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A guide to maximizing the therapeutic potential of protein-polymer conjugates by rational design

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Abstract

Proteins are an important class of therapeutics that have advantages including high target specificity, but challenges to their use include rapid clearance and low physical stability. Conjugation of synthetic polymers is an effective approach to address the drawbacks and enhance other properties such as solubility. In this review, we present various considerations in synthesizing protein-polymer conjugates for therapeutic applications with a focus on the choice of polymer, protein, and conjugation method, as well as characterization and evaluation of the resulting conjugate in order to maximize the therapeutic potential of the protein drug.

1. Introduction

Ever since the Food and Drug Administration (FDA) approved the first human recombinant protein drug, insulin, in 1982, many proteins have been developed as therapeutics. Proteins are often more specific than small molecule drugs and have fewer side effects from off-target activity.[1,2] Also, proteins can be used to target interactions (*e.g.*, protein-protein interactions) that may not be easily targeted by small molecule drugs. From a business perspective, protein therapeutics are more likely to receive FDA approval than small molecules.[1,2] Reflecting on these advantages, protein drugs accounted for 31% (62 drugs) of the top 200 drugs by sales in 2016, increasing almost 6-fold from 2006.[3] Given this rapid growth, proteins are expected to play an even more important role as therapeutics in the future.

However, proteins are quickly cleared or inactivated in the body through metabolism, excretion, and other pathways.[4] Most are also highly unstable and their precisely defined three-dimensional structure can be easily disrupted by heat, freezing, purification, and many other conditions found during manufacturing, storage, and transport. These limitations require modification of proteins and/or addition of excipients to enable clinical use.

One of the approaches to enhance protein properties such as pharmacokinetics is to replace amino acids or to create fusion proteins by using recombinant DNA technology.[1] Another approach is the conjugation of synthetic polymers. The most common example is

Conflicts of interest

There are no conflicts to declare.

poly(ethylene glycol) (PEG), which has shown to be effective in increasing circulation half-life of proteins and there are fourteen FDA-approved PEG-protein conjugates in the market.[5] In addition to circulation half-life, synthetic polymer conjugation can improve storage stability,[6,7] control ligand binding,[8] and protect the protein in the gastrointestinal tract.[9]

Despite these advantages, covalently linking a synthetic macromolecule with a biomacromolecule is not always straightforward. Selection of the protein and polymer combination that yields the maximal benefit requires consideration of multiple factors such as the intended disease and protein property to be enhanced. The design becomes even more complicated due to the conjugation chemistry, which requires mild reaction conditions to prevent the protein from denaturing and selective chemistries to inhibit unwanted side reactions with non-target amino acids. The conjugation chemistry must also be highly efficient, since proteins and polymers have significantly lower reaction rates than small molecules. Purification, analysis, and evaluation can also be more difficult for conjugates than for unmodified proteins.

This review serves as a guide to developing protein-polymer conjugates with a focus on therapeutic applications. It is intended for researchers who are new to the field of protein-polymer conjugates to become accustomed to the available techniques, decision processes, and considerations in conjugate synthesis. It should also serve as a useful reference for experts in the field when planning a new project involving protein-polymer conjugates. The review is organized into four parts: selection of the polymer, selection of the protein target, choice of the conjugation chemistry, and *in vitro* and *in vivo* characterization of the conjugate. Although non-covalent protein-polymer conjugates and polymeric nanoparticles are also promising methods for protein delivery, this review will focus on covalently-linked, linear protein-polymer conjugates.

2. Selection of the polymer to be conjugated

There are two possible scenarios at the outset of designing a protein-polymer conjugate. In the first, the research group has a protein that requires polymer conjugation to address the requirements for a particular application. In such an instance, this section will provide a starting point to selecting the appropriate polymer for conjugation. Alternatively, the research group may have developed a novel polymer for improving certain characteristics of protein drugs, and is searching for a model protein to conduct a proof-of-concept study. Section 3 provides considerations in selecting the protein and a list of potential therapeutic protein targets. In both cases, the types of polymers available for conjugation may be limited by the conjugation method, which in turn is dictated by the identity of the protein and the conjugation handles that are available. Therefore, the design of a protein-polymer conjugate involves a dynamic assessment of all three components (the protein, the polymer, and the conjugation technique) rather than a simple sequential process.

2.1 Identity of the polymer

2.1.1 PEG and PEG analogues.—Table 1 shows a selection of polymers that have been used in protein-polymer conjugates. The most widely used polymer is PEG, which increases the hydrodynamic radius of the conjugated protein and also helps immunogenic

proteins to evade the immune system through steric shielding.[5] PEG is commercially available with various functional groups, is biocompatible, and PEG conjugates with therapeutic proteins are FDA approved.[5] The demonstration of safety in humans and extensive data on this polymer is beneficial to the development of PEG conjugates as therapeutics.

However, some humans have been shown to develop anti-PEG antibodies, and as a result PEGylated proteins can be more rapidly cleared *in vivo* for these patients.[5] For example, 40% of patients treated with PEGylated uricase developed an anti-PEG antibody, and the antibody level was strongly correlated with the loss of the drug efficacy that occurred around 6 weeks after the start of the treatment.[10] For applications that require repeated administration such as in a replacement therapy (see Section 3), PEG-brush polymers may provide the same enhancement in circulation half-life as PEG but without inducing an anti-PEG antibody response. Poly(poly(ethylene glycol methyl ether methacrylate)) (p(PEGMA)), a methacrylate polymer with oligo(ethylene glycol) side-chains, has been used in various protein-polymer conjugates.[7,11–13] The brush architecture was hypothesized to prevent the immune system from recognizing the PEG side chains to generate anti-PEG antibodies, provided the oligo(ethylene glycol) side chains were short.[13] A PEG-functionalized norbornene polymer synthesized by ring-opening metathesis polymerization (ROMP) has also been used in conjugates to reduce the immunogenicity of viral capsid particles.[14]

Poly(*N*-(2-hydroxypropyl)methacrylamide) (p(HPMA)) is another water soluble and biocompatible polymer that can be used to increase *in vivo* half-life similar to PEG without the associated immunogenicity. It was originally developed by Kopecek and co-workers as a small-molecule drug carrier, and it has since been employed in several protein-polymer conjugates.[15]

2.1.2 Stimuli-responsive polymers.—In addition to improved pharmacokinetics, polymer conjugation can impart new properties to the protein. Some polyacrylamides such as poly(*N*-isopropylacrylamide) (p(NIPAAm)) possess a lower critical solution temperature (LCST) such that the polymer precipitates as the temperature is raised. They have been conjugated to streptavidin to modulate ligand binding with temperature[8] and to insulin to control the particle size.[16] Less explored are the conjugates containing pH-responsive ionic polymers such as poly(acrylic acid)[9] and poly((*N,N*-dimethylamino)ethyl methacrylate) (p(DMAEMA)).[17] Use of stimuli-responsive polymers in conjugates may enable interesting applications such as hyperthermia-mediated cancer therapy, but the potential toxicity especially for cationic polymers should be taken into account.[18]

2.1.3 Biomimetic polymers.—Polymers inspired by molecules found in nature have shown to be effective in imbuing the desirable properties of the natural molecule along with the advantages of synthetic polymers. Our group has been inspired by the natural disaccharide trehalose, which stabilizes various biomacromolecules and cells in nature to extreme conditions. We found that synthetic polymers containing trehalose as a side chain have superior stabilizing ability than the trehalose itself likely due to the multivalency and

higher local concentration.[6,19] The polymer as a simple excipient, conjugate, or hydrogel stabilizes proteins,[5,19] and covalent conjugation also improves protein *in vivo* half-life.[6]

Heparin-mimicking polymers are another example of biomimetic polymers. Fibroblast growth factor 2 (FGF2) is a protein with potential for therapeutic wound healing, but it is highly unstable. It is stabilized in nature by heparin, but heparin is heterogeneous and prone to contamination.[7] We employed poly(styrene sulfonate) and poly(vinyl sulfonate) as synthetic heparin mimics, with styrene sulfonate stabilizing FGF2 and vinyl sulfonate helping FGF2 bind to its receptor. The block copolymer of both monomers conjugated to FGF2 increased protein stability and also produced a superagonist compared to the native protein. These examples highlight the versatility of synthetic polymer conjugates since it is easy to combine multiple properties by making a copolymer of respective monomers.

2.1.4 Degradable polymers.—The polymers described above are not degradable and could accumulate in the body.[5] There is currently a strong drive to develop degradable polymers for biomedical usage. Cyclic ketene acetals (CKA) are monomers that undergo radical ring opening to generate hydrolytically degradable backbone ester bonds. CKAs can be copolymerized with vinyl monomers to yield degradable polymers. Our group has conjugated a copolymer of 5,6-benzo-2-methylene-1,3-dioxepane (BMDO), a commonly used CKA, and PEGMA to lysozyme whose amines have been thiolated *via* disulfide exchange.[20] The resulting conjugate was active and the polymer was degradable under a basic condition (5% KOH), which suggests that the polymer could be degraded by ester cleavage *in vivo.*

Other polymers used in conjugates include natural polymer analogues such as polypeptides and hydroxyethyl starch (HES). Various proteins have been conjugated to synthetic polypeptides,[9] and although these polypeptides are hydrolytically stable they may be degraded *in vivo* by proteases. Similarly, HES is degraded by a-amylase in the plasma and its conjugates have been extensively investigated for therapeutic uses.[5]

2.1.5 Other polymers with potential to be used in conjugates.—Although some polymers have yet to be used in conjugates, they have great potential for therapeutic uses (Table 2). Degradable polymers can be synthesized using CKAs, but CKAs copolymerize rather poorly with most vinyl monomers and the resulting polymers have large dispersity.[20] To address these concerns, we have synthesized allyl substituted caprolactones that can be installed with sugars, zwitterion, or oligoPEG on the side chain.[19] The polymers had low molecular weight dispersity (most had D = 1.1–1.2), and both the polymers and the degradation products were not cytotoxic. Caprolactones modified with trehalose and zwitterion were the most effective in stabilizing proteins as excipients, and these show promise to be used in protein-polymer conjugates.

ROMP monomers have been used to create densely functionalized brush polymers equipped with cytotoxic drugs and imaging agents.[21] Although these polymers have been used to make polymeric nanoparticles, they may potentially be employed for protein-polymer conjugates for theranostics and other applications that require multifunctional materials.

2.2 Toxicity of the polymer

Unless the polymer has been previously tested for toxicity, *in vitro* and *in vivo* toxicological testing is necessary. Such evaluation should be conducted as early as possible, as safety concerns that arise further along the development pipeline can be very costly. For example, toxicity of HES was extensively tested yet serious side effects were clinically observed during the use of HES alone as plasma volume expander, thus raising concern for their use in protein-polymer conjugates despite significant investment that had been made.[5]

2.3 Molecular weight of the polymer

A major motivation for polymer conjugation to proteins is to increase its circulation half-life. Since renal filtration is a major clearance pathway especially for small proteins, larger polymers will generally increase the half-life.[4] However, larger polymers will be prone to accumulation[4] as renal filtration is around 30 to 50 kDa, depending on the polymer charge and architecture. Therefore, one should choose the minimum polymer size that will increase the circulation half-life of the protein and/or desired properties to a level required for the intended treatment.

More often than not, the pharmacokinetic data for the intended polymer is not available and comparison needs to be made to a different polymer. Data on PEGylated proteins are readily available, and thus new polymers are frequently compared to PEG based on molecular weight or the degree of polymerization (DP). However, the hydrodynamic radius of a polymer is the most relevant value since it is more likely to correlate to the distribution of the polymer in the body after injection (or pharmacokinetics). Indeed, two polymers with the same molecular weight or DP may have very different hydrodynamic radii. For instance, 10 kDa norbornene with PEG side chains occupies approximately one- third hydrodynamic volume when compared to 10 kDa PEG,[14] which is expected since brush and branched polymers have smaller radii than similar linear polymers when they have the identical molecular weight.[22]

Another consequence of polymer size is its effect on bioactivity. Larger polymers often yield conjugates with lower bioactivity, most likely by non-specific steric hindrance. For example, insulin PEGylated at PheBl had *in vivo* bioactivity that decreased with PEG size (98% for 600 Da, 83% for 2000 Da, and 71% for 5000 Da) even when it was conjugated site selectively.[23] This is another reason that the smallest possible polymer that will achieve the desired effect should be used.

2.4 Other considerations

Monomers and polymers that are commercially available or easy to synthesize will greatly facilitate the preparation of materials for biological evaluation. For example, many functionalized PEGs are available and can be readily conjugated to various proteins. When designing new polymers, the synthetic route should be simple and high yielding. For example, in our group, the trehalose monomer (Section 2.1.3) was synthesized in two steps avoiding protecting groups,[6] and the degradable poly(caprolactones) with various side chains were synthesized in a modular manner using the efficient thiol-ene chemistry.[19]

Polymer architecture may have a profound effect on the conjugation yield and properties of the resulting conjugate. Linear, brush, or branched polymers can be used in conjugates. In general, linear polymers are easier to access synthetically, but brush polymers can have distinct advantages. For example, linear PEGs are known to induce long-term immunogenicity, but Chilkoti, Matyjaszewski, and co-workers observed that brush PEG with methacrylate backbones alleviated the immune response for the duration of the study.[13] However, brush and branch polymers have more steric load and tend to have lower conjugation efficiencies. Therefore, one should be aware that when adopting conjugation methods from the literature, the conjugation yields may significantly vary depending on the polymer structure, architecture, and the protein *(e.g.,* if the intended reactive site is buried vs. surface exposed).

In addition, polymer solubility may not be sufficient for the intended conjugation method if the chemistry is not highly efficient and requires very high polymer equivalents. For example, conjugation is typically conducted at the protein concentration range of 1–5 mg mL^{-1}. Since the polymer is often used in excess (from 10 to 200 molar equivalents), this may correspond to polymer concentrations of 10–1000 mg mL^{-1}. General solubility limit of polymers discussed above are around 100–500 mg mL^{-1}, depending on the polymer type and the molecular weight. Diluting the reaction mixture may not always work, since conjugation is a bimolecular reaction and the kinetics is highly dependent on the concentration. Moreover, high polymer concentration may destabilize some proteins. For these reasons, it is advisable to choose the most efficient conjugation chemistry that is available for the given protein and polymer pair, and conduct conjugation at the lowest polymer equivalents possible.

2.5 Overview of polymer selection

Fig. 1 shows a flowchart that summarizes the polymer selection process. It should be again emphasized that there are many other factors that go into the selection and the choice of the protein and the conjugation chemistry may limit the types of polymers that can be used. Therefore, the flowchart should serve as an initial guide only, particularly for new and untested polymers.

First, the molecular weight of the polymer should be determined. If 30 kDa or larger polymer is required for the desired application, a degradable polymer will be necessary to minimize accumulation in the body (see Section 2.3); however, smaller polymers may also benefit from degradability and faster clearance from the body. Next, it should be determined if only the half-life extension is desired for the conjugate. In such a case, linear PEGs can be used for many applications; a wide variety of monofunctional PEGs are commercially available, and this strategy has proven effective for many FDA-approved conjugates.[5] It should be noted that although rare, some patients may have pre-formed anti-PEG antibodies and exhibit immediate allergic reaction to PEG-conjugate even when used for the first time.[24] For chronic use, polymers with reduced immunogenicity should be considered since upon repeated injections over a patient's lifetime, the patient can form antibodies against PEG.[5] Other polymers can be chosen for imbuing the conjugate with desired attributes. For

example, trehalose[6] and zwitterionic polymers[19] improve storage stability, and stimuli responsive polymers may be useful for environment-responsive protein drug delivery.

3. Selection of the protein target

In many cases, the research group will have a protein target in mind, and only need a reference for selecting the polymer (Section 2) or conjugation method (Section 4). In other instances, especially for researchers developing new polymers and bioconjugation methods, a selection of an appropriate protein target is important to demonstrate the utility of the polymer or the conjugation method. The first step is to identify the rationale for attachment of synthetic polymer. Many proteins benefit from increased serum half-life. Non-human proteins may also be immunogenic, and polymer conjugation can help reduce immune recognition.[5] Protein drugs also have varying stability that may not be addressed by drug formulation alone. Moreover, most protein drugs require refrigeration and cannot be used in areas without the cold chain.[1] Therefore, increasing the storage stability is a strong motivation for polymer conjugation. In addition, a researcher may want to impart a particular property such as alteration of charge, increase in solubility, or responsiveness to a particular stimulus.

Another factor is that the benefits from polymer conjugation have to outweigh the significant increase in the cost of the drug. A survey of protein drugs on the market shows that for many PEGylated proteins the benefits of increased pharmacokinetics justify the cost of the conjugate. Fragment crystallizable (Fc) domain fusion can also increase pharmacokinetics,[25] but a polymer that can provide additional benefits such as enhanced storage stability may make polymer conjugation an attractive alternative to Fc domain-fusion protein drugs, as well as eliminating the need to form a new protein construct.

Projects that demonstrate a new concept and/or polymer frequently use bovine serum albumin (BSA) and lysozyme. BSA has only one free cysteine residue (Cys34), simplifying the conjugate synthesis and characterization. Lysozyme is also commonly employed due to its relatively high stability and easy activity assay. However, if the ultimate objective is to synthesize protein-polymer conjugates for therapeutic application, it is desirable to use a clinically relevant protein. For example, insulin is a small protein (5.8 kDa) that can be easily characterized *in vitro* and *in vivo,* reactivity of its functional groups are well-studied (for amines, GlyAl > LysB29 ≫ PheBl at neutral pH), and the effect of conjugation at different sites has been reported.[6,23,26] For these reasons, it is frequently used to test new polymers and conjugation methodologies.[6,16,27] Using approved protein drugs for conjugation is advantageous since their therapeutic benefits are proven and they are usually well characterized *(e.g.,* pharmacokinetics, mechanism of action). As such, the data available through pre-clinical reports, clinical trials, and patents facilitate the design of conjugation strategy and *in vitro/in vivo* experiments. Table 3 shows the 50 protein drugs from the top 200 sales drug list (excluding 12 duplicate drugs sold by different companies and peptide drugs with less than 50 amino acids). Several notable features are that about 40% are antibodies, in accordance with significant interest in antibody therapy from the pharmaceutical companies, in part due to the success of cancer immunotherapeutics. Another notable class is the replacement therapy drugs that replace defective endogenous

hormones/enzymes in patients with genetic disease with exogenous proteins. Insulin and its analogues are the most widely used protein drugs in this category due to the prevalence of diabetes, while coagulation factors and other enzymes are used to treat rare diseases caused by defective endogenous proteins. There are also 4 fusion proteins with Fc domain, which improves protein half-life through Fc receptor-mediated recycling.[25]

Also apparent in Table 3 is the high abundance of lysines and very low abundance of free cysteines, which are the two amino acids most frequently used as conjugation handles. Conjugation methods will be discussed in Section 4, but at this stage it should be determined whether non-selective conjugation to lysine is sufficient, or site-selective conjugation is necessary and if the protein requires amino acid substitution or unnatural amino acid incorporation. The native protein can be used in the former case, but in the latter, protein engineering may require significant time and resources.

Although FDA-approved proteins are usually chosen for protein-polymer conjugates, the resulting conjugate will be considered a new molecular entity (NME) and subject to extensive safety evaluation for approval by regulation agencies. As mentioned above, the motivation for polymer conjugation to a protein, whether approved or not, must be strong in order for the industry to become interested and invest significant time and resource to bring the conjugate to the clinic, and the conjugate should demonstrate significantly better efficacy and/or properties compared to the existing treatment in order to outweigh the risk of failure. For these reasons, at this stage it is advisable for chemistry-based research groups to collaborate or at least consult with biologists or clinicians who understand mechanism of action, possible side effects, and/or clinical design requirements that may not be obvious to chemists.

4. Choosing the right conjugation chemistry

In general, polymer conjugation to a protein faces significantly higher steric and entropic barrier than small molecule coupling reactions. Therefore, conjugation methods commonly used are highly efficient and use large excess of the polymer to maximize the conjugate yield. The choice of the conjugation chemistry also depends on the requirements for site selectivity, availability of target residues on the protein, and the compatibility of the protein-reactive functional group with the polymerization technique. Additionally, the choice of grafting-to and grafting-from approach may dictate which conjugation chemistry would be the most effective for synthesizing the desired conjugate.

4.1 Grafting-to and grafting-from approaches

Traditionally, polymers were directly conjugated to proteins in what is now referred to as the grafting-to approach. Although most polymers are compatible with this approach, it tends to have low conjugation efficiency especially for polymers that are sterically demanding. It may also be very challenging to purify the conjugate from the mixture that contains three types of macromolecules (unreacted protein, excess free polymer, and conjugate) since the entities could be similar in size. Additionally, mass spectrometry characterization of the conjugate to determine the conjugation site may be difficult for polymers that do not readily ionize. For example, our group could not directly analyze an insulin-trehalose polymer

conjugate by mass spectrometry, and it was necessary to cleave the polymer by treatment with a strong acid.[6] Fragments containing the short residual linker were detected, thereby determining the conjugation site.

To address these problems, our group developed the grafting-from technique by modifying the protein with an initiator followed by polymerization off of the protein macroinitiator. In our original report in 2005, we used biotin modified with an atom transfer radical polymerization (ATRP) initiator that binds to streptavidin to generate a protein macroinitiator, which was used for polymerization of NIPAAm at ambient temperature.[11] Since then, the approach has been adopted by various research groups and routinely used for synthesis of protein-polymer conjugates.[5,12,13,16,26,30]

The grafting-from approach requires a very mild polymerization condition. Therefore' it can be used with polymers synthesized by ROMP or radical polymerization such as ATRP and reversible addition-fragmentation chain-transfer (RAFT) polymerization.[5] As such' the grafting-from approach for vinyl polymers is typically used with (meth)acrylate- and (meth)-acrylamide-type monomers' since the monomer is sufficiently reactive for polymerization near ambient temperature.

4.2 Reactive group on the protein

Fig. 2 provides an overview of the commonly targeted protein conjugation handles. Proteins or enzymes with small substrates usually retain activity better than proteins that bind to other proteins *(e.g.'* hormones and cytokines) or have large substrates *(e.g.* polysaccharides)' and non-specific modification at lysines may be first attempted (Section 4.2.1).

For other cases' site-selective conjugation is beneficial or required to achieve sufficient bioactivity. In general' the conjugation site should be away from the active site or binding motif in order to maximally preserve protein activity.[5,7,23] However' the protein may still have significantly reduced bioactivity even when the conjugation site is away from the active/binding site' and the effect of conjugation on the activity may be hard to predict (refer to the following report[31] for current understanding). Another attractive strategy is traceless conjugation using responsive and self-immolative linkers' which release unmodified proteins upon exposure to a triggering stimulus.[32]

Free cysteines are among the rarest residues in proteins[33] and are the first conjugation handles that should be targeted for site-selective conjugation (Section 4.2.2). However, cysteine residues are usually involved in disulfides bonds or are not solvent accessible, and cysteines are often engineered into proteins *via* recombinant technique. Native disulfide bonds may also be reduced to make cysteines available for conjugation (Section 4.2.3).

Terminus modification is also commonly used for site-selective modification. For monomeric proteins, N- and C-termini are unique and guarantee true site-specific conjugation. N-Terminus modification (Section 4.2.4) is better established and more techniques are available compared to C-terminus modification (Section 4.2.5).

Tyrosine has also been used for polymer conjugation and is briefly covered (Section 4.2.6). Other methods such as unnatural amino acid incorporation combined with subsequent

polymer conjugation using bioorthogonal chemistry such as strain-promoted azide-alkyne cycloaddition (SPAAC) are also effective.[34] However, unnatural amino acid incorporation requires some expertise, and thus this review will focus on methods that can be readily adapted by research groups not adept with that technique.

4.2.1 Lysine.—Along with serine, lysine is the most abundant amino acid on protein surface,[33] and is usually the first residue to be attempted for non-selective conjugations. Neutral to basic pH is required for the ε-amino group to be sufficiently nucleophilic (pK_a ~ 10.5),[35] which may limit the usefulness for some proteins that are unstable at high pH.

Lysine is not often used for site-selective conjugation, but recent efforts have focused on achieving site selectivity by understanding lysine reactivity. Russell, Matyjaszewski, and co-workers studied conjugation of ATRP initiators to lysozyme and chymotrypsin, and concluded that a lysine must be sufficiently exposed; in addition, the microenvironment such as the presence of adjacent lysine and local charge affects the reactivity.[36] Bernardes, Jiménez-Osés, and co-workers used a sulfonyl acrylate reagent to selectively modify a single lysine in five different proteins (human serum albumin, synaptotagmin-I C2A domain, lysozyme, annexin-V, and trastuzumab).[37] High reactivity of the reagent allowed the most acidic lysine to be selectively modified at pH 8, and the conjugation reached complete conversion using equimolar amounts of the reagent. Although the conjugate is labeled with a small-molecule acrylic group that would require a second conjugation with a polymer, such site-selective methods seem promising.

4.2.2 Cysteine.—Conjugation sites for cysteines are better defined than lysines due to their low abundance. Moreover, their high nucleophilicity allows easy modification. However, free cysteines are rare and often buried in hydrophobic pockets such that they are unavailable. If protein engineering tools are readily available, amino acid substitution to install a cysteine at a desired site is an effective strategy for site-selective conjugation.[7,38] However, there is no *a priori* guarantee that the modified protein will properly fold and will not form the undesired disulfide dimer. Alternatively, natural disulfide bonds can be reduced to obtain free cysteines (Section 4.2.3).

Other modern methods for cysteine modification have been covered in other reviews,[34] and thus only a few examples will be mentioned here. Pentelute and co-workers identified a tetrapeptide sequence (referred to as the π-clamp) that reacts with perfluoroaryl compounds.[39] The reaction is selective for the p-clamp cysteine even in the presence of other cysteines, and its rate (0.73 M^{-1} s^{-1}) is on par with some of the fast strain-promoted azide-alkyne cycloadditions (0.96 M^{-1} s^{-1} for biarylazacyclooctynone or BARAC[40]). Buchwald, Pentelute, and co-workers developed palladium reagents for cysteine arylation in proteins, which occurred quantitatively within a few minutes under dilute and mild conditions.[41] Inspired by this approach, Spokoyny and our group recently used gold(III) reagents for cysteine arylation specific to aryl iodide bonds.[42]

4.2.3 Disulfide bond.—Disulfide bonds are present in most proteins (Table 3), and they can be useful handles for residue-specific conjugation. For large proteins that contain many disulfide bonds, partially reducing them may allow conjugation to the reduced cysteines

without disrupting the tertiary and quaternary structures. Although they are small-molecule conjugates, antibody-drug conjugates such as brentuximab vedotin (Adcetris®, Seattle Genetics) are synthesized through this route by partial disulfide reduction and conjugation with a maleimide-functionalized anti-cancer drug.[43] The use of this method for the production of clinically used therapeutics serves as a testimony to the robustness and efficiency of the chemistry, provided the reduction is reproducible and scalable.

For smaller proteins with only a few disulfide bonds, the above approach may lead to destabilization of the tertiary structure. Several methods have been developed to address this by replacing the native disulfide bonds with synthetic disulfide rebridging linkers to conjugate small molecules and polymers at the disulfide sites while maintaining the disulfide bridge (Section 4.3.6).[44]

4.2.4 N-Terminus.—The N-terminal amine has a significantly lower pK_a (6–8) than lysines (~10.5),[35] and the pH-dependent difference in reactivity is often leveraged for selective N-terminus modification. An example is pegfilgrastim (Neulasta®), which is granulocyte colony-stimulating factor (G-CSF) conjugated to a single PEG chain at the N-terminus. Reductive amination at pH 5 yields mostly N-terminus PEG conjugate at a high conversion (92%).[45] This strategy is most effective for relatively small proteins with only a few lysine residues; larger proteins such as antibodies contain large excess of lysine residues that would compete with the N-terminus amino group and result in significant off-target conjugations.

Chemical reagents have also been developed to selectively modify the N-terminus (Section 4.3.7). Enzymatic labeling of the N-terminus can also be achieved using sortase A (SrtA) from *Staphylococcus aureus.* The molecule to be conjugated is modified with the peptide tag LPXTG (Leu-Pro-any-Thr-Gly), and SrtA ligates the tag (with removal of Gly) to the N-terminus.[35] Several challenges of this approach include the need to modify the polymer or initiator with the tag and the difficulty in purifying the conjugate from SrtA in the conjugation mixture.

4.2.5 C-Terminus.—The C-terminal carboxylate group is difficult to chemically distinguish from the side-chain carboxylates (Asp/Glu), and thus specific amino acid sequences are installed at the C-terminus for conjugation by native chemical ligation or enzymatic ligation. For enzymatic ligation, SrtA can be used on proteins with LPXTG/A engineered at the C-terminus. Chilkoti and co-workers combined sortase-mediated conjugation with the grafting-from approach to install a peptide-tagged ATRP initiator at the C-terminus of green fluorescent protein (GFP), and PEGMA was polymerized from the macroinitiator.[30] Farnesyl transferase may also be used with proteins modified with CaaX (Cys-aliphatic-aliphatic-any) on the C-terminus and farnesyl pyrophosphate-tagged polymer or initiator.[46]

Enzymatic labeling is highly specific, but it requires protein engineering. Unlike N-terminal modification, an efficient C-terminus conjugation method for an unmodified protein is lacking. Recently, MacMillan and co-workers used photoredox catalysis to modify the C-terminal carboxylate, which has a lower oxidation potential ($E^{red}_{1/2}$ ~ 0.95 V) compared to

other carboxylates ($E^{red}_{1/2}$ ~ 1.25 V).[27] Although common metal photocatalysts were ineffective, organic flavins catalyzed the conjugation of small molecules and an oligo(ethylene glycol) linker to the C-termini of peptides and insulin. While significant challenges remain to be overcome *(e.g.,* moderate conjugation efficiency at 31–52% and the sensitivity to O_2), the mild reaction conditions and unique selectivity of photoredox chemistry warrants further investigation.

4.2.6 Tyrosine.—Although less utilized than the above conjugation handles, tyrosine is an amino acid with a moderate surface abundance (4.8%)[33] and several conjugation methods have been developed (Section 4.3.8). Compared with other site-selective conjugation methods, they tend to have some cross-reactivity with nucleophilic residues such as histidine[47] and have lower efficiency.

4.3 Reactive group on the polymer

Table 4 shows commonly used protein-reactive functional groups for conjugation, which are electrophilic reagents that react with the nucleophilic lysine, cysteine, or tyrosine residues. Typically these functional groups are placed at one end of the polymer chain. For simplicity, the discussion below will assume a grafting-to approach, since the only major difference for the grafting-from approach will be with regards to the selection of monomers that can be polymerized from the protein.

4.3.1 Activated esters and carbonates.—*N*-Hydroxysuccinimide (NHS)-modified polymers are widely used and many are commercially available. However, NHS hydrolyzes fast within few hours at neutral pH and may not be suited for all protein conjugations, especially when the lysine is not easily accessible. In such cases, pentafluorophenyl (PFP) esters may lead to a more efficient conjugation. De Geest and co-workers compared NHS and PFP ester end groups on poly(2-hydroxyethyl acrylate) for conjugation to BSA and ovalbumin, and found that the PFP-modified polymer has almost two-fold higher conjugation than the NHS-modified one.[48] For the grafting-to approach, the activated ester or carbonate end group may hydrolyze during purification of some polymers that require aqueous dialysis during purification.[6] In such a case, an alternative two-step modification may be necessary (see Section 4.3.3).

4.3.2 Aldehydes.—Aliphatic and aromatic aldehydes are used for reductive amination to lysines and unmodified N-termini. The reaction occurs in three steps: (1) nucleophilic attack on the aldehyde carbon by an amine, (2) dehydration to form an imine, and (3) reduction of the imine. The first step is slow at low pH while the second step is slow at high pH,[49] and thus the optimal pH is around 5, although conjugation can occur at a pH as high as 8 at least for aromatic aldehydes.[6] Reductive amination is commonly used for selective N-terminal conjugation at low pH.

Aliphatic aldehydes are prone to degradation processes such as condensation and oxidation during both synthesis and storage,[50] and are often protected as an acetal. Aromatic benzaldehydes are more stable and functional group tolerant, and can be left unprotected during polymerization and dialysis in water.[6] Benzaldehydes condense with amines slower

than aliphatic aldehydes, but the conjugation better stabilizes the imine intermediate.[49] Both aliphatic and aromatic aldehydes have been successfully used for conjugation of polymers to proteins.[6,45]

4.3.3 Amine-reactive thiolating reagents.—Although lysines are most often used for non-selective conjugation, conversion of lysines to thiols using reagents such as the Traut's reagent (2-iminothiolane) or *N*-succinimidyl-*S*-acetylthiopropionate (SATP)[20] may be helpful in some instances. If the polymer requires aqueous purification *(e.g.,* dialysis) prior to use, it is difficult to use activated esters and carbonates for direct conjugation and thus lysines may be converted to thiols so that cysteine conjugation chemistry can be used. Another motivation is that lysine conjugation may not have satisfactory yields due to steric hindrance from two macromolecules (protein and polymer) coming together. The grafting-from approach can be one solution as mentioned in Section 4.1. Alternatively, thiolation of lysines extends the conjugation handle further out from the surface to increase its accessibility, and subsequent Michaeltype reaction with maleimides or disulfide exchange with pyridyl disulfides has higher conjugation yields compared to using NHS esters.[48] Such two-step conjugation strategy can be used with even more efficient methods such as tetrazine ligation to achieve higher conjugation yields.[51]

4.3.4 Michael acceptors.—Maleimides are the most frequently used reagents for cysteine conjugation. They have very fast reaction kinetics (10^3–10^4 M^{-1} s^{-1} at pH 7.5),[41] and the conjugates are generally stable. Due to the efficiency of the Michael-type addition and the conjugate stability, maleimides are used in clinically approved antibody-drug conjugates.[43] An important consideration, however, is that the maleimide-thiol bond is reversible *in vivo.* For example, the anti-CD30 antibody-auristatin E conjugate cleaves *in vivo* with $t_{1/2}$ ~ 5 days.[52] More recently, Lyon and co-workers at Seattle Genetics designed a self-stabilizing maleimide with an adjacent amine group that spontaneously undergoes ring hydrolysis to yield a stable conjugate to overcome this issue.[52]

Vinyl sulfones are also frequently used Michael acceptors. They are slightly less reactive than maleimides, but they are hydrolytically more stable especially at higher pH and may be preferred over maleimides for some applications.

It should be also noted that free cysteines are very frequently oxidized and direct conjugation yield can be low. In some cases, the conjugation yield can be significantly increased by treating the protein with reducing agents such as tris-(2-carboxyethyl)phosphine (TCEP) or dithiothreitol (DTT) immediately before the conjugation.[38]

4.3.5 Disulfide exchange.—Another efficient cysteine conjugation is reaction with pyridyl disulfide (PDS)-modified polymers or initiators. The PDS end group is stable during radical polymerization.[7] PDS is very efficient and resulted in higher conjugation yield with ovalbumin and BSA compared to the analogous maleimide-functionalized polymer.[48] The protein and the polymer are then linked *via* a disulfide bond, which is relatively stable but can be cleaved under reducing conditions. This can be useful for characterization of the conjugate and for releasing the protein in the reducing environment inside the body. A factor

to consider for *in vivo* applications is that the sterics around the disulfide bond can be optimized to vary the time to disulfide cleavage during blood circulation if so desired.[53]

4.3.6 Disulfide rebridging.—As discussed in Section 4.2.3, disulfide rebridging linkers are better suited for disulfide conjugation in smaller proteins. Most approaches involve bis-alkylation of electrophiles such as bis-sulfones and disubstituted maleimides.[44] These reagents create a three-carbon (bis-sulfone) or a two-carbon (maleimide) bridge between two cysteines, thereby minimally perturbing the spacing of the disulfide bond.

Although these reagents are highly efficient, many are also reactive towards free cysteines.[44] Also, solubility may need to be considered for some reagents. For example, the bis-sulfone is the most established rebridging agent, but it is hydrophobic. Yet, attachment of a hydrophilic polymer chain can minimize this issue. Furthermore, the approach requires mild reducing conditions and this could be disruptive to the structures of more sensitive proteins.[44]

4.3.7 N-Terminal conjugating reagents.—Francis and co-workers have developed many N-terminus modification methods, and select examples are presented here (refer to the following review on N-terminus modification for a comprehensive picture[35]). Pyridoxal-5'-phosphate (PLP) was used to install a ketone group under mild conditions, which can subsequently be selectively conjugated with aminooxy-modified polymers *via* oxime linkage. They also developed a more facile one-step approach using 2-pyridinecarboxaldehyde (2PCA). The aldehyde (modified with a polymer chain or a small molecule) condenses with the N-terminus amino group to form an imine that spontaneously cyclizes with the adjacent amide group that is absent in the e-amino group of lysine. While selective, these methods require a large excess of the reagent and exhibit slow to moderate kinetics, and also work better for some amino acids over others (for example, methionine shows the best reactivity for the 2PCA conjugation).

4.3.8 Tyrosine conjugation.—Diazonium and Mannich-type coupling are some of the more frequently employed tyrosine conjugation methods. Diazonium salts were used by the Francis group and the Barbas group to install a ketone or a benzaldehyde group in tyrosines that was then conjugated with end-functionalized PEG.[34] Francis and co-workers have also developed a Mannich-type reaction using aniline and an aldehyde as a tool for tyrosine conjugation.[34] More recently, these two tyrosine conjugation methods were compared using BSA.[54] For diazonium coupling, anilines were converted to diazoniums in *situ,* and thus incomplete diazonium formation seems to have contributed to lower efficiency compared to the Mannich-type coupling. Although Mannich-type coupling was efficient for small aniline derivatives (67–100% of tyrosines modified), PEG-aniline derivative was attached to only 24% of tyrosines most likely due to increased steric hindrance.

Barbas and co-workers also demonstrated that 4-phenyl-1,2,4-triazole-3,5-dione (PTAD) rapidly reacts with tyrosines over a wide pH range (2 through 10).[34] Due to the highly electrophilic nature of PTAD, the reduced form is attached as an end group throughout synthetic manipulations and oxidized to PTAD immediately before use. PTAD quickly decomposes to isocyanate in water and reacts with lysines to a significant extent, but this

side reaction can be suppressed by using tris(hydroxymethyl)aminomethane (Tris), which is a commonly used buffer component.

4.4 Other considerations—While selectivity and efficiency are the main factors for selection of a conjugation technique, toxicity of the residual reagents and/or the remaining linkage could potentially be important. Transition-metal catalyzed conjugation methods are receiving attention,[34] but the residual metal removal and toxicity is a concern. Fortunately, there are ample methods to remove the metal such as metal adsorbent[26] and liquid chromatography,[13,41] and ways to accurately determine residual metal concentration such as inductively coupled plasma mass spectrometry (ICP-MS).[41] Other organic reagents and electrophilic conjugation partners *(e.g.*, formaldehyde) may possess toxicity and must be thoroughly removed from the conjugate. This may not be trivial if the electrophile is on the polymer. Toxicity should be also considered for eventual metabolism/degradation byproducts of the polymer as well as the conjugation linker, especially if they are newly developed and have not been evaluated for their safety.

It is also important to consider the compatibility of the conjugation chemistry with the synthesis of some polymers. For example, azide-alkyne cycloaddition has been used for conjugation of polymers to proteins,[34] but if the azide or alkyne polymer is to be synthesized by ROMP, the azide or alkyne must be added post-polymerization or protected, respectively. Therefore, one must ensure that all components of the conjugation handles will remain intact throughout the polymer/initiator synthesis and purification, and similarly the conjugation chemistries are compatible with the polymer functionalities and the polymer functional groups do not interfere with the conjugation reaction.

4.5 Overview of conjugation chemistry selection

Although many other factors need to be considered, Fig. 3 provides a general outline for selecting the conjugation chemistry. For the simplest case of non-specific conjugates, NHS and PFP-modified polymers or initiators can be used (Section 4.3.1). If the conjugation yield is too low even for PFP esters, then lysines can be thiolated (Section 4.3.3) for thiol-modification reactions that are highly efficient (Section 4.3.4). For the same reason, free cysteines should be first attempted for site-selective conjugation when available and away from the active/binding site, or they can be installed by protein engineering (Section 4.3.4). If protein engineering is not an option, N-terminal modification methods are the most well-established alternatives. Reductive amination may be used for proteins with only a few lysines (Section 4.3.2), or other N-terminal selective reagents can be used (Section 4.3.7). For proteins that do not have a free N-terminus nor cysteines, disulfide rebridging (Section 4.3.6) and tyrosine conjugation (Section 4.3.8) can be attempted.

5. Characterization of the protein-polymer conjugates

5.1 Conjugate purification

Purification of protein-polymer conjugates presents a unique challenge different from small molecule and polymer purification. For the grafting-to approach, the polymer with a protein-reactive end group is almost always used in excess and the conjugation yield is often less

than 100%. Therefore, the mixture will often contain three types of macromolecules - the protein, the polymer, and the conjugate. For very small and stable proteins, high-performance liquid chromatography (HPLC) may be used for the purification. However, use of organic solvent and the high pressure inside the column will denature the tertiary structure of most proteins. Similarly, size exclusion chromatography (SEC) will work for only a small subset of conjugates because it is best for separation of two materials with a size difference higher than two-fold, which is further complicated by the molecular weight dispersity inherent to synthetic polymers. Other chromatography methods such as ion exchange[45] and hydrophobic interaction[52] chromatography are often better options, and they are used for the production of protein drugs in the clinic. The grafting-from approach has distinct advantage in terms of purification, since the small molecule initiator can be easily purified from the modified protein.

5.2 Conjugate characterization

Common characterization methods for conjugates include gel electrophoresis, mass spectrometry, SEC, and dynamic light scattering (DLS). No one method is definitive, and multiple methods should be used for characterization.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separates proteins based on size, and the protein that is conjugated with a polymer will show a shift (and often smeared due to polymer dispersity). A native PAGE without the surfactant, in which the protein mobility will be dependent on both the size and the isoelectric point, may also provide useful information. The gels are then stained with a Coomassie dye, silver, or iodide (the latter of which stains PEG). Silver stain is more involved, but it only requires nanograms of materials as opposed to micrograms for Coomassie stain. Also, the stain specificity may be concentration dependent and appropriate controls (free protein and free polymer) should always be analyzed together with the conjugates. If an antibody for the protein is available, western blot only stains the protein and can be used to verify the conjugation with high specificity. It is possible, but likely very rare, that the polymer inhibits antibody binding.

Mass spectrometry is also an important characterization method that should be conducted whenever possible. It is especially useful for determining the conjugation site when coupled with protein digestion or tandem mass spectrometry techniques. The analysis can be hindered for polymers that inhibit ionization. In this case, the conjugate may be derivatized to enable ionization, or if the conjugation linker has a labile bond, the polymer may be cleaved from the protein.[6] Again, the grafting-from approach greatly facilitates the conjugation site analysis by mass spectrometry since the number and location of the small molecule initiators on the protein is usually easily detectable.

SEC and DLS also help to verify polymer modification by increased hydrodynamic radius of the conjugate compared to the free protein.[16,26,30] SEC is helpful for the grafting-from approach to measure the polymer dispersity and if required, to improve the control in polymerization by changing the conditions, *e.g.,* catalyst loading and ligand amounts.[16,26] For the grafting-from approach, the protein may be digested with a protease in order to characterize the polymer itself.[26]

5.3 Biological evaluation of the conjugate

After purification and characterization, the conjugate should be tested for its activity (and the polymer toxicity if it has not yet been determined). The *in vitro* activity assays for the protein can range from cell proliferation assays for growth factors[7,19] to enzyme-linked immunosorbent assay (ELISA) for antibodies. Structural integrity of the protein attached to a polymer can also be checked by circular dichroism (CD),[12] DLS (to check for potential aggregation), and ELISA. After polymer conjugation, the protein activity typically decreases to about 20 to 80%, although it can be as low as 7%.[47] As mentioned in Section 2.3, the activity often decreases with increasing molecular weight and is dependent on the conjugation site.

If the protein activity is not satisfactory, it can be improved by site-selective conjugation. For example, our group first synthesized an insulin-trehalose polymer conjugate by non-specific reductive amination to two of the three amine sites on insulin.[6] As intended, trehalose polymer conjugation significantly improved the circulation half-life compared to free insulin (Fig. 4a) and was statistically comparable to PEGylated insulin. Also, the free trehalose polymer added in excess (10 weight equivalents) helped insulin retain its activity after heating, and the conjugate had even higher retention of activity even though it only had one to two polymer chains attached (Fig. 4b). However, the conjugate required five-fold higher dose to achieve similar blood glucose decrease in mice compared to free insulin, likely due to the non-specific conjugation.[23] To enhance the conjugate activity, we used the grafting-from approach to preferentially attach an ATRP initiator at LysB29 by conjugation at high pH followed by purification of the macroinitiator and *in situ* polymerization to yield the conjugate (Fig. 4c).[26] Of the three possible conjugation sites (GlyAl, PheBl, and LysB29), LysB29 was chosen because GlyAl conjugate has significantly lower bio-activity than others and LysB29 is more reactive than PheBl.[23,26] This site-specific conjugate only required three-fold higher dose and thus significantly higher activity than the previous approach.

Toxicity and immunological evaluations of the conjugate are important and should be undertaken as early as possible in developing protein-polymer conjugates as therapeutic drugs. *In vitro* cytotoxicity tests should be conducted with multiple cell lines including normal human cell lines since compatibility with a single cell line does not guarantee safety. After positive *in vitro* results, *in vivo* acute toxicity experiments should be conducted at a dose that is at least 5 to 10 fold higher than the therapeutic dose, although higher concentrations up to the solubility limit would provide a larger safety window. Toxicity may potentially arise from residual metal catalysts or end-group functionalities (such as in RAFT), which can be addressed by metal removal and end-group removal. If the polymer or the linker itself is cytotoxic,[4,5] it must be switched with a different non-toxic polymer/end group or conjugation linker. Similarly, immune response and antibody generation against the polymer can reduce the effectiveness of the drug over repeated injection or cause safety concerns for chronic use and this should be investigated. As covered in Section 2.1.1, polymer architecture may possibly be modified to reduce the polymer immunogenicity. On the other hand, immunogenic proteins from a non-human source may be safely delivered as conjugates, when the polymer shields the protein from detection by the immune system.[5]

Finally, *in vivo* biodistribution of the conjugate can have significant consequences in terms of its *in vivo* bioactivity. For example, antibody-drug conjugates with higher drug loadings were cleared quicker *in vivo* likely due to the increased hydrophobicity, even though they fully retained the bioactivity *in vitro*.[43] Biodistribution study is often conducted by fluorescence imaging, but the optical modality is subject to artifacts from tissue heterogeneity and the results should be analyzed with caution.[55] Radiotracing imaging such as positron emission tomography (PET) is a more quantitative method for studying biodistribution. As an example, Li and co-workers used PET imaging to compare the biodistribution of dimeric antibody fragments (diabodies) modified with PEGs with different architecture and molecular weights.[38] They engineered a cysteine at the C-terminus of the diabody specific for the tumor-associated antigen 5T4. The diabody was reacted with a maleimide- functionalized linear and branched PEG ranging from 5 to 40 kDa, yielding conjugates with exactly two PEGs (Fig. 5a and b). Serum half-life of free diabody was only 40 min, while the half-life of the conjugate increased with PEG molecular weight (4 to 44 h). For PET, the unmodified and PEGylated diabodies were nonspecifically conjugated with the metal chelator desferrioxamine containing the amine-reactive isothiocyanate group (NCS-DFO), yielding conjugates with an average of 2.4 DFO. Interestingly, the linear 20 kDa PEG conjugate had larger hydrodynamic radius than the branched 20 kDa PEG conjugate, but faster kidney clearance and slightly lower tumor uptake by PET (Fig. 5c). Moreover, the 40 kDa branched PEG conjugate had longer half-life but slower tumor uptake than the 20 kDa branched PEG conjugate, demonstrating the importance of PEG size and branching on reducing renal clearance while allowing the conjugate to diffuse into the tumor tissue.

6. Conclusions

In this review, various aspects of protein-polymer conjugates were surveyed, the most commonly used polymers, proteins, and conjugation methods were discussed, and examples of *in vitro* and *in vivo* characterization were provided. Developments in controlled polymerization techniques and bioconjugation methods in the last few decades provide us with a diverse array of tools for synthesizing protein-polymer conjugates under the stringent requirements for manipulation of sensitive biomolecules. Perhaps the most important factor in a protein-polymer conjugate project is the strong motivation for polymer conjugation for the desired application, whether it be to increase serum half-life, improve storage stability, or reduce immunogenicity of the protein. Driven by this motivation, the researcher can dynamically evaluate the pros and cons of different components and approaches, which may require several iterations to optimize and improve the performance of the conjugate. With growing attention on protein therapeutics, protein-polymer conjugates will play an increasingly important role in overcoming the weaknesses of protein therapeutics (instability and rapid *in vivo* clearance) and augmenting their utility as an essential class of therapeutics. This will be especially true as researchers continue to improve protein bioactivity retention despite polymer modification by utilizing new conjugation chemistries, linkers, and smart design. Thus, the future is abound with exciting opportunities for scientists to improve on Nature’s highly evolved machinery towards advancing human health.

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Biography

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Jeong Hoon Ko (JK) is a postdoctoral researcher at the California Institute of Technology. He received his BSE degree in biomedical engineering and AB in chemistry from Duke University in 2013, and his PhD degree in chemistry from the University of California, Los Angeles in 2018 for work with Professor Heather Maynard. His doctoral research was focused on using glycol-polymers to stabilize therapeutically and industrially important proteins. He is currently researching with Professor Robert H. Grubbs to develop polymers for medical applications.

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Key learning points

1. Motivations for synthesizing protein-polymer conjugates
2. Selection of the polymer based on the desired enhancement
3. Selection of the protein based on its characteristics
4. Choice of the bioconjugation method
5. *In vitro* and *in vivo* methods for the characterization and evaluation of the conjugates

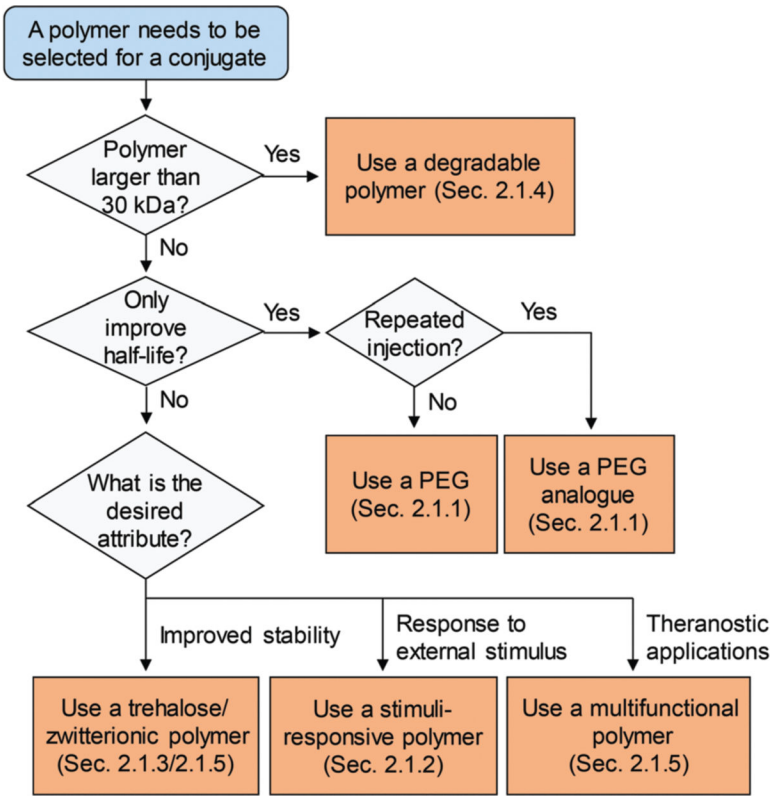

Fig. 1.
A summary of the polymer selection process.

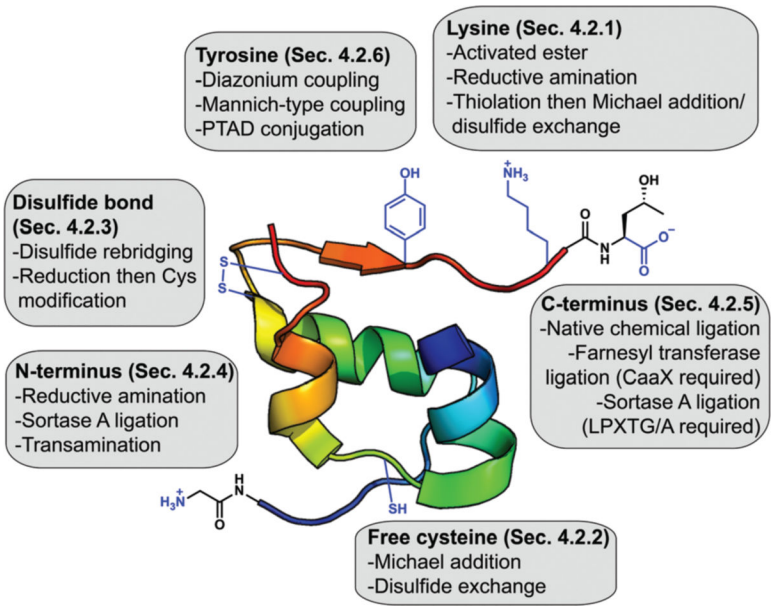

Fig. 2.

Commonly used conjugation chemistries for different protein residues (PDB ID: 4INS).

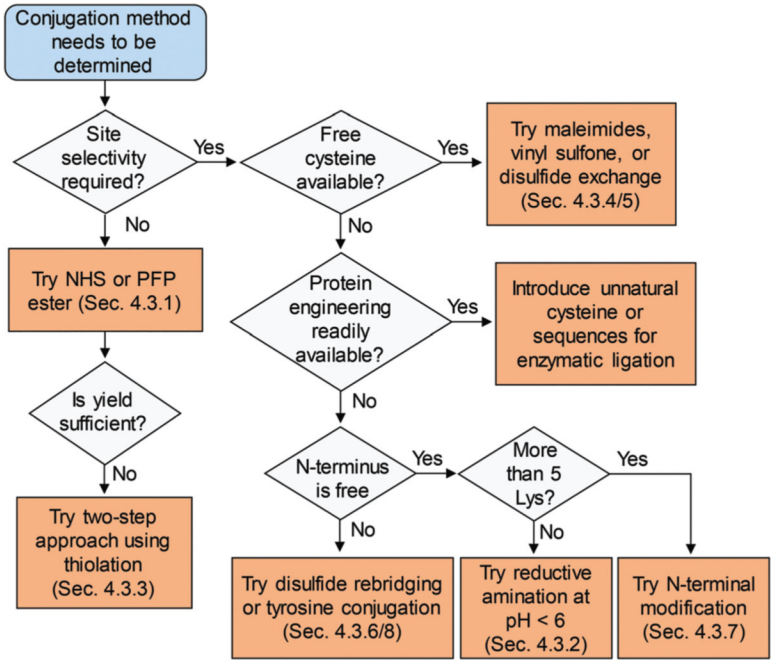

Fig. 3.

A summary of the conjugation chemistry selection process. Adapted with permission from ref. 46. Copyright 2011 Springer Nature.

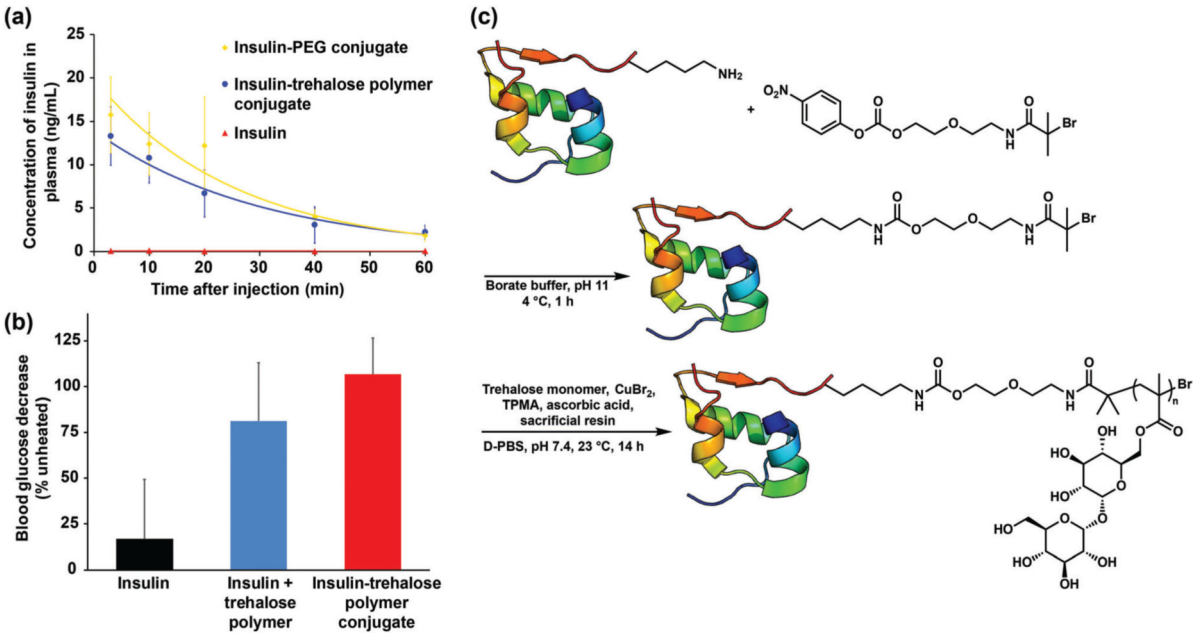

Fig. 4.

Insulin-trehalose polymer conjugate. The conjugate synthesized by non-selective route was used for (a) *in vivo* pharmacokinetics ($n = 5$) and (b) bioactivity in mice after heating (90 °C, 30 min) ($n = 5$). (c) Synthesis of LysB29 selective conjugate by the grafting-from approach. Adapted with permission from ref. 6 and 26. Copyright 2017–2018 American Chemical Society.

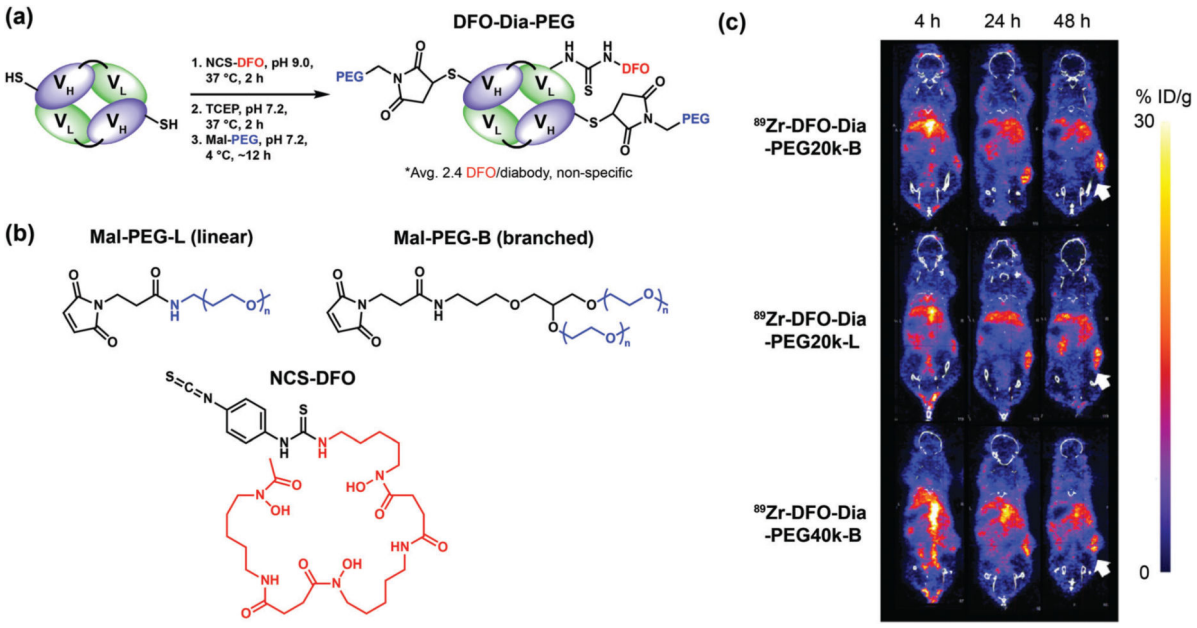

Fig. 5.

Imaging biodistribution of PEGylated diabody using PET. (a) Conjugation of PEG and metal chelator to diabody (Dia). (b) Structures of maleimide PEG, linear (Mal-PEG-L) and branched (Mal-PEG-B), and metal chelator used (isothiocyanate desferrioxamine, NCS-DFO). (c) PET-CT images obtained from a mouse injected with ^{89}Zr-DFO-Dia-PEG20k-B, ^{89}Zr-DFO-Dia-PEG20k-L, or ^{89}Zr-DFO-Dia-PEG40k-B, with a white arrow indicating the location of the tumor. Adapted with permission from ref. 38. Copyright 2018 Elsevier.

Table 1

Polymers that have been used in protein-polymer conjugates (structure, properties, and representative conjugates)

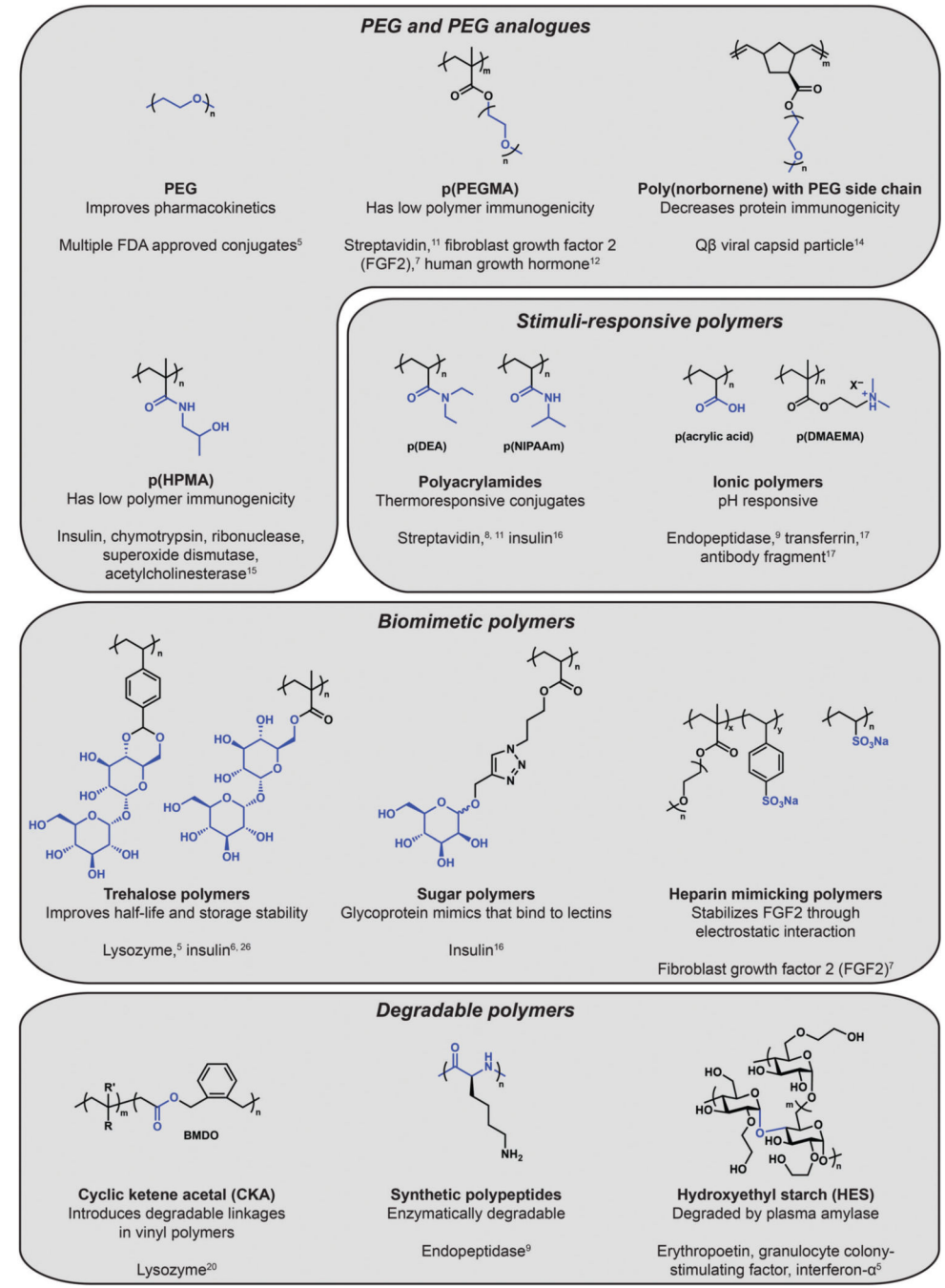

Table 2

Polymers with potential to be used in protein-polymer conjugates

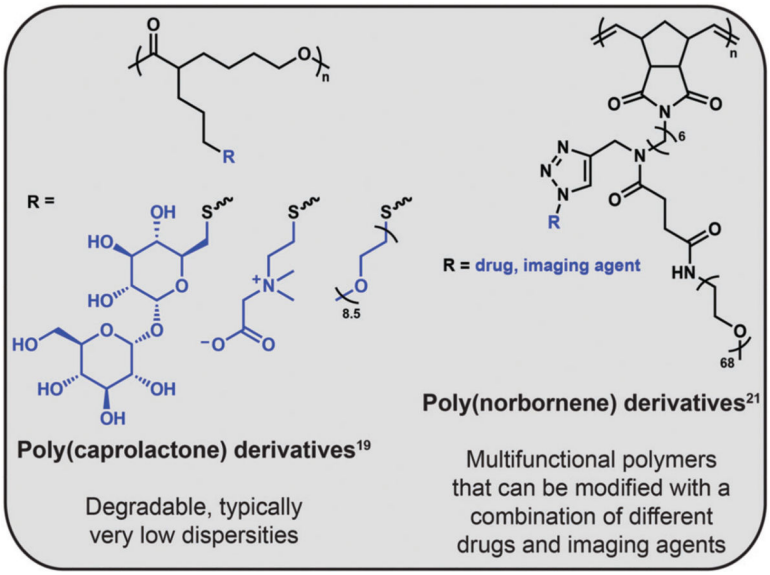

Table 3

Protein therapeutics from the top 200 drugs by retail sales in 2016[3,28,29]

Generic (marketed) name ***Class*** **[Sales (10^6 USD)][a]**	**Function; target disease**	**MW (kDa)**	**pI**	**# Lys[b]**	**#Cys (non-S-S)[c]**	**#S-S bonds**	**Route[d]**	**$t_{1/2}$**
Adalimumab (Humira)	TNF-α inhibitor; arthritis	148	7.94–9.14	88	0	16	S.C.	10–20 d
Antibody [16 078]								
Etanercept (Enbrel)	TNF-α inhibitor; arthritis	150	7.89	54	0	29	S.C.	70 h
Fusion (TNF receptor with Fc) [8875]								
Infliximab (Remicade)	TNF-α inhibitor; autoimmune diseases	149	7.3–7.6	N/A	N/A	N/A	I.V.	8–10 d
Antibody [8234]								
Rituximab (Rituxan)	CD20 binder; autoimmune disease, cancer	144	8.68	98	0	16	I.V., S.C.	30–400 h
Antibody [7227]								
Insulin glargine (Lantus)	Basal insulin; diabetes	6.1	6.7	1	0	3	S.C.	N/A; 30 h *in vitro*
Hormone [6745]								
Bevacizumab (Avastin)	VEGF-A inhibitor; cancer, macular degeneration	149	8.3	90	0	16	I.V., I.Vit.	20 d
Antibody [6715]								
Trastuzumab (Herceptin)	HER2 inhibitor; breast cancer	146	8.45	90	0	16	I.V., S.C.	2–12 d
Antibody [6714]								
Aflibercept (Eylea)	VEGF inhibitor; macular degeneration	115	8.2	70	0	10	I.V., I.Vit.	7 d I.Vit. 5–6 d I.V.
Fusion (VEGF receptor with Fc) [5046]								
Pegfilgrastim (Neulasta)	Stimulates white blood cell synth.; post-chemotherapy	~40	6.1	4	1	2	S.C.	15–80 h
Cytokine/hormone [4648]								
Insulin aspart (Novomix, novorapid/novolog)	Fast acting insulin; diabetes	5.8	5.1	1	0	3	I.V., S.C.	81 min S.C.
Hormone [4259]								
Nivolumab (Opdivo)	Anti-PD-1 checkpoint inhibitor; cancer	144	N/A	80	0	16	I.V.	27 d
Antibody [3774]								
Ustekinumab (Stelara)	Blocks IL-12 and IL-23; psoriasis	149	N/A	N/A	N/A	N/A	I.V., S.C.	15–32 d
Antibody [3232]								

Generic (marketed) name *Class* [Sales (10^6 USD)][a]	Function; target disease	MW (kDa)	pI	# Lys[b]	#Cys (non-S-S)[c]	#S-S bonds	Route[d]	$t_{1/2}$
Ranibizumab (Lucentis)	VEGF-A inhibitor; eye disease	48	N/A	52	0	10	I.Vit.	9d
Antibody fragment [3227]								
Denosumab (Prolia)	RANKL inhibitor; osteoporosis	145	N/A	86	0	16	S.C.	25 d
Antibody [3164]								
Eculizumab (Soliris)	Complement protein inhibitor; autoimmune diseases	148	N/A	N/A	N/A	N/A	I.V.	8–15 d
Antibody [2843]								
Botulinum toxin A (Botox)	Acetylcholine release inhibitor; muscle disorders, cosmetics	149	6.06	103	5	2	I.M.	N/A
Neurotoxin [2786]								
Insulin lispro (Humalog)	Fast acting insulin; diabetes	5.8	5.4	1	0	3	I.V., I.M., S.C.	1 h S.C.
Hormone [2769]								
Golimumab (Simponi)	TNF-α inhibitor; arthritis	147	N/A	N/A	N/A	N/A	I.V., S.C.	11–14 d
Antibody [2511]								
Insulin detemir (Levemir)	Basal insulin; diabetes	5.9	N/A	1	0	3	S.C.	5–7 h
Hormone [2392]								
Epoetin alfa (Epogen)	Stimulates red blood cell synthesis; anemia	30	8.75	8	0	2	I.V., S.C.	4h
Cytokine [2387]								
Omalizumab (Xolair)	IgE binder; allergic asthma, hives	145	7.03	86	N/A	N/A	S.C.	26 d
Antibody [2318]								
Interferon beta-1a (Avonex)	Maintains inflammatory response 20 balance; multiple sclerosis	20	8.93	11	1	1	S.C., I.M.	10 h
Cytokine [2314]								
Abatacept (Orencia)	Inhibits T cell activation; arthritis	92	4.5–5.5	46	0	9	I.V., S.C.	13–17 d
Fusion (CTLA-4 with Fc) [2265]								
Darbepoetin alfa (Aranesp)	Stimulates red blood cell synthesis; anemia	38	8.75	8	0	2	I.V., S.C.	21 h
Cytokine [2093]								
Natalizumab (Tysabri)	α4 integrin inhibitor; multiple sclerosis, Crohn's disease	149	N/A	N/A	N/A	N/A	I.V.	7–15 d
Antibody [1964]								
Pertuzumab (Perjeta)	Inhibits HER dimerization; breast cancer	148	N/A	88	0	16	I.V.	18 d

Chem Soc Rev. Author manuscript; available in PMC 2019 December 21.

Generic (marketed) name *Class* [Sales (10^6 USD)][a]	Function; target disease	MW (kDa)	pI	# Lys[b]	#Cys (non-S-S)[c]	#S-S bonds	Route[d]	$t_{1/2}$
Antibody [1828]								
Tocilizumab (Actemra)	IL-6 receptor inhibitor; arthritis	148	N/A	N/A	N/A	N/A	I.V., S.C.	11–23 d
Antibody [1680]								
Insulin (Human insulins)	Fast acting and basal insulins; diabetes	~6	N/A	1	0	3	N/A	N/A
Hormone [1553]								
Human coagulation factor VIIa (NovoSeven)	Promotes blood coagulation; hemophilia	45	6.09	17	2	11	I.V.	4–6 h
Enzyme [1466]								
Palivizumab (Synagis)	Binds to respiratory syncytial virus; prevent infection	148	N/A	N/A	N/A	N/A	I.M.	18–20 d
Antibody [1407]								
Pembrolizumab (Keytruda)	Anti-PD-1 checkpoint inhibitor; cancer	149	6.8–6.9	86	0	16	I.V.	26 d
Antibody [1402]								
Insulin isophane (Humulin)	Intermediate acting insulin; diabetes	5.8	5.4	1	0	3	S.C.	N/A
Hormone [1366]								
Antihemophilic factor, recombinant (Kogenate)	Promotes blood coagulation; hemophilia	265	6.97	158	N/A	N/A	I.V.	8–19 h
Coagulation factor [1236]								
Somatropin (Norditropin)	Growth hormone; children growth disorders	22	5.27	9	0	2	I.M., S.C.	0.6 h I.V. 2 h S.C./I.M.
Hormone [1228]								
Vedolizumab (Entyvio)	Integrin $\alpha_4\beta_7$ inhibitor; inflammatory bowel disease	147	7.6	94	0	16	I.V.	14–15 d
Antibody [1167]								
Secukinumab (Cosentyx)	IL-17A inhibitor; psoriasis	148	N/A	82	2	16	S.C.	22–31 d
Antibody [1128]								
Alteplase/tenecteplase (Activase/TNKase)	Removes blood clots; heart attacks	59/59	7.6/7.6	21/20	1/1	17/17	I.V.	90–130 min
Enzyme [1097]								
Ipilimumab (Yervoy)	Anti-CTLA-4 checkpoint inhibitor; melanoma	148	N/A	84	0	16	I.V.	15 d
Antibody [1053]								
Dulaglutide (Trulicity)	GLP-1 agonist; diabetes	60	N/A	38	N/A	N/A	S.C.	5d
Fusion (GLP-1 with Fc) [926]								

Generic (marketed) name *Class* [Sales (10^6 USD)][a]	Function; target disease	MW (kDa)	pI	# Lys[b]	#Cys (non-S-S)[c]	#S-S bonds	Route[d]	$t_{1/2}$
Trastuzumab emtasine (Kadcyla) *Antibody-drug conjugate [823]*	HER2 inhibitor linked to tubulin inhibitor; breast cancer	149	N/A	~87	0	16	I.V.	4d
Imiglucerase (Cerezyme) *Enzyme [793]*	Hydrolyzes glycolipid; Gaucher's disease	59	7.41	22	3	2	I.V.	4–10 min
Interferon beta-1b (Betaseron) *Cytokine [778]*	Maintains inflammatory response balance; multiple sclerosis	20	9.02	11	0	1	S.C.	8 min-4 h
Alglucosidase alfa (Myozyme) *Enzyme [769]*	Glycogen hydrolysis; Pompe disease	105	N/A	15	1	6	I.V.	2.3 h
Filgrastim (Neupogen) *Cytokine/hormone [765]*	Stimulates white blood cell synth.; post-chemotherapy	19	5.7	4	1	2	I.V., S.C.	4h
Pancrelipase (Creon) *Enzyme mixture [730]*	Digestive enzymes; pancreatic disorder	N/A	N/A	N/A	N/A	N/A	Oral	N/A
Agalsidase beta (Fabrazyme) *Enzyme [714]*	Hydrolyzes glycolipid; Fabry disease	100	5.17	34	N/A	N/A	I.V.	45–102 min
Coagulation factor IX (Benefix) *Enzyme [712]*	Promotes blood coagulation; hemophilia	55	5.20	26	0	11	I.V.	14–24 h
Cetuximab (Erbitux) *Antibody [687]*	EGFR inhibitor; cancer	146	8.48	88	0	16	I.V.	114 h
Complement C1 esterase inhibitor (Cinryze) *Enzyme inhibitor [680]*	Complement protein inhibitor; hereditary angioedema	105	N/A	N/A	N/A	N/A	I.V., S.C.	56–62 h I.V. 200 h S.C.
Dornase alfa (Pulmozyme) *Enzyme [678]*	Cleaves DNA; cystic fibrosis	29	4.58	6	0	2	Inh.	3–4 h I.V. 11 h Inh.

[a] Sales for a same generic drug with different marketed name (by different company) are combined.

[b] Note that the lysines in this count may be post-translationally modified or not surface accessible.

[c] Cysteines are often buried inside hydrophobic pocket and unreactive.

[d] I.V.: intravenous, S.C.: subcutaneous, I.M.: intramuscular, I.Vit.: intravitreal, Inh.: inhalation.

Table 4

Protein-reactive functional groups commonly used for conjugation (PDB ID: 1GFL, residues drawn do not reflect actual sequence)