amino groups on proteins.[37] In the presence of thiols, thiol−thioester exchange at the thioester trigger exposes a free thiol, which in turn initiates its self-immolation by cyclization. The authors suggest that the release rate can be controlled via the steric hindrance of the thioester, as it does for thiol−disulfide exchange. In model studies, a series of thioester terminated PEGs with increasing steric hindrance were shown to be degraded monoexponentially in a phosphate buffered glutathione (5 mM) solution (Figure 2). Increased steric hindrance of the thioester functionality prolonged the half-lives from 0.4 to 9.7 h. The authors PEGylated lysozyme and tumor necrosis-related apoptosis inducing ligand (TRAIL) and demonstrated the release of the native protein by mass spectrometry. The cytotoxicity of the regenerated TRAIL was fully regenerated upon release.

5. 1,6-BENZYL ELIMINATION LINKERS

5.1. Esterase-Triggered. One of the most investigated rPEGylation strategies relies on the 1,6-benzyl elimination of a linker molecule to release fully unmodified amino groups on proteins. This process is initiated by activation of a trigger group, as illustrated in Figure 3. Lee et al.[11] have exploited this chemistry for the preparation of a series of lysozyme−mPEG

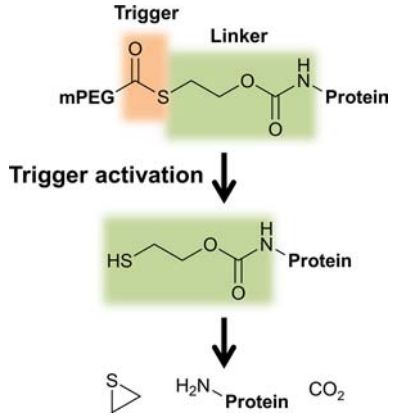

Figure 2. rPEGylation achieved by thiol−thioester exchange. Thiol−thioester exchange exposes a free thiol which initiates the release of the free protein by cyclization. De-PEGylation of conjugated peptide via thioester bond under reducing environment.[37]

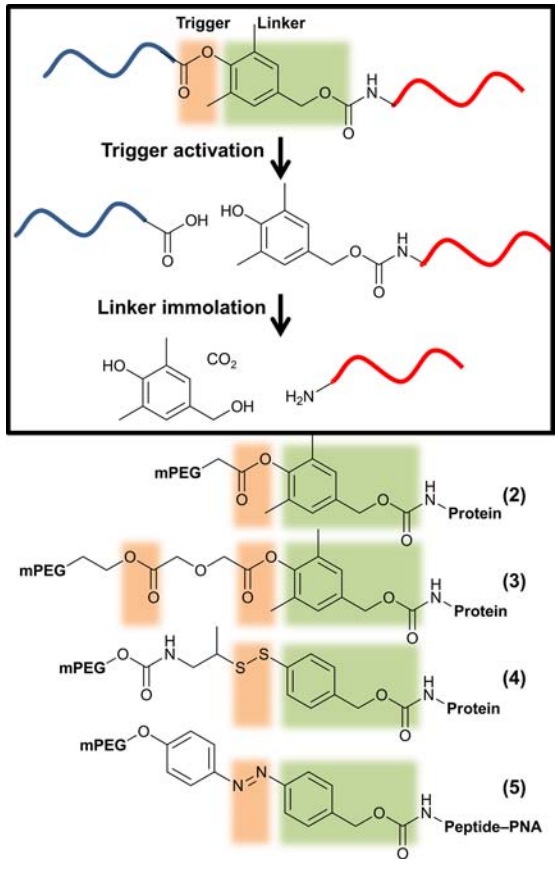

Figure 3. rPEGylation employing 1,6-benzyl elimination linkers. In the box, an example of the steps involved in protein release is given. Examples of esterase-triggered linkers,[11,38] thiol−disulfide exchange-triggered linker,[40] and azoreductase-triggered linker.[41]

conjugates. De-PEGylation was triggered in rat plasma by the enzymatic cleavage of the ester bond trigger. While the conjugates themselves were stable in PBS pH 7.4, release was observed with a half-life of ∼6 h in this buffer. The authors noted that de-PEGylation occurred three times more slowly than for a comparably rPEGylated small-molecule drug, suggesting the role of steric hindrance in enzyme-triggered reconversion. Complications could therefore potentially be observed for highly PEGylated proteins, due to restricted access

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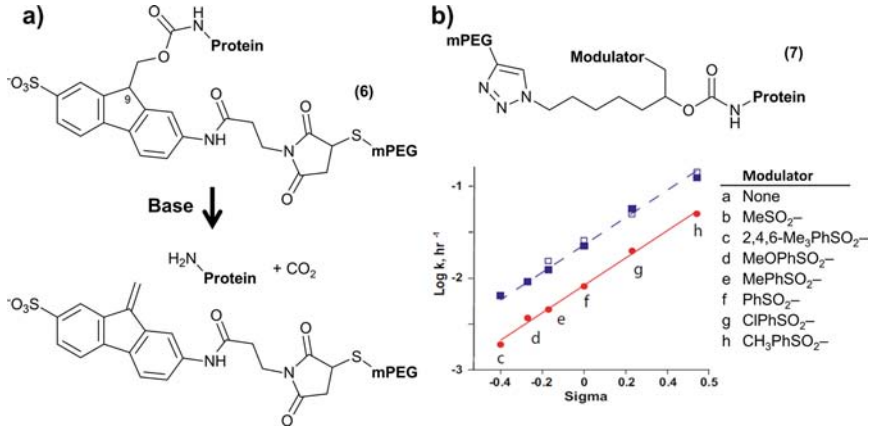

Figure 4. rPEGYlation employing β-eliminative linkers. (a) De-PEGylation of sulfonated fluorenyloxycarbonyl *N*-hydroxysuccinimide ester linkers is solely dependent on pH. (b) De-PEGylation of β-eliminative linkers is controlled in vitro and in vivo by a modulator group. An excellent Hammett correlation was observed, suggesting predictability in adjusting the rate of de-PEGylation by additional modification of the modulator group, using σ constants for the substituents (note: σ constant for *b* is not available). (in vitro (–●–), in rats (- -■- -), and mice (- -□- -). Adapted from original contributions with permission.[49]

to the trigger. To increase the rate of reconversion, Greenwald et al. developed more rapidly hydrolyzing linkers based on the concept of anchimeric assistance (intramolecular assistance to hydrolysis).[38] The authors designed a trigger consisting of an ester of a primary alcohol (**3** in Figure 3), which was more easily hydrolyzed than the ester trigger above (**2** in Figure 3). Hydrolysis of the ester produced a carboxylate anion which in turn facilitated the rapid hydrolysis of the distal hindered ester link of the trigger, via a six-membered ring transition state. The rate of de-PEGylation was not affected by the presence of methyl substituents on the aromatic ring. The authors suggested that this is possibly because the anchimeric effect removed some of the steric effects on trigger activation. More recently, Xie et al.[39] reported the synthesis of an α,ω-heterobifunctional PEG bearing at one extremity the immolative linker **3**, and a second reactive group for decoration of the conjugate with ligands such as fluorophores peptides, antibodies, or other proteins. This report, however, does not describe the conjugation of the reactive polymer to a protein.

5.2. Disulfide-Triggered. Zalipsky et al.[40] examined a variant of the approach above in which the trigger is changed from an ester to a disulfide (**4** in Figure 3). Thiol–disulfide exchange or reduction of the disulfide led to an unstable *p*-mercaptobenzyl carbamate intermediate, which broke down via 1,6-benzyl elimination and decarboxylation to release the unmodified protein. The authors prepared an mPEG–lysozyme conjugate which spontaneously de-PEGylated upon exposure to thiols. However, a putative *p*-mercaptobenzyl carbamate lysozyme intermediate remained detectable in the reaction medium, indicating that self-immolation of the linker occurred relatively slowly. Following intravenous administration, the pharmacokinetics of mPEG–lysozyme conjugates was identical whether a cleavable or noncleavable linker was employed. This suggests that the low concentration of blood thiols may be insufficient in the given configuration of the disulfide to effectively trigger reconversion. Interestingly, after subcutaneous injection, a significantly higher area-under-the-curve was observed for the cleavable conjugate, suggesting disulfide trigger activation by thiols. Methods to alter the rate of thiol–disulfide exchange, which could be used to tune the rate of de-PEGylation, might be of use to optimize the rate of de-PEGylation. To further characterize the nature of thiols potentially involved in reconversion of the conjugate in vivo, the authors showed that overnight incubation of the conjugate in an albumin solution triggered disassembly. This suggests that this macromolecular thiol, with a moderately solvent-exposed cysteine residue, was able to interact with the disulfide trigger, despite expected steric hindrance considerations. Future work may be needed to accelerate the rate of immolation of the linker upon activation of the disulfide triggering, as this process appears to be slow at physiological pH.

5.3. Azoreductase-Triggered. In a further adaptation of this linker design, Lee et al.[41] recently prepared an activatable cell-penetrating peptide conjugate platform for the delivery of a peptide nucleic acid (PNA) drug to the colon (**5** in Figure 3). The concept relied on the rPEGylation of lysine residues of the cell-penetrating peptide (CPP) with a self-immolative aminobenzyl carbamate containing an azobenzene trigger. In its intact form, the PEGylated CPP is inactive, though reductive cleavage of the azobenzene in the colon by bacterial azoreductase is expected to initiate 1,6-benzyl elimination. This releases the active CPP, which can then promote cellular uptake at this location. De-PEGylation releases 4-aminobenzyl alcohol, a byproduct reported to have low cytoxicity.[42] Since active azoreductases are not commercially available, the reconversion of the rPEGylated CPP–PNA was studied with a surrogate reducing agent, sodium dithionite. After incubation with this compound, disappearance of the PEGylated conjugate was observed with reappearance of the free CPP–PNA. No evidence of a residual tag on the CPP was evidenced by mass spectrometry, indicating that under these conditions the kinetics of linker immolation are rapid in comparison to the kinetics of trigger activation. Leriche et al.[43] recently conducted an extensive reactivity study to determine the key structural features that favor the dithionite-triggered reductive cleavage of the azo-arene group. Their work suggests that diazo triggers might potentially be tunable for controlling the rate of release by bacterial azoreductases via substitutions to the aromatic ring. This, however, remains to be examined experimentally.

6. β-ELIMINATIVE LINKERS

Shechter and co-workers have developed a releasable linker for protein rPEGylation based on the β-elimination of a water-soluble sulfonated fluorenyloxycarbonyl *N*-hydroxysuccinimide

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ester (Figure 4a). This compound reacts with amino groups on proteins/peptides and also bears a maleimide group for connection to mPEG-SH by Michael addition. In a substantial body of work, bioconjugates of exendin-4, human growth hormone, interferon-α2a, peptide YY_{3-36}, atrial natriuretic peptide, and insulin have been prepared and evaluated in vitro and in vivo.[44−48] The linker molecule itself absorbs light at 320 nm, which is convenient for determining the level of mPEG conjugation. The rate of de-PEGylation of the bioconjugates was measured at pH 8.5, 37 °C which, for this type of linker, provided comparable cleavage results to that in serum. The peptides/proteins were released in a homogeneous manner with an in vitro half-life of 12 h and retention of biological activity. The constant rate of hydrolysis of the linker was due to the β-elimination reaction, which occurred at position 9 of the fluorenyl moiety, and is solely dependent on the pH of the surrounding medium. Furthermore, the released sulfonated fulvene moiety was reported to be nontoxic. While no effort to modulate the intrinsic rate of de-PEGylation has been attempted, such a modulation may be achievable for multiply PEGylated proteins by varying the degree of polymer grafting.

Recently, Santi et al.[49] developed a platform of β-eliminative linkers which incorporate a series of sulfone modulators that control the rate of de-PEGylation (Figure 4b). Release occurred on the basis of a nonenzymatic β-elimination reaction with preprogrammed and highly tunable cleavage rates. The linkers bore a succinimidyl carbonate group for attachment to an amine-containing peptide/protein and a tunable modulator that controls the rate of β-eliminative cleavage. The linkers provided predictable, tunable release rates of ligands from macromolecular conjugates both in vitro/vivo with half-lives spanning hours to over one year at physiological pH. The rate of elimination was only modestly dependent on the basicity of the aliphatic amine component of the carbamate, suggesting only a small influence of the local chemical environment at the conjugation site. The coproduct of the β-elimination is a PEGylated β-alkenyl sulfone, which is a Michael acceptor. The authors observed, however, that the reaction rates of such compounds with serum nucleophiles, such as 0.5 mM glutathione, were either competitive or slower than the rates of clearance of the polymer from the body. They further reported an excellent experimental Hammett correlation with σ constants for the substituents on the phenyl sulfone modulator (reported for pK_a values of substituted phenols), both in vitro and in vivo, using a model fluorophore rather than a protein. Release rates were 2- to 3-fold faster in vivo. Predictable fine-tuning of in vitro β-elimination rates can thus be achieved by varying the substituent on the phenyl sulfone modulator by exploiting the availability of sigma constants for over 60 substituted phenols. More recently, the authors expanded this linker design for the modification of phenol groups rather than amino groups, though this has not yet been tested for peptide/protein−polymer conjugation.[50]

7. BICIN LINKERS

Aliphatic esters structures synthesized from *N*-modified bis-2-hydroxyethylglycinamide (bicin) have been examined as self-immolating linkers for rPEGylation. Zhao et al. have prepared linear (**8** in Figure 5) and branched (**9** in Figure 5) mPEG bioconjugates of both lysozyme and interferon β1b (Figure 5).[51] Bicin linkers de-PEGylate by cyclization after hydrolysis of an ester trigger. The rate of protein release from the linear PEG

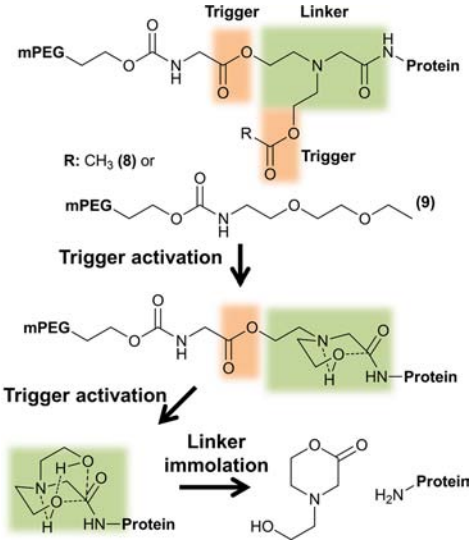

Figure 5. rPEGylation achieved using bicin linkers. Hydrolysis of the ester triggers induces release of the protein, via cyclization of bicin.[51]

conjugate was faster than for the branched counterpart. This phenomenon could be due to reduced hindrance of the linear PEG conjugates toward esterase or nonenzymatic hydrolysis. The kinetics of protein release for proteins bearing multiple mPEG chains in rat plasma and in vivo correlated with the number of polymer chains, and the half-lives observed spanned from hours to days, within a therapeutically relevant window. The authors mentioned that the bicin linker bearing two mPEG chains sequentially release one mPEG after another, which could complicate the analysis of the resulting pharmacokinetics. Filpula et al. pursued work with this linker by PEGylating immunotoxins.[52] Immunotoxins produce potent antitumor responses, but the toxin domain may exhibit nonspecific binding to normal tissues. In addition, neutralizing antibodies can be formed resulting in dose-limiting toxicity or diminished therapeutic potency. Also, in contrast to antibodies, these systems do not possess an inherently long circulation time, due to the lack of an Fc domain. The authors examined conjugates containing the linear and branched bicin linkers. The conjugates exhibited prolonged blood residency time and greatly expanded the therapeutic exposure while reducing the nonspecific toxicity of the immunotoxin. In addition to controlling the rate of protein release via the number of conjugated mPEG chains, in principle, altering the structure of the ester trigger could be used to control the intrinsic rate of de-PEGylation, which would be valuable in the case of mono-PEGylated bioconjugates.

8. β-ALANINE LINKERS

Pasut et al. reported an rPEGylation strategy based on a β-alanine spacer between mPEG and the protein.[53] Following conjugation of mPEG to human growth hormone, partial de-PEGylation was observed over a 3−5-day period in phosphate buffer, due to hydrolysis of the amide bond between the linker and the protein. In a follow-up study, the authors used model peptides to determine that the PEGylation of lysine and tyrosine residues using this linker is permanent. However, histidine residues were modified reversibly.[54] The use of this approach for effective rPEGylation and full release of the protein may therefore require that temporary protecting groups

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be installed on protein amino and phenol groups, so that the polymer is selectively conjugated to histidine residues.

9. BIODEGRADABLE POLYMER

In 2008, Duncan and co-workers introduced an interesting strategy to recover protein activity from polymer-modified bioconjugates. This approach, referred to as PUMPT for polymer-masking-unmasking-protein-therapy, resides in the use of a polymer that can be effectively hydrolyzed in biological fluids by enzymes to ultimately release the protein.[55] The polymer consisted of the polysaccharide dextrin, a linear α-1,4-poly(glucose), which is degraded to maltose and isomaltose by α-amylases. This polymer can be modified by succinoylation for both conjugation to proteins, and to slow the rate of enzymatic degradation. The authors grafted this polymer to trypsin as well as melanocyte stimulating hormone, and demonstrated that coincubation with amylases partially regenerated protein activity. However, the extent of regeneration of protein/peptide activity in these in vitro models is complex to interpret because of the variability associated with the hydrolysis phenomenon. Subsequently, the authors developed a bioresponsive dextrin–phospholipase A_2 (PLA_2) conjugate which could reduce PLA_2 systemic toxicity but still retain its antitumor activity upon α-amylase-triggered release of the protein in the tumor interstitium.[56] In addition, Ferguson et al. investigated the replacement of dextrin with hyaluronic acid (HA), and prepared bioconjugates of trypsin with enhanced stability.[57] Restoration of biological activity was observed following exposure to hyaluronidase. HA conjugation did not alter trypsin's activity significantly, though incubation of the conjugate with HAase increased its activity to 145% compared to that of free enzyme. The underlying reason for this increase of activity remains to be determined, though the results suggested that only partial removal of HA was occurring. Recently, biodegradable analogues of mPEG have been developed by incorporating hydrolyzable linkers within the polymer's main chain.[58−60] Such an innovation represents an interesting opportunity for PUMPT of proteins using synthetic polyethers such as mPEG, rather than intrinsically biodegradable polymers such as those discussed above.

10. E1CB ELIMINATION LINKERS

Göfperich and co-workers have examined phenyl carbamates as hydrolyzable linkers for conjugation of linear mPEG and 4-arm PEG to lysozyme and fluorescent bovine serum albumin (Figure 6).[61,62] The hydrolysis of phenyl carbamates in neutral and basic solutions proceeds by an E1cb elimination reaction involving the intermediate formation of an unstable isocyanate, which then disintegrates into a primary amine and carbon dioxide. This process releases the native protein from the polymer. The authors implemented this conjugation approach into the design of a hydrogel by mixing the multiarm polymer with protein. De-PEGylation induced disassembly of the network and ultimately the release of the native protein. In a model study, the rate of de-PEGylation was assessed using linear mPEG conjugated to lysozyme. For a lysozyme–mPEG conjugate bearing 3–5 mPEG chains, almost complete release of lysozyme was achieved within 24 h at 50 °C. Three linker structures were modified to control the rate of de-PEGylation. Electron-withdrawing carbamoyl groups in the *ortho*- or *para*-position (**10** in Figure 6) stabilize the phenolate ion by resonance and accelerate hydrolysis compared to the non-

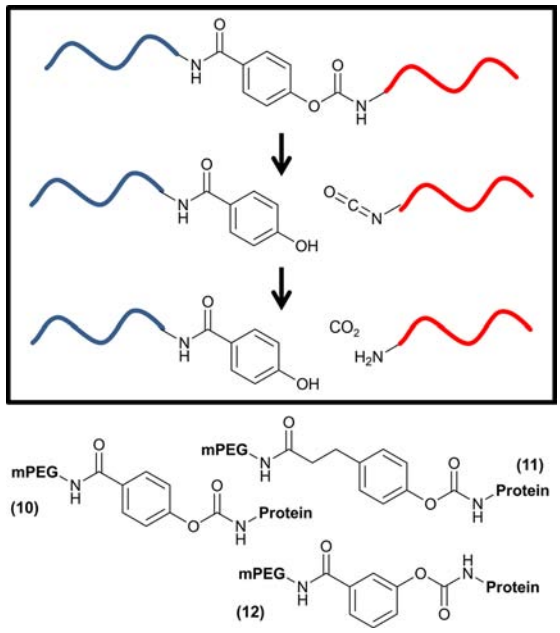

Figure 6. rPEGylation achieved with E1cb linkers. In the box, an example of the steps involved in protein release is given. An intermediate isocyanate is formed, which may be responsible for side-reactions. Structures of linkers used are in ref 61.

substituted analogue. On the other hand, when the carbamoyl group was located in the *meta*-position (**12** in Figure 6), only a minor influence on rate of hydrolysis was observed. However, when an electron-releasing alkyl group was placed in the *para*-position (**11** in Figure 6), destabilization of the phenolate ion dramatically slowed hydrolysis kinetics. Unfortunately, analysis of the de-PEGylation process revealed only partial release of lysozyme. The authors suggested that this could result on one hand from contaminants in the PEGylation reagent that permanently modify the protein, or from the reaction of nucleophiles on the protein with the isocyanate produced during the hydrolysis of the phenyl carbamate, which also produces stable chemical bond. The latter hypothesis was supported by evidence of protein dimer formation, due to reaction of nucleophiles on one protein with an isocyanate on another. However, self-aggregation of lysozyme could not be discounted. This side-reaction could become problematic in the more complex environment of biological fluids due to the presence of numerous natural nucleophiles. This challenge should be further investigated and alterations to the carbamate implemented to minimize this phenomenon. An interesting aspect of this work is that, within the hydrogel, proteins act as cross-linking agents which are connected to multiple polymer chains. While de-PEGylation of the hydrogel network can ultimately release a fully unmodified protein, it can also release partially PEGylated ones into circulation. The latter should have an extended circulation lifetime due to the attached polymer chains. This particularity may offer an addition degree of freedom for achieving sustained blood concentration of protein therapeutics from hydrogel implants. However, pharmacokinetics may be more complex, and this aspect should be investigated in future work.

11. PHOTOIMMOLATIVE LINKER

Light is a unique nonphysiological stimulus for releasing proteins from PEGylated adducts. Georgianna et al. have

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developed a photocleavable linker based on *ortho*-nitrobenzene that responds to nonphototoxic UV light (365 nm) to release the native protein (Figure 7).[63] Full release of lysozyme from a

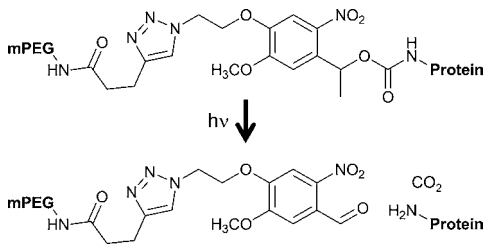

Figure 7. rPEGylation achieved by a photoimmolative linker. Exposure to light (365 nm) for 30 min leads to release of the protein.[63]

multi-PEGylated conjugate was achieved after 30 min irradiation, using a 25 W source. However, the latter only possessed 50% of the activity expected of the protein. The authors indicated that an incomplete restoration of enzymatic activity is not uncommon in the light-activation of biological processes. While photocontrol of reconversion of mPEG–protein conjugates may be interesting for, e.g., localized therapies, pulsatile or controlled release, translation of this concept to in vivo studies will require that the opacity of biological tissues to UV light be addressed and the mechanism of protein deactivation be re-examined.

12. ZYMOGEN ACTIVATION

In an interesting example, Østergaard et al. described an rPEGylation strategy based on the activation of a protein zymogen.[64] Selective glyco-PEGylation was achieved using the substrate promiscuity of the sialyl transferase ST3Gal3, which allowed for the transfer of cytidine 5′-monophosphosialic acid-6′-mPEG (40 kDa) to the terminal galactoses of the *N*-glycans in the activation peptide of the desialylated recombinant Factor IX protein (FIX) (Figure 8). FIX is a vitamin K-dependent glycoprotein and an essential protease of the hemostatic system. FIX is converted to the 2-chain activated form by the tissue factor–factor VIIa complex or factor XIa. Activation occurs by limited proteolysis at Arg145 and Arg180 in the protease domain and liberates a 35-amino-acid activation peptide that carries the only 2 N-linked glycans in the protein.

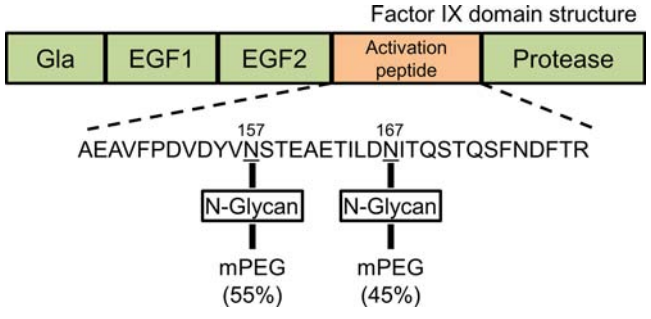

Figure 8. rPEGylation achieved by zymogen activation. Domain structure of FIX (Gla: γ-carboxyglutamic acid domain; EGF-1 and EGF-2: epidermal growth factor domains; Protease: serine protease domain). EGF-2 connects to the serine protease domain through a linker peptide that is required for a proper orientation and folding of serine proteases. To have a physiologically active FIX, two cleavages must occur to remove a 35-amino-acid region that precedes the catalytic site.

The attached mPEG moiety is thus present only on the circulating zymogen form of the bioconjugate, and release of the final protein remains subject to normal physiology regulation, while benefiting from enhanced circulation due to the mPEG component.

13. PROTEOLYSIS

Nollmann et al.[65] reported a strategy for the rPEGylation of proline-rich antimicrobial peptides by *N*-terminal extension with three residues (glycine-alanine-arginine) and an mPEG chain. The active peptide is rationalized to be released by trypsin-like activity in the body, due to digestion at the tripeptide. Interestingly, peptides were released at a similar rate, independently of the molecular weight of the mPEG moiety (0.75 and 5 kDa). This indicated that for this range of mPEG molecular weights, recognition of the cleavable peptide sequence was not hampered by the polymer. In addition, altering the sequence of the peptide linker was shown to control the rate of release. For instance, a second linker, glycine-alanine-arginine-serine-glycine, could be cleaved with a half-life of 40 min in mouse serum (37 °C), which is faster than the corresponding linker above. In another example, Zhang et al. conjugated poly(aspartic acid) to a cell-penetrating peptide via a matrix metalloproteinase (MMP)-cleavable peptide (PLGVR).[66] Upon exposure to MMP at tumor sites, the polyanion is expected to be removed by proteolysis, which would lead to exposure of cell-penetrating peptide and concurrent cellular uptake of the system. In vitro, the authors demonstrated the implication of MMP on release. In general, because of the simplicity of solid-phase peptide synthesis, the approaches discussed in this section are easily implemented and adaptable. The rate of release in the biological milieu may be complicated by variability in the expression levels of the enzymes responsible for release of the peptide.

14. HOST–GUEST INTERACTIONS

To the extent of our knowledge, the first example of rPEGylation based on host–guest interactions was from Bontempo et al.[67] who described the synthesis of poly(*N*-isopropylacrylamide) possessing a terminal biotin unit by ATRP. This polymer readily conjugated to the corresponding binding sites on streptavidin, a process which could be reversed in *N*,*N*-dimethylformamide/water at 90 °C within 1 h. More recently, Mueller et al.[68] reported a traceless rPEGylation approach based on hydrophobic guests interacting with natural hydrophobic guest binding sites on proteins. mPEG bearing a single or two dansyl groups was prepared, which when added to a salmon calcitonin solution decreased the susceptibility to aggregation of this protein. This effect was observed for protein/mPEG ratios from 100/1 to 1/1, but deteriorated at higher mPEG content. An mPEG with a molecular weight of 2 kDa was found to be optimal for preventing aggregation, and no added benefit between a single or two dansyl units was observed. This observation was suggested to result from hindered interaction between the protein and polymer, due to steric hindrance of the polymer. Unfortunately, binding constants between the two macromolecules were not reported.

15. ION COMPLEXATION

In addition to exploiting reversible covalent coupling approaches for rPEGylation, recent efforts have been directed toward exploiting coordination chemistry. Early work by

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Kapanidis et al. has shown that nitrilotriacetic acid (NTA) has a k_d of 10 μM toward hexa-histidine tags on proteins.[69] NTA is able to associate with the imidazole side-chain of histidine residues in proteins through a reversible coordination bond, mediated by a metal ion. Szoka and co-workers pursued this finding and developed an optimized tri-NTA agent with a k_d of 20 nM for a hexa-histidine peptide.[70] In an interesting adaptation of this approach, Mero et al. investigated the NTA-mediated rPEGylation of proteins not possessing hexa-histidine tags.[71] The authors synthesized an eight-arm PEG modified at its eight extremities with NTA, for cooperative complexation of isolated solvent-exposed histidine residues. It was rationalized that this structure would have sufficient flexibility to coordinate individual natural histidine residues dispersed on the protein surface. The authors examined their hypothesis with five different proteins, though only two efficiently complexed the PEG. Granulocyte colony stimulating factor strongly associated with the PEG in the presence of copper with a k_d of 4.3 nM, but the complex did not show a half-life prolongation in vivo. The authors attributed this result from either competition with plasma proteins or displacement in vivo. In vitro, human serum albumin did not compete for the polymer or cause de-PEGylation. More recently, Kim et al.[72] synthesized a bis-NTA mPEG reagent for the modification of hexa-histidine tagged proteins, a process which was mediated by Ni^{2+}. Using this reagent, the authors PEGylate hexa-histidine-tagged TRAIL (k_d 0.27 μM). Improved in vivo properties were observed yielding 3- to 4-fold improved efficacy over native TRAIL in terms of solution stability and half-life versus the native protein. The authors observed that the bioactivity of the protein was not strongly deactivated by the polymer due to the benefits of the site-specific PEGylation. Future adaptations of the structure of the NTA ligand may potentially permit tuning of the rate of disassembly of the bioconjugate.

16. OUTLOOK

This contribution highlights that numerous opportunities exist for the reversible PEGylation of peptides/proteins and several platforms already exist that permit their predictable and tunable release in biological milieu. De-PEGylation is typically triggered by hydrolysis, enzymatic processing, or reaction/exchange with endogenous nucleophiles. Future work may be directed toward identifying new types of dynamic covalent bonds that could exploit alternative stimuli or disease-associated stimuli. This could enable the targeted release of the proteins to specific regions in the body. In addition, one of the major challenges of this field is the development of self-immolating linkers which remove themselves from the protein upon activation. Rapid self-immolation is sometimes difficult to achieve, and can lead to the released protein possessing residual tags that persist and initiate unwanted side-reactions. This is a shared concern with nontraceless rPEGylation approaches. Additionally, most rPEGylation strategies have focused thus far on the modification of amino groups on proteins and, arguably, strategies for the modification of other residues (or glycans) remain limited. Future work may be directed to the rPEGylation of other types of amino acids prone to degradation or associated with immune peptide sequences. This may enable the design of better protected rPEGylated protein therapeutics. Furthermore, while most work in this field has been performed using mPEG as polymer, recent studies have shown that structural and functional complexity may confer new and exciting properties to protein bioconjugates.[73−77] Adapting rPEGylation to new polymer platforms may thus offer new opportunities for the development of functional bioconjugates. Finally, while the specific case of reversible conjugation of polymers to peptides/proteins is discussed herein, lessons learned herein can be adapted for the reversible modification of other entities (drugs, surfaces, nanoparticles, etc.) with polymers.

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Notes

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NOTE ADDED AFTER ASAP PUBLICATION

This paper was published ASAP on February 9, 2015, with incorrect structures in Figure 3. The corrected version was reposted on June 2, 2015.

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