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: substituted maleic anhydride and succinates, disulfides, 1,6-benzyl-elimination, host–guest interactions, bicin, β-elimination, biodegradable polymers, E1cb elimination, β-alanine, photo-immolation, coordination chemistry, zymogen activation, proteolysis, and thioesters.

Keywords: Protein; Peptide; Polymer; Cleavable; Linker; Traceless; Activity; Binding; Supramolecular; Host–guest; mPEG; rPEGylation; Bio-conjugate

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 re-folding 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 bio-conjugates 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.

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

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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 (non-traceless). 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 bio-conjugate. 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, etc., 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 hours at 25 ºC 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 nineties, 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 drop-wise 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 bio-conjugate. 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 bio-conjugate. 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 <u>Figure 1</u>) produced by atom transfer radical polymerization, 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 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 bio-conjugation 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 >22000-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 sub-

set 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.
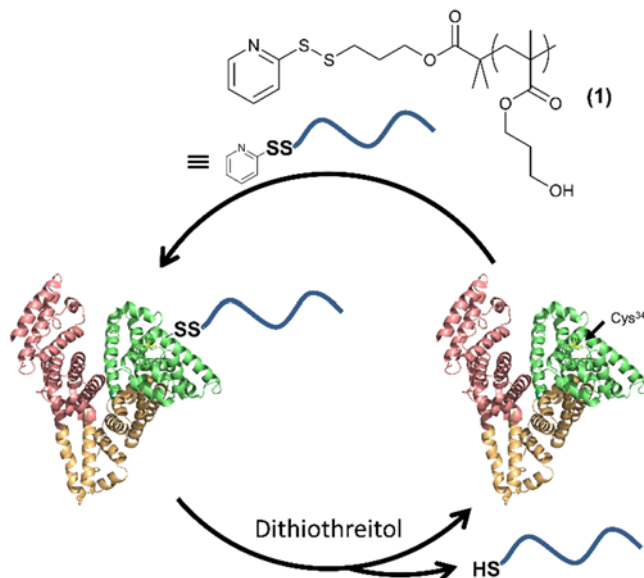

Figure 1| **rPEGylation via thiol–disulfide exchange**. Bontempo et al.[32] have synthesized a thiol-reactive pHEMA from a functional initiator for ATRP. This polymer was conjugated to cysteine-34 of bovine serum albumin, and could be removed by exposure to reducing agents, such as dithiothreitol.

4. Thiol–thioester exchange

Recently, Chen et al. have 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 mono-exponentially 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 hours. The authors PEGylated lysozyme and TRAIL and demonstrated release of the native protein by mass spectrometry. The cytotoxicity of the regenerated TRAIL was fully regenerated upon release.
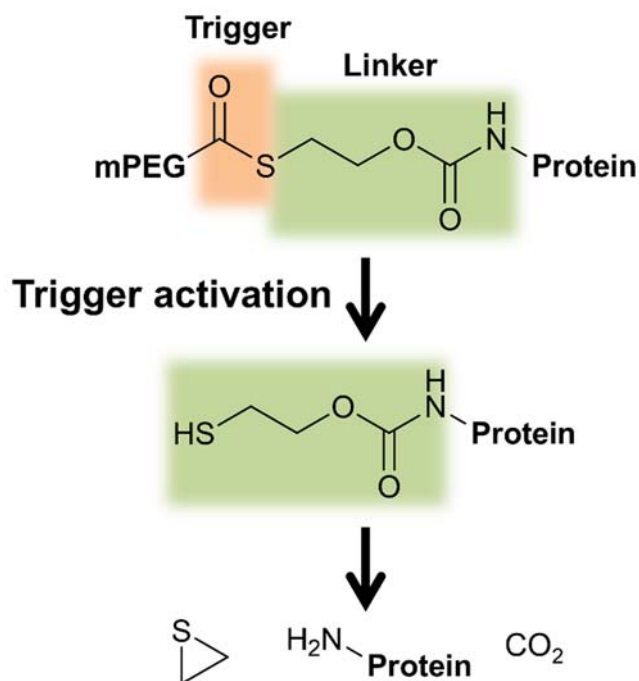
Figure 2| **rPEGylation achieved by thiol–thioester exchange**. Thiol–thioester exchange exposes a free thiol which initiates the release of the free protein by cyclization. De-PEGylation of conjugated peptide via thioester bond under reducing environment.[37]

5. 1,6-Benzyl elimination linkers

5.1. Esterase-triggered

One of the most investigated rPEGylation 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 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 slower 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 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] have reported the synthesis of a α,ω-hetero-bifunctional 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 and non-cleavable 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] have 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 by-product 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] have recently conducted an extensive reactivity study to determine the key structural features that favour 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.
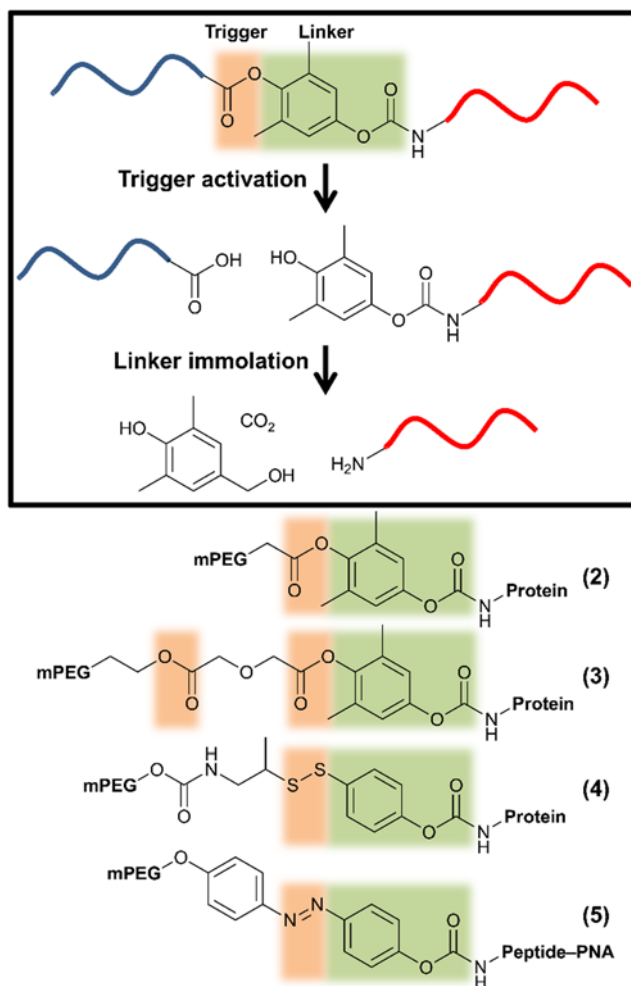

Figure 3| **rPEGylation employing 1,6-Benzyl elimination linkers**. In the box, an example of the steps involved in protein release is given. Examples of esterase-triggered linkers,[11, 38] thiol–disulfide exchange-triggered linker[40], and azoreductase-triggered linker.[41]

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 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, bio-conjugates 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 bio-conjugates 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 homogenous manner with an *in vitro* half-life of 12 hours 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 non-toxic. 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] have 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 non-enzymatic β-elimination reaction with pre-programmed 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 co-product 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, are 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 two to threefold 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 has not yet been tested for peptide/protein–polymer conjugation.[50]
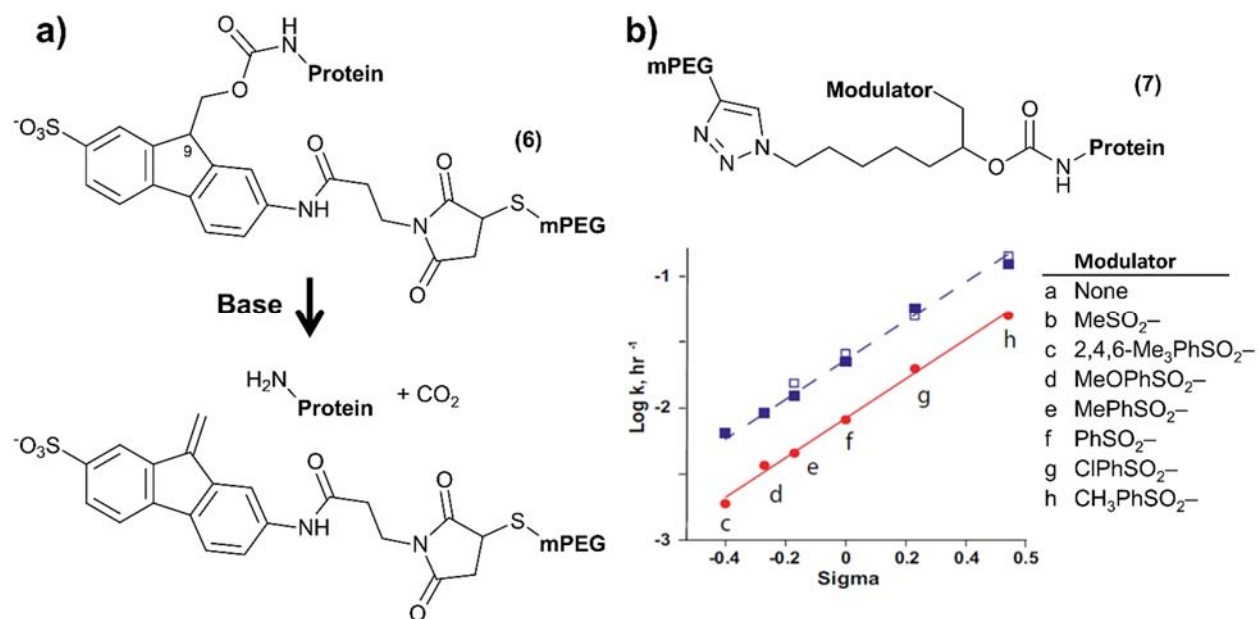
Figure 4| **rPEGYlation employing β-eliminative linkers**. (**a**) De-PEGylation of sulfonated fluorenyloxycarbonyl *N*-hydroxysuccinimide ester linkers is solely dependent on pH. (**b**) De-PEGylation of β-eliminative linkers is controlled *in vitro* and *in vivo* by a modulator group. An excellent 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]

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 bio-conjugates 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 conjugate was faster than for the branched counterpart. This phenomenon could be due to reduced hindrance of the linear PEG conjugates toward esterase or non-enzymatic 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 non-specific 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 non-specific 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 bio-conjugates.
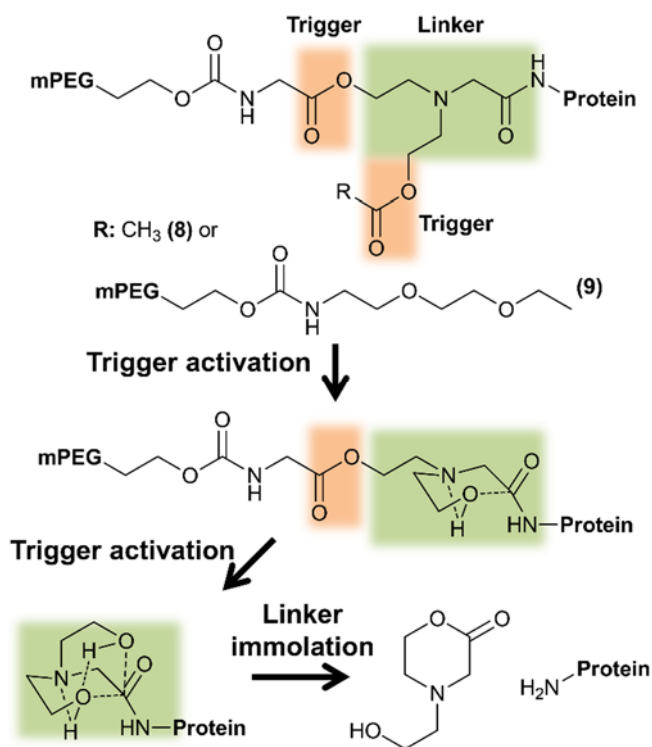

Figure 5| **rPEGylation achieved using bicin linkers**. Hydrolysis of the ester triggers induces release of the protein, via cyclization of bicin.[51]

8. β-Alanine linkers

Pasut et al. have 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 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 in interesting strategy to recover protein activity from polymer-modified bio-conjugates. 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 co-incubation 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 bio-responsive 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 bio-conjugates 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 analogs of mPEG have been developed by incorporating hydrolysable 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 hydrolysable 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 multi-arm 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 hours at 50 ºC. 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-substituted analog. 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 to which are connected to multiple polymer chains. While de-PEGylation of the hydrogel network can ultimately release a fully unmodified protein, is 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.
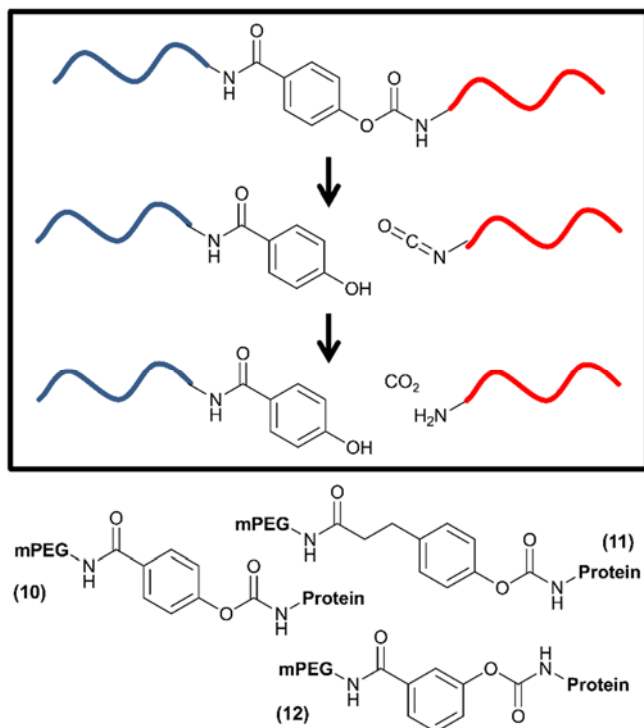

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Figure 6| **rPEGylation achieved with E1cb linkers**. In the box, an example of the steps involved in protein release is given. An intermediate isocyanate is formed, which may be responsible for side-reactions. Structures of linkers used in Ref.[61]

11. Photo-immolative linker

Light is a unique non-physiological stimulus for releasing proteins from PEGylated adducts. Georgianna et al. have developed a photocleavable linker based on *ortho*-nitrobenzene that responds to non-phototoxic UV light (365 nm) to release the native protein (Figure 7).[63] Full release of lysozyme from a multi-PEGylated conjugate was achieved after 30 minute 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 photo-control 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.
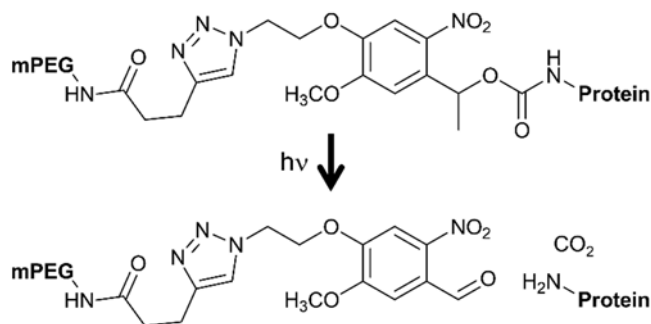

Figure 7| **rPEGylation achieved by a photo-immolative linker**. Exposure to light (365 nm) for 30 min leads to release of the protein.[63]

12. Zymogen activation

In an interesting example, Ostergaard 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 de-sialylated 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. The attached mPEG moiety is thus present only on the circulating zymogen form of the bio-

conjugate, and release of the final protein remains subjected to normal physiology regulation, while benefiting from enhanced circulation due to the mPEG component.
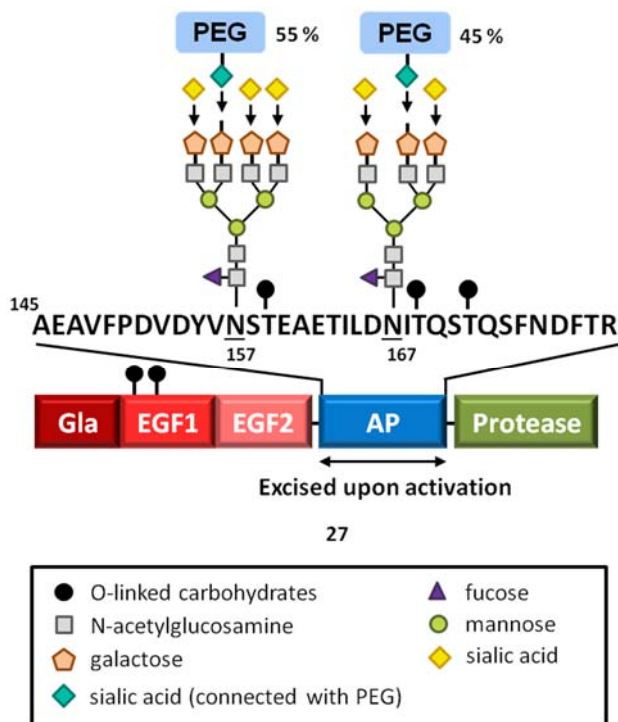

Figure 8| **rPEGylation achieved by zymogen activation**. Domain structure of FIX (Gla: γ-carboxyglutamic acid domain; EGF-1 and EGF-2: epidermal growth factor domains; Protease: serine protease domain). EGF-2 connects to the serine protease domain through a linker peptide that is required for a proper 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. Reproduced with permission.[64]

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 and is 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 ℃), which is faster than the corresponding linker above. In another example, Zhang et al. conjugated poly(aspartic acid) to a cell-penetrating peptide

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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 biological milieu may be complication 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 atom transfer radical polymerization. This polymer readily conjugated to the corresponding binding sites on streptavidin, a process which could be reversed in *N*,*N*-dimethylformamide/water at 90 ºC 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 were 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, neither binding constants between the two macromolecules were reported.

15. Ion Complexation

In addition to exploiting reversible covalent coupling approaches for rPEGylation, recent efforts have been directed towards exploiting coordination chemistry. Early work by Kapanidis et al. have shown that nitrilotriacetic acid (NTA) has a k_d of 10 μM towards 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 a 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. have investigated the NTA-mediated rPEGylation of proteins not possessing hexa-histidine tags.[71] The authors synthesized an 8-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 tumor necrosis-related apoptosis inducing ligand (TRAIL, k_d 0.27 µM). Improved *in vivo* properties were observed yielding three- to four-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 bio-conjugate.

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 towards 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 non-traceless 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 structurally and functionally complex may confer new and exciting properties to protein bio-conjugates.[73-77] Adapting rPEGylation to new polymer platforms may thus offer new opportunities for the development of functional bio-conjugates. 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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References

(1)   Veronese, F. M. (2001) Peptide and protein PEGylation: a review of problems and solutions. *Biomaterials 22*, 405-417.

(2)   Kozlowski, A., and Milton Harris, J. (2001) Improvements in protein PEGylation: pegylated interferons for treatment of hepatitis C. *Journal of Controlled Release 72*, 217-224.

(3)   Roberts, M. J., Bentley, M. D., and Harris, J. M. (2002) Chemistry for peptide and protein PEGylation. *Advanced Drug Delivery Reviews 54*, 459-476.

(4)   Jevševar, S., Kunstelj, M., and Porekar, V. G. (2010) PEGylation of therapeutic proteins. *Biotechnology Journal 5*, 113-128.

(5)   Reinders, M. K., and Jansen, T. L. T. A. (2010) New Advances in the Treatment of Gout: Review of Pegloticase. *Journal of Therapeutics and Clinical Risk Management 6*, 543-550.

(6)   Pasut, G., and Veronese, F. M. (2007) Polymer–drug conjugation, recent achievements and general strategies. *Progress in Polymer Science 32*, 933-961.

(7)   Shannon, D. A., and Weerapana, E. (2015) Covalent protein modification: the current landscape of residue-specific electrophiles. *Current Opinion in Chemical Biology 24*, 18-26.

(8)   Gauthier, M. A., and Klok, H.-A. (2008) Peptide/protein-polymer conjugates: synthetic strategies and design concepts. *Chemical Communications*, 2591-2611.

(9)   Gauthier, M. A., and Klok, H.-A. (2010) Arginine-Specific Modification of Proteins with Polyethylene Glycol. *Biomacromolecules 12*, 482-493.

(10)   Gauthier, M. A., Ayer, M., Kowal, J., Wurm, F. R., and Klok, H.-A. (2011) Arginine-specific protein modification using [small alpha]-oxo-aldehyde functional polymers prepared by atom transfer radical polymerization. *Polymer Chemistry 2*, 1490-1498.

(11)   Lee, S., Greenwald, R. B., McGuire, J., Yang, K., and Shi, C. (2001) Drug Delivery Systems Employing 1,6-Elimination: Releasable Poly(ethylene glycol) Conjugates of Proteins. *Bioconjugate Chemistry 12*, 163-169.

(12)   Heredia, K. L., Tao, L., Grover, G. N., and Maynard, H. D. (2010) Heterotelechelic polymers for capture and release of protein-polymer conjugates. *Polymer Chemistry 1*, 168-170.

(13)   Shaunak, S., Godwin, A., Choi, J.-W., Balan, S., Pedone, E., Vijayarangam, D., Heidelberger, S., Teo, I., Zloh, M., and Brocchini, S. (2006) Site-specific PEGylation of native disulfide bonds in therapeutic proteins. *Nat Chem Biol 2*, 312-313.

(14)   Balan, S., Choi, J.-w., Godwin, A., Teo, I., Laborde, C. M., Heidelberger, S., Zloh, M., Shaunak, S., and Brocchini, S. (2006) Site-Specific PEGylation of Protein Disulfide Bonds Using a Three-Carbon Bridge. *Bioconjugate Chemistry 18*, 61-76.

(15)   Bailon, P., Palleroni, A., Schaffer, C. A., Spence, C. L., Fung, W.-J., Porter, J. E., Ehrlich, G. K., Pan, W., Xu, Z.-X., Modi, M. W., et al. (2001) Rational Design of a Potent, Long-Lasting Form of Interferon: A 40 kDa Branched Polyethylene Glycol-Conjugated Interferon α-2a for the Treatment of Hepatitis C. *Bioconjugate Chemistry 12*, 195-202.

(16)   Greenwald, R. B., Choe, Y. H., McGuire, J., and Conover, C. D. (2003) Effective drug delivery by PEGylated drug conjugates. *Advanced Drug Delivery Reviews 55*, 217-250.

(17)   Filpula, D., and Zhao, H. (2008) Releasable PEGylation of proteins with customized linkers. *Advanced Drug Delivery Reviews 60*, 29-49.

(18)   Ikeda, Y., and Nagasaki, Y. (2012) PEGylation Technology in Nanomedicine, in *Polymers in Nanomedicine* (Kunugi, S., and Yamaoka, T., Eds.) pp 115-140, Springer Berlin Heidelberg.

(19)   Pasut, G., and Veronese, F. M. (2012) State of the art in PEGylation: The great versatility achieved after forty years of research. *Journal of Controlled Release 161*, 461-472.

(20)   Pasut, G. (2014) Polymers for Protein Conjugation. *Polymers 6*, 160-178.

(21)   Shechter, Y., Mironchik, M., Saul, A., Gershonov, E., Precido-Patt, L., Sasson, K., Tsubery, H., Mester, B., Kapitkovsky, A., Rubinraut, S., et al. (2007) New Technologies to Prolong Life-time of Peptide and Protein Drugs In vivo. *International Journal of Peptide Research and Therapeutics 13*, 105-117.

(22)   Shim, M. S., and Kwon, Y. J. (2012) Stimuli-responsive polymers and nanomaterials for gene delivery and imaging applications. *Advanced Drug Delivery Reviews 64*, 1046-1059.

(23)   Ikeda, Y., and Nagasaki, Y. (2014) Impacts of PEGylation on the gene and oligonucleotide delivery system. *Journal of Applied Polymer Science 131*, n/a-n/a.

(24)   Hanai, R., and Wang, J. C. (1994) Protein footprinting by the combined use of reversible and irreversible lysine modifications. *Proceedings of the National Academy of Sciences 91*, 11904-11908.

(25)   Garman, A. J., and Barret Kalindjian, S. (1987) The preparation and properties of novel reversible polymer-protein conjugates 2-ω-Methoxypolyethylene (5000) glycoxymethylene-3-methylmaleyl conjugates of plasminogen activators. *FEBS Letters 223*, 361-365.

(26)   Roberts, M. J., and Milton Harris, J. (1998) Attachment of degradable poly(ethylene glycol) to proteins has the potential to increase therapeutic efficacy. *Journal of Pharmaceutical Sciences 87*, 1440-1445.

(27)   P J Butler, J. I. H., B S Hartley, R Lebeman (1969) The use of maleic anhydride for the reversible blocking of amino groups in polypeptide chains. . *Biochemical Journal 112*, 679-689.

(28)   Woghiren, C., Sharma, B., and Stein, S. (1993) Protected thiol-polyethylene glycol: A new activated polymer for reversible protein modification. *Bioconjugate Chemistry 4*, 314-318.

(29)   Pomroy, N. C., and Deber, C. M. (1998) Solubilization of Hydrophobic Peptides by Reversible Cysteine PEGylation. *Biochemical and Biophysical Research Communications 245*, 618-621.

(30)   Azarkan, M., Maes, D., Bouckaert, J., Thi, M.-H. D., Wyns, L., and Looze, Y. (1996) Thiol pegylation facilitates purification of chymopapain leading to diffraction studies at 1.4 Å resolution. *Journal of Chromatography A 749*, 69-72.

(31)   Azarkan, M., Matagne, A., Wattiez, R., Bolle, L., Vandenameele, J., and Baeyens-Volant, D. (2011) Selective and reversible thiol-pegylation, an effective approach for purification and characterization of five fully active ficin (iso)forms from Ficus carica latex. *Phytochemistry 72*, 1718-1731.

(32)   Bontempo, D., Heredia, K. L., Fish, B. A., and Maynard, H. D. (2004) Cysteine-Reactive Polymers Synthesized by Atom Transfer Radical Polymerization for Conjugation to Proteins. *Journal of the American Chemical Society 126*, 15372-15373.

(33)   Wang, L., Yuan, L., Wang, H., Liu, X., Li, X., and Chen, H. (2014) New Strategy for Reversible Modulation of Protein Activity through Site-Specific Conjugation of Small Molecule and Polymer. *Bioconjugate Chemistry 25*, 1252-1260.

(34)   Kellogg, B. A., Garrett, L., Kovtun, Y., Lai, K. C., Leece, B., Miller, M., Payne, G., Steeves, R., Whiteman, K. R., Widdison, W., et al. (2011) Disulfide-Linked Antibody−Maytansinoid Conjugates: Optimization of In Vivo Activity by Varying the Steric Hindrance at Carbon Atoms Adjacent to the Disulfide Linkage. *Bioconjugate Chemistry 22*, 717-727.

(35)   Wu, C., Belenda, C., Leroux, J.-C., and Gauthier, M. A. (2011) Interplay of Chemical Microenvironment and Redox Environment on Thiol–Disulfide Exchange Kinetics. *Chemistry – A European Journal 17*, 10064-10070.

(36)   Wu, C., Wang, S., Brülisauer, L., Leroux, J.-C., and Gauthier, M. A. (2013) Broad Control of Disulfide Stability through Microenvironmental Effects and Analysis in Complex Redox Environments. *Biomacromolecules 14*, 2383-2388.

(37)   Chen, J., Zhao, M., Feng, F., Sizovs, A., and Wang, J. (2013) Tunable Thioesters as "Reduction" Responsive Functionality for Traceless Reversible Protein PEGylation. *Journal of the American Chemical Society 135*, 10938-10941.

(38)   Greenwald, R. B., Yang, K., Zhao, H., Conover, C. D., Lee, S., and Filpula, D. (2003) Controlled Release of Proteins from Their Poly(Ethylene Glycol) Conjugates: Drug Delivery Systems Employing 1,6-Elimination. *Bioconjugate Chemistry 14*, 395-403.

(39)   Xie, Y., Duan, S., and Forrest, M. L. (2010) Alkyne- and 1,6-elimination- succinimidyl carbonate - terminated heterobifunctional poly(ethylene glycol) for reversible "Click" PEGylation. *Drug discoveries & therapeutics 4*, 240-245.

(40)   Zalipsky, S., Mullah, N., Engbers, C., Hutchins, M. U., and Kiwan, R. (2007) Thiolytically Cleavable Dithiobenzyl Urethane-Linked Polymer–Protein Conjugates as Macromolecular Prodrugs: Reversible PEGylation of Proteins. *Bioconjugate Chemistry 18*, 1869-1878.

(41)   Lee, S. H., Moroz, E., Castagner, B., and Leroux, J.-C. (2014) Activatable Cell Penetrating Peptide–Peptide Nucleic Acid Conjugate via Reduction of Azobenzene PEG Chains. *Journal of the American Chemical Society 136*, 12868-12871.

(42)   Greenwald, R. B., Pendri, A., Conover, C. D., Zhao, H., Choe, Y. H., Martinez, A., Shum, K., and Guan, S. (1999) Drug Delivery Systems Employing 1,4- or 1,6-Elimination: Poly(ethylene glycol) Prodrugs of Amine-Containing Compounds. *Journal of Medicinal Chemistry 42*, 3657-3667.

(43) Leriche, G., Budin, G., Brino, L., and Wagner, A. (2010) Optimization of the Azobenzene Scaffold for Reductive Cleavage by Dithionite; Development of an Azobenzene Cleavable Linker for Proteomic Applications. *European Journal of Organic Chemistry 2010*, 4360-4364.

(44) Tsubery, H., Mironchik, M., Fridkin, M., and Shechter, Y. (2004) Prolonging the Action of Protein and Peptide Drugs by a Novel Approach of Reversible Polyethylene Glycol Modification. *Journal of Biological Chemistry 279*, 38118-38124.

(45) Peleg-Shulman, T., Tsubery, H., Mironchik, M., Fridkin, M., Schreiber, G., and Shechter, Y. (2004) Reversible PEGylation: A Novel Technology To Release Native Interferon α2 over a Prolonged Time Period. *Journal of Medicinal Chemistry 47*, 4897-4904.

(46) Shechter, Y., Tsubery, H., Mironchik, M., Rubinstein, M., and Fridkin, M. (2005) Reversible PEGylation of peptide YY3-36 prolongs its inhibition of food intake in mice. *FEBS Letters 579*, 2439-2444.

(47) Nesher, M., Vachutinsky, Y., Fridkin, G., Schwarz, Y., Sasson, K., Fridkin, M., Shechter, Y., and Lichtstein, D. (2007) Reversible Pegylation Prolongs the Hypotensive Effect of Atrial Natriuretic Peptide. *Bioconjugate Chemistry 19*, 342-348.

(48) Shechter, Y., Mironchik, M., Rubinraut, S., Tsubery, H., Sasson, K., Marcus, Y., and Fridkin, M. (2008) Reversible pegylation of insulin facilitates its prolonged action in vivo. *European Journal of Pharmaceutics and Biopharmaceutics 70*, 19-28.

(49) Santi, D. V., Schneider, E. L., Reid, R., Robinson, L., and Ashley, G. W. (2012) Predictable and tunable half-life extension of therapeutic agents by controlled chemical release from macromolecular conjugates. *Proceedings of the National Academy of Sciences 109*, 6211-6216.

(50) Schneider, E. L., Robinson, L., Reid, R., Ashley, G. W., and Santi, D. V. (2013) β-Eliminative Releasable Linkers Adapted for Bioconjugation of Macromolecules to Phenols. *Bioconjugate Chemistry 24*, 1990-1997.

(51) Zhao, H., Yang, K., Martinez, A., Basu, A., Chintala, R., Liu, H.-C., Janjua, A., Wang, M., and Filpula, D. (2006) Linear and Branched Bicin Linkers for Releasable PEGylation of Macromolecules: Controlled Release in Vivo and in Vitro from Mono- and Multi-PEGylated Proteins. *Bioconjugate Chemistry 17*, 341-351.

(52) Filpula, D., Yang, K., Basu, A., Hassan, R., Xiang, L., Zhang, Z., Wang, M., Wang, Q.-c., Ho, M., Beers, R., et al. (2007) Releasable PEGylation of Mesothelin Targeted Immunotoxin SS1P Achieves Single Dosage Complete Regression of a Human Carcinoma in Mice. *Bioconjugate Chemistry 18*, 773-784.

(53) Pasut, G., Caboi, F., Schrepfer, R., Tonon, G., Schiavon, O., and Veronese, F. M. (2007) New active poly(ethylene glycol) derivative for amino coupling. *Reactive and Functional Polymers 67*, 529-539.

(54) Pasut, G., Mero, A., Caboi, F., Scaramuzza, S., Sollai, L., and Veronese, F. M. (2008) A New PEG−β-Alanine Active Derivative for Releasable Protein Conjugation. *Bioconjugate Chemistry 19*, 2427-2431.

(55) Duncan, R., Gilbert, H. R. P., Carbajo, R. J., and Vicent, M. J. (2008) Polymer Masked−Unmasked Protein Therapy. 1. Bioresponsive Dextrin−Trypsin and −Melanocyte Stimulating Hormone Conjugates Designed for α-Amylase Activation. *Biomacromolecules 9*, 1146-1154.

(56) Ferguson, E. L., and Duncan, R. (2009) Dextrin−Phospholipase A2: Synthesis and Evaluation as a Bioresponsive Anticancer Conjugate. *Biomacromolecules 10*, 1358-1364.

(57)   Ferguson, E. L., Alshame, A. M. J., and Thomas, D. W. (2010) Evaluation of hyaluronic acid–protein conjugates for polymer masked–unmasked protein therapy. *International Journal of Pharmaceutics 402*, 95-102.

(58)   Reid, B., Tzeng, S., Warren, A., Kozielski, K., and Elisseeff, J. (2010) Development of a PEG Derivative Containing Hydrolytically Degradable Hemiacetals. *Macromolecules 43*, 9588-9590.

(59)   Dingels, C., Müller, S. S., Steinbach, T., Tonhauser, C., and Frey, H. (2012) Universal Concept for the Implementation of a Single Cleavable Unit at Tunable Position in Functional Poly(ethylene glycol)s. *Biomacromolecules 14*, 448-459.

(60)   Lundberg, P., Lee, B. F., van den Berg, S. A., Pressly, E. D., Lee, A., Hawker, C. J., and Lynd, N. A. (2012) Poly[(ethylene oxide)-co-(methylene ethylene oxide)]: A hydrolytically degradable poly(ethylene oxide) platform. *ACS Macro Letters 1*, 1240-1243.

(61)   Brandl, F., Hammer, N., Blunk, T., Tessmar, J., and Goepferich, A. (2010) Biodegradable Hydrogels for Time-Controlled Release of Tethered Peptides or Proteins. *Biomacromolecules 11*, 496-504.

(62)   Hammer, N., Brandl, F. P., Kirchhof, S., and Goepferich, A. M. (2014) Cleavable carbamate linkers for controlled protein delivery from hydrogels. *Journal of Controlled Release 183*, 67-76.

(63)   Georgianna, W. E., Lusic, H., McIver, A. L., and Deiters, A. (2010) Photocleavable Polyethylene Glycol for the Light-Regulation of Protein Function. *Bioconjugate Chemistry 21*, 1404-1407.

(64)   Østergaard, H., Bjelke, J. R., Hansen, L., Petersen, L. C., Pedersen, A. A., Elm, T., Møller, F., Hermit, M. B., Holm, P. K., Krogh, T. N., et al. (2011) *Prolonged half-life and preserved enzymatic properties of factor IX selectively PEGylated on native N-glycans in the activation peptide*, Vol. 118.

(65)   Nollmann, F. I., Goldbach, T., Berthold, N., and Hoffmann, R. (2013) Controlled Systemic Release of Therapeutic Peptides from PEGylated Prodrugs by Serum Proteases. *Angewandte Chemie International Edition 52*, 7597-7599.

(66)   Zhang, J., Yuan, Z.-F., Wang, Y., Chen, W.-H., Luo, G.-F., Cheng, S.-X., Zhuo, R.-X., and Zhang, X.-Z. (2013) Multifunctional Envelope-Type Mesoporous Silica Nanoparticles for Tumor-Triggered Targeting Drug Delivery. *Journal of the American Chemical Society 135*, 5068-5073.

(67)   Bontempo, D., and Maynard, H. D. (2005) Streptavidin as a Macroinitiator for Polymerization: In Situ Protein−Polymer Conjugate Formation. *Journal of the American Chemical Society 127*, 6508-6509.

(68)   Mueller, C., Capelle, M. A. H., Arvinte, T., Seyrek, E., and Borchard, G. (2011) Noncovalent pegylation by dansyl-poly(ethylene glycol)s as a new means against aggregation of salmon calcitonin. *Journal of Pharmaceutical Sciences 100*, 1648-1662.

(69)   Kapanidis, A. N., Ebright, Y. W., and Ebright, R. H. (2001) Site-Specific Incorporation of Fluorescent Probes into Protein: Hexahistidine-Tag-Mediated Fluorescent Labeling with (Ni2+:Nitrilotriacetic Acid)n−Fluorochrome Conjugates. *Journal of the American Chemical Society 123*, 12123-12125.

(70)   Huang, Z., Hwang, P., Watson, D. S., Cao, L., and Szoka, F. C. (2009) Tris-Nitrilotriacetic Acids of Subnanomolar Affinity Toward Hexahistidine Tagged Molecules. *Bioconjugate Chemistry 20*, 1667-1672.

(71) Mero, A., Ishino, T., Chaiken, I., Veronese, F., and Pasut, G. (2011) Multivalent and Flexible PEG-Nitrilotriacetic Acid Derivatives for Non-covalent Protein Pegylation. *Pharmaceutical Research 28*, 2412-2421.

(72) Kim, T. H., Swierczewska, M., Oh, Y., Kim, A., Jo, D. G., Park, J. H., Byun, Y., Sadegh-Nasseri, S., Pomper, M. G., Lee, K. C., et al. (2013) Mix to Validate: A Facile, Reversible PEGylation for Fast Screening of Potential Therapeutic Proteins In Vivo. *Angewandte Chemie 125*, 7018-7022.

(73) Fuhrmann, G., Grotzky, A., Lukić, R., Matoori, S., Luciani, P., Yu, H., Zhang, B., Walde, P., Schlüter, A. D., Gauthier, M. A., et al. (2013) Sustained gastrointestinal activity of dendronized polymer–enzyme conjugates. *Nat Chem 5*, 582-589.

(74) Liu, M., Tirino, P., Radivojevic, M., Phillips, D. J., Gibson, M. I., Leroux, J.-C., and Gauthier, M. A. (2013) Molecular Sieving on the Surface of a Protein Provides Protection Without Loss of Activity. *Advanced Functional Materials 23*, 2007-2015.

(75) Liu, M., Johansen, P., Zabel, F., Leroux, J.-C., and Gauthier, M. A. (2014) Semi-permeable coatings fabricated from comb-polymers efficiently protect proteins in vivo. *Nat Commun 5*.

(76) Nguyen, T. H., Kim, S.-H., Decker, C. G., Wong, D. Y., Loo, J. A., and Maynard, H. D. (2013) A heparin-mimicking polymer conjugate stabilizes basic fibroblast growth factor. *Nat Chem 5*, 221-227.

(77) Keefe, A. J., and Jiang, S. (2012) Poly(zwitterionic)protein conjugates offer increased stability without sacrificing binding affinity or bioactivity. *Nat Chem 4*, 59-63.

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