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Perspective

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Therapeutic Protein–Polymer Conjugates: Advancing Beyond PEGylation

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ABSTRACT: Protein–polymer conjugates are widely used as therapeutics. All Food and Drug Administration (FDA)-approved protein conjugates are covalently linked to poly(ethylene glycol) (PEG). These PEGylated drugs have longer half-lives in the bloodstream, leading to less frequent dosing, which is a significant advantage for patients. However, there are some potential drawbacks to PEG that are driving the development of alternatives. Polymers that display enhanced pharmacokinetic properties along with additional advantages such as improved stability or degradability will be important to advance the field of protein therapeutics. This perspective presents a summary of protein–PEG conjugates for therapeutic use and alternative technologies in various stages of development as well as suggestions for future directions. Established methods of producing protein–PEG conjugates and new approaches utilizing controlled radical polymerization are also covered.

1. INTRODUCTION

Protein–polymer conjugates display a unique combination of properties derived from both the biologic and synthetic materials, which can be individually tuned to elicit a desired effect. Inherent protein biorecognition can be used in therapies to replace deficient or absent natural proteins, upregulate existing metabolic pathways, or inhibit molecules and organisms.[1] Proteins may function in chemotherapeutic delivery devices as targeting agents. Additionally, enzymes can be used to catalyze chemical reactions both in vivo and in vitro. Synthetic polymers exhibit high thermal and chemical stabilities and can be synthesized with controlled molecular weight and low dispersity (i.e., narrow molecular weight distribution). Moreover, synthetic polymers allow for the incorporation of desired functional groups and can be designed to respond to biological and nonbiological stimuli, including changes in pH, temperature, redox potential, or analyte concentration. This fusion of biological properties and chemical stability or reactivity gives protein–polymer conjugates a unique position at the intersection of chemistry, biotechnology, nanotechnology, and medicine. The subject of protein–polymer conjugates has been extensively reviewed,[2−10] with details on synthetic methods and comprehensive summaries of reported work. This perspective will provide a brief overview of the history of poly(ethylene glycol) (PEG) conjugates, the current status of therapeutic protein–polymer conjugates, advanced conjugates for biomedical use, and an outlook to the future.

PEG Conjugates. In 1977, Abuchowski and co-workers demonstrated the first conjugation of monomethoxy-PEG (mPEG) to bovine serum albumin (BSA) through the use of a cyanuric chloride coupling agent.[11] This BSA–PEG conjugate displayed a lower immunogenic response in animal models relative to the native protein. They later reported that PEGylated proteins increased circulation times in animal models relative to native proteins.[11,12] Two years later, Kanamaru and co-workers reported increased biocirculation of the chromoprotein neocarzinostatin when it was covalently coupled to a polystyrene–maleimide copolymer.[13] Together, these discoveries prompted a flurry of interest in conjugating polymers to proteins for therapeutic use (Figure 1). In Japan, the protein–polymer conjugate SMANCS (zinostatin stimalamer), which is made up of the antitumor chromoprotein neocarzinostatin and a styrene–maleic acid copolymer, was approved by the Ministry of Health and Welfare (MHW) for the treatment of hepatocellular cancer.[14] However, the majority of the focus for medical use has been on the conjugation of PEG to proteins, also known as PEGylation, and only PEG has been attached in all of the Food and Drug Administration (FDA)-approved protein conjugates.[4]

PEG is generally regarded as safe by the FDA and is known to increase protein half-life through multiple mechanisms. Conjugation of PEG to protein or small-molecule therapeutics increases their molecular weight, which reduces kidney clearance and increases biocirculation time. PEG is also well-known as a "stealth" molecule; this allows PEG therapeutic conjugates to avoid phagocytosis and removal from the bloodstream. One process for clearing foreign material from the body requires opsonization, or the coverage of exogenous particles by opsonin proteins, and subsequent activation of phagocytes, which then remove the foreign bodies via endocytosis and degradation.[15] Opsonization relies on attractive forces between the exogenous body and the opsonins and is typically increased for hydrophobic particles[16,17] and charged species[18] as a result of enhanced adsorbability. Because PEG is a hydrophilic, neutral, and nonfouling molecule, it exhibits low opsonization rates and longer biocirculation. Additionally, PEGylation adds steric bulk and nonfouling properties, which may reduce the immunological response to a protein by disruption of antibody binding or breakdown of the biomolecule by enzymes. There are currently 10 FDA-approved PEG–protein conjugates and one PEG–aptamer conjugate with diverse applications as therapeutics.[19,20] These conjugates function as replacement therapies for native enzyme

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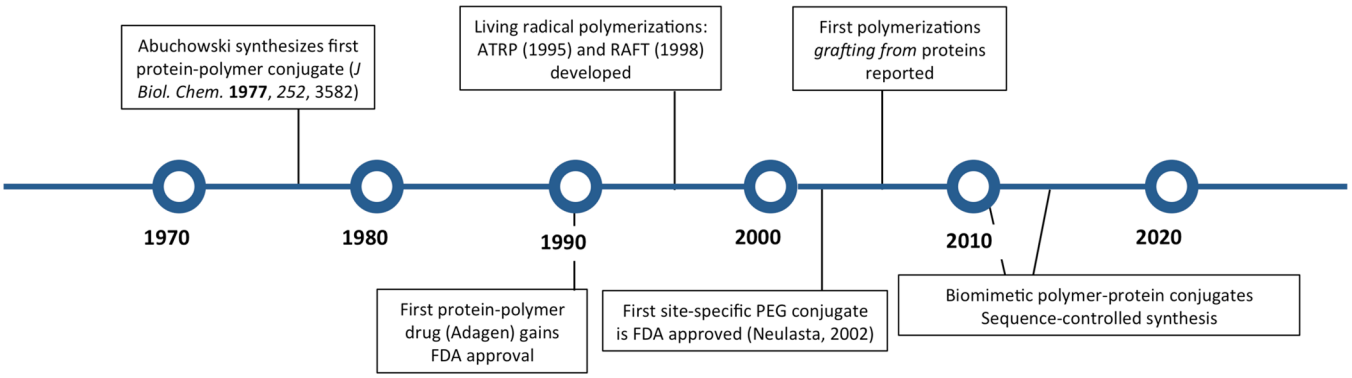

Figure 1. Advances in protein–polymer conjugation chemistry.

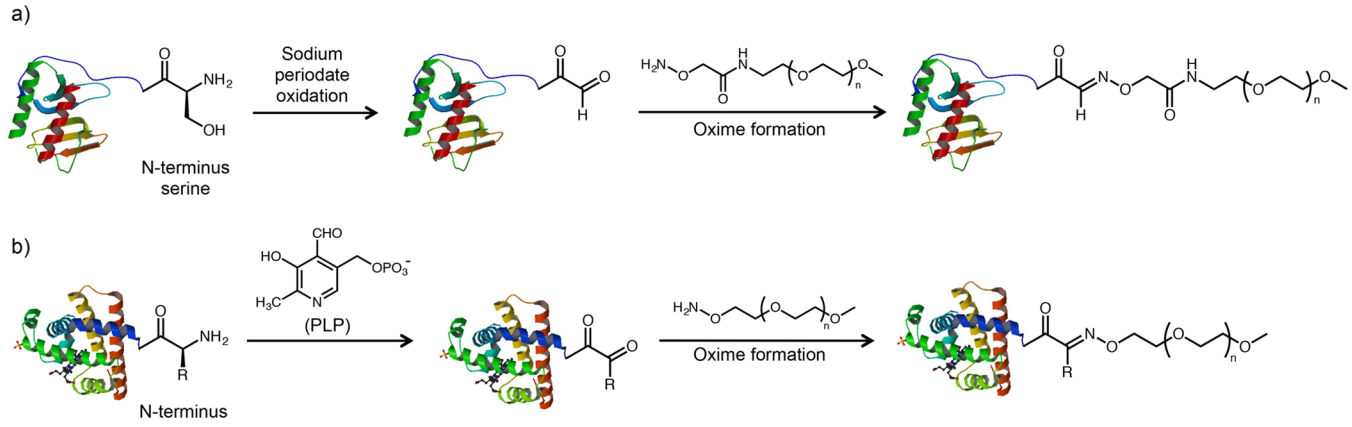

Figure 2. Examples of second-generation site-selective protein PEGylation using oxime "click" chemistry: (a) Gaertner and Offord;[22] (b) Gilmore et al.[23] The protein structure of interleukin 8 in (a) is from PDB entry 1IL8, and the protein structure of myoglobin in (b) is from PDB entry 1WLA.

deficiencies, stimulate immune responses, increase production of red or white blood cells, and neutralize overactive cytokines or receptors.[4] They have been used to treat diseases such as hepatitis C or Crohn's disease and to stimulate regrowth of white blood cells after chemotherapy. As a result, protein–polymer conjugates are an important class of biologics, generating more than 5 billion dollars in 2010.[1]

2. ADVANCES IN PROTEIN CONJUGATION AND POLYMER CHEMISTRY

Straight-Chain PEG Conjugates. PEG conjugation techniques have greatly improved over the past 40 years. First-generation PEGylation methods used semitelechelic mPEG to modify lysine side chains. The mPEG hydroxyl groups were activated through the formation of derivatives such as mPEG dichlorotriazine, mPEG succinimidyl carbonate, mPEG benzotriazol, activated esters, and mPEG tresylate, but these methods were inefficient.[2] They suffered from cross-linking due to contamination with bisfunctional PEG, unstable and easily cleavable protein–polymer linkages, alterations in protein charges, and lack of selectivity, all of which led to heterogeneity of the PEGylated proteins.[5,21] Most of these early protein conjugations relied on postpolymerization modifications or coupling reactions with the terminal hydroxyl group of PEG that required multiple steps or difficult purification procedures.[2,11]

Second-generation techniques focused on chemical transformations of mPEG to derivatives such as mPEG propionaldehyde, which could produce more stable linkages as well as more selective PEGylation, resulting in increases in bioactivity. For example, Neulasta was prepared by PEGylation of the N-terminus of granocyte colony-stimulating factor (G-CSF) utilizing this chemistry.[4] In other examples, it was shown that the N-terminal amine of interleukin 8 (IL-8) may be selectively oxidized with sodium periodate under mild conditions to form a glyoxyl group, which can subsequently react with an aminooxy PEG to form a conjugate via oxime bond formation (Figure 2a).[22] A transamination reaction was introduced wherein the N-terminus could be selectively modified with pyridoxal-5-phosphate (PLP) to yield an oxoamide (Figure 2b).[23] A variety of hydroxylamine-functionalized molecules or polymers including PEG were conjugated to the resulting N-terminus carbonyls via an oxime bond. However, the downsides of these techniques are the possibility of nonselective oxidation affecting other amino acids and low yields in the transamination reaction. Second-generation techniques also can minimize the negative effects on biological activity caused by conjugation. For instance, PEGylation of G-CSF and other proteins resulted in less protein aggregation when the positive charge on the N-terminal methionine residue was retained after conjugation.[24]

Free cysteines in the desired protein have also been targeted using thiol-reactive groups such as maleimide, vinyl sulfone, iodoacetamide, and pyridyl disulfide.[25,26] FDA-approved CIMZIA was prepared utilizing maleimide PEG addition to antitumor necrosis factor α (anti-TNFα).[4] Recently, newer chemistries to target free cysteines have been reported. For example, disulfide bridges may be reduced and conjugated in situ with a bridging moiety to retain tertiary structure while incorporating a linear or comb PEG chain.[27−29] In another

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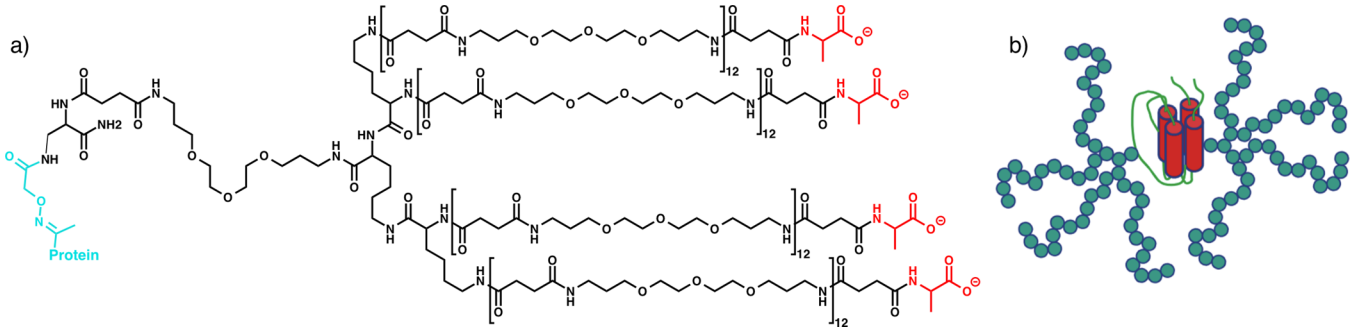

Figure 3. (a) Synthetic erythropoiesis protein with the structure of a branched, negatively charged PEG-like polymer. (b) Schematic of the resulting PEGylated protein. Reprinted with permission from ref 33. Copyright 2003 American Association for the Advancement of Science.

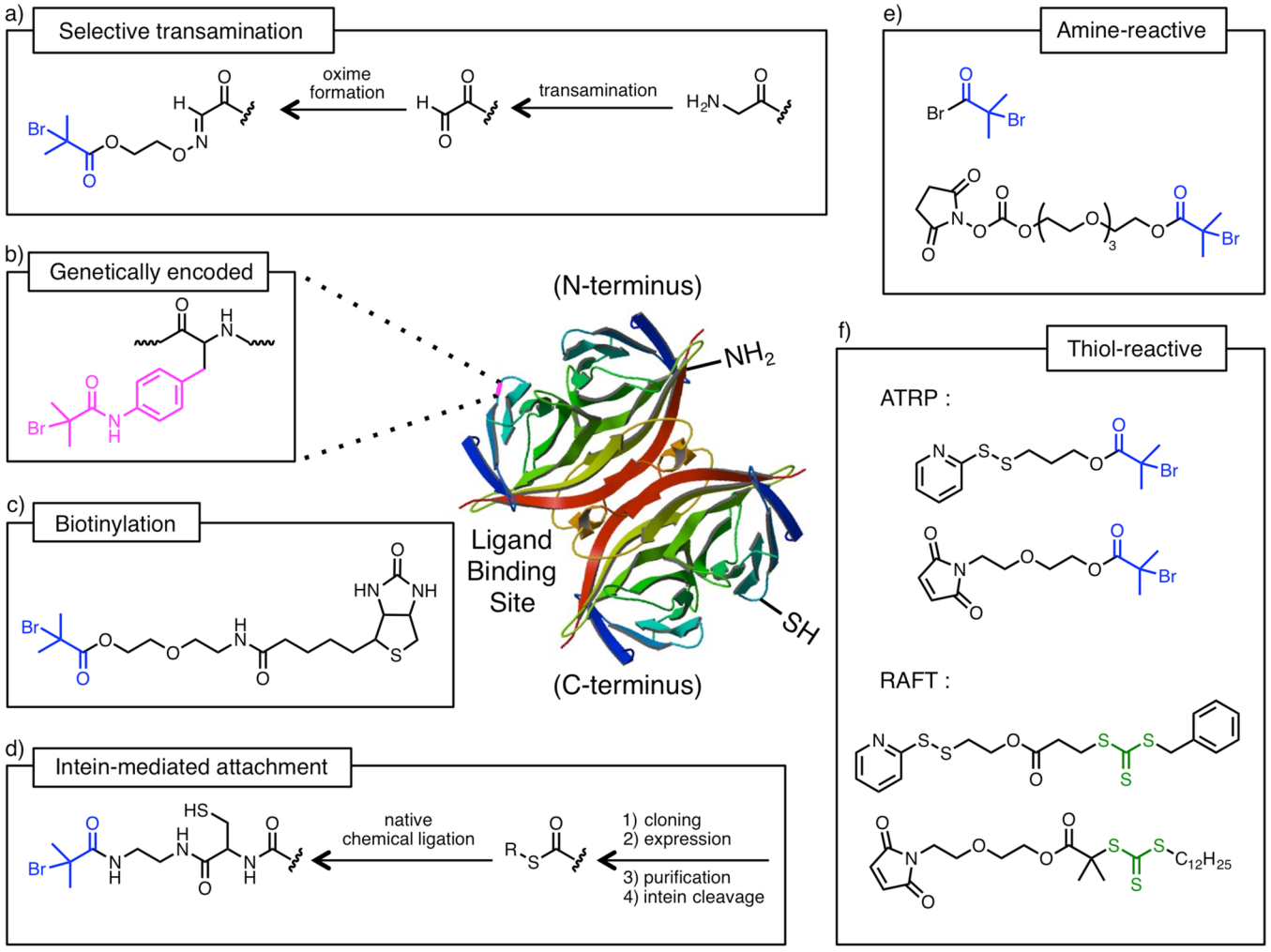

Figure 4. Schematic presentation of significant "grafting from" methods (the protein structure of strepavidin is from PBD entry 1N4J). References: (a) Gao et al.;[38] (b) Peeler et al.;[39] c) Bontempo and Maynard;[40] d) Gao et al.;[41] (e) Lele et al.;[42] Magnusson et al.;[43] (f) Heredia et al.,[44] Liu et al.,[45], and De et al.[46]

example, cross-metathesis was utilized to functionalize a protein with a short ethylene glycol. Specifically, a native cysteine was modified to contain an *S*-allyl group, which was then able to undergo cross-metathesis with a variety of alkenes, including carbohydrate groups and an alkene-functionalized tetra-(ethylene glycol).[30]

For proteins that do not contain free cysteines or where non-natural amino acids are desired, these can be inserted through genetic engineering.[31,32] Other ways to incorporate reactive handles include the preparation of synthetic proteins using native chemical ligation.[34] For example, erythropoiesis protein (EPO) was made by synthesizing polypeptide chains containing levulinyl ester-modified lysine residues (Figure 3).[33] Branched PEG-like polymers with negatively charged end groups were then site-specifically conjugated via oxime chemistry, and the resulting PEGylated EPO had bioactivity similar to that of the native protein with enhanced pharmacokinetics. The inclusion of reactive functional groups in the peptide sequence allows for

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site-specific PEGylation by a multitude of chemistries as well as retention of native protein bioactivity provided that the conjugated polymer does not inhibit access to the active site. However, of the above-mentioned PEGylation techniques, only five linker chemistries have been utilized in the FDA-approved protein conjugates: activated carbonyls such as *N*-hydroxysuccinimidyl (NHS) ester, *p*-nitrophenol carbonate, and NHS carbonate as well a thiol-reactive maleimide and an amine-reactive aldehyde.[4]

New Polymeric Methods for Bioconjugation. Just as the development of novel techniques for PEG conjugation has greatly improved the field of PEGylated conjugates, the development and use of novel monomers and new polymerization methods have greatly enhanced the potential to broaden the scope of therapeutic protein−polymer conjugates. Controlled radical polymerization (CRP) is currently the most popular technique to prepare conjugates with polymers other than PEG. Atom transfer radical polymerization (ATRP), in which a halogenated initiator undergoes a reversible redox reaction mediated by a transition-metal catalyst,[35,36] and reversible addition−fragmentation chain transfer (RAFT) polymerization, which utilizes a chain-transfer agent (CTA) to mediate the reversible chain-transfer process,[37] are the two CRP techniques commonly used for this purpose. Both techniques tolerate a wide range of functional groups, solvents, and reaction conditions and allow for the introduction of functional end groups. Importantly for therapeutic applications, these techniques provide polymers with narrow molecular weight distributions. This can be important for FDA approval as well as for uniformity in resulting properties.

These new polymerization techniques have also led to new methods of protein conjugation.[3,6,7] *Grafting to* is the covalent attachment of a synthetic polymer to a protein using a protein-reactive handle. CRP techniques using protein-reactive initiators or CTAs can eliminate postpolymerization modifications and synthesis steps and facilitate more efficient conjugations. For example, pyridyl disulfide and maleimide are commonly incorporated directly into initiators and CTAs, resulting in protein-reactive polymers by ATRP or RAFT without postpolymerization modification.[47−49] *Grafting from* involves the polymerization of monomers from an initiating site on the protein, which offers clear benefits in minimal steric hindrance and higher efficiency in the initiator−protein conjugation as well as easier purification and characterization. Initial reports focused on biotinylated ATRP initiators that could bind streptavidin noncovalently and generate a protein macroinitiator upon association with streptavidin.[40] After polymerization of various monomers, the protein was modified at the biotin binding sites. Shortly thereafter, pyridyl disulfide-functionalized, maleimide-functionalized, and activated ester ATRP initiators[42,44] and RAFT agents[45,50] were used and conjugated to free cysteines to generate protein macroinitiators. ATRP initiators have also been introduced in proteins through other chemistries such as oxime formation, genetic encoding of unnatural amino acids, and intein-mediated attachment.[38,39,41] Figure 4 outlines a representative sampling of grafting from methods that have been developed in this past decade.

Together, these polymeric methods greatly enhance the available chemical space of the polymers while minimizing the number of synthesis steps. But a drawback to the use of CRP methods is the possibility of cytotoxicity: ATRP uses toxic copper catalysts, and the trithiocarbonate and dithioester moieties found in RAFT CTAs have shown degradation and cytotoxicity in vitro.[51] However, both of these issues can be solved by removal of the metal or by postpolymerization modification steps to remove the end groups. Additionally, at this stage there is a general lack of long-term studies on the fate of these synthetic polymers in the body. Limited in vivo studies have showed promise as to the safety and efficacy of CRP polymer−protein conjugates,[38,41,43,52−56] but more thorough studies will need to be conducted. For example, little is known about their pharmacokinetic properties, safety, and long-term fate in the body.

3. ALTERNATIVES TO PEG IN DEVELOPMENT

An advantage of the new polymer and conjugation chemistries described above is the ability to prepare alternative polymers to PEG; this is especially important because of the potential side effects of the polymer that have been observed. PEG has been shown to cause hypersensitivity and immunological responses, accumulation in tissues, and accelerated blood clearance upon repeated exposure. The recognition of PEG by the body and the formation of anti-PEG antibodies were first reported in 1984.[57] Reports of differing levels of PEG antibodies in the general population vary widely with detection technique, from 4% using general enzyme-linked immunosorbent assay (ELISA)[58] up to 25% using a combination of serology and flow cytometry.[59] These PEG antibodies were shown to increase clearance rates of PEGylated conjugates.[60,61] Since the goal of protein PEGylation is to reduce immunogenicity and increase biocirculation time, the occurrence of these antibodies detracts from their utility in protein therapies. Hypersensitivity has been observed in other cases; potential allergic reactions to PEG detract from its usefulness in some patient populations.[62] The non-biodegradability of PEG is another main drawback; PEG has been shown to form vacuoles in organs such as the liver, kidney, and spleen after protein−PEG conjugate administration.[63−67] While non-biodegradable, PEG has shown degradation under light, heat, and mechanical stress with the possibility of toxic side product buildup during storage.[68] These deficiencies have led researchers to investigate alternative polymers for protein conjugation.

Known Biocompatible Polymers. While PEG remains the only polymer conjugate to be FDA-approved, many other polymers are widely recognized as biocompatible in other contexts (such as small-molecule drug carriers) and have been used to produce protein−polymer conjugates with improved in vitro and in vivo properties.

Statistical copolymers of *N*-(2-hydroxypropyl)methacrylamide (HPMA) and a monomer containing a pendant drug-reactive group (such as an ester or carboxylic acid) have been thoroughly studied in many clinical trials.[69] Although this polymer is well-established as a biocompatible drug carrier and promising as a PEG replacement, protein conjugates of HPMA are less developed. HPMA conjugates have demonstrated improved stability against heat and autolysis with model[70,71] and therapeutically relevant proteins.[72−74] The synthesis of end-functionalized HPMA by CRP using a protein-reactive thiazolidine-2-thione-functionalized CTA has been reported.[53,70,71]

Poly(vinylpyrrolidone) (PVP) has also been explored in vivo as a nontoxic, hydrophilic PEG alternative for conjugation to proteins[75,76] and has been synthesized by RAFT polymerization.[77] Studies comparing the immune response of protein conjugates in mice revealed that both PVP and poly(*N*-acryloylmorpholine) (pNAcM) conjugates with uricase stimu-

lated antibody production against the polymers after the first dose.[78] This result underscores the effect of conjugation on the immunological response to polymers and may limit the use of these polymers since antibody production is already a concern for PEG.

A set of promising alternatives are poly(2-oxazolines), which exhibit stealth behavior similar to that of PEG and easy renal clearance.[79,80] These polymers are easily modifiable and thermoresponsive, and they have been widely promoted as biocompatible PEG alternatives for use as polyplexes, conjugates, and micelles.[81−83] Experiments have shown that the conjugation of poly(2-ethyl-2-oxazoline) to G-CSF through reductive amination or enzyme-mediated acyl transfer resulted in conjugates that are bioactive in vivo.[84]

Degradable PEG Alternatives. The polymers above share with PEG the disadvantage of non-biodegradability. For those patients that use protein conjugates as replacement therapies, these polymers, like PEG, may accumulate in the body. This provides a strong motivation to develop polymers that can eventually degrade. Degradable main-chain PEG-like polymers have been developed that offer exciting alternatives to existing PEGylation strategies (Figure 5). For example, redox-sensitive

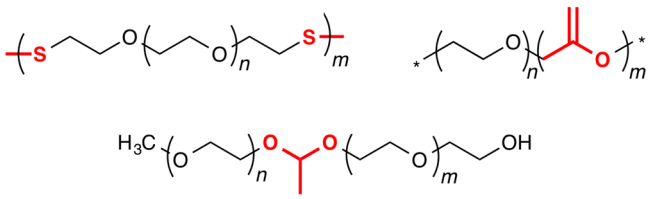

Figure 5. Structures of PEG-like polymers with degradable backbones.[85,87,88]

degradable PEG has been produced by the introduction of disulfide bridges between oligomeric units.[85,86] More recently, poly[(ethylene oxide)-*co*-(methylene ethylene oxide)] was prepared through postpolymerization elimination of epichlorohydrin monomer.[87] The resulting copolymer was degradable at physiological pH and temperature yet stable under storage at 6 °C. Acid-labile acetals have also been incorporated into the backbone of functional PEGs.[88] These polymers have not yet been conjugated to proteins or biologically evaluated, although the approach of preparing main-chain-degradable PEGs is promising.

Other degradable PEG alternatives in development have been studied in vivo, including hydroxyethyl starch (HES), polysialic acid, and dextrin (Figure 6). HES polymer conjugates are one of the most advanced of these alternatives.[89] HESylation of EPO by the pharmaceutical company Fresenius-Kabi showed in vivo and in vitro bioactivities comparable to those of Mircera (the PEGylated protein currently on the market) with a 3-fold increase in half-life over the wild-type protein.[89] Other proteins, including G-CSF and interferon-α (IFNα), have also been HESylated with comparable results.[89] HES has the advantage that it can be degraded by α-amylase, although degradation slows with increasing hydroxyethylation.[89] While HES was commonly used as a volume expander for patients with severe blood loss, currently the safety of HES is under controversy. On the scale used for fluid therapy, HES has been shown to accumulate in the liver, kidney, and bone marrow, leading to increased risk of kidney injury and death for critically ill patients.[90] While HESylated proteins exhibit good pharmacokinetic properties, the side effects associated with the repeated use of HES may be a hindrance to their adoption as conjugates for all patient populations. Conjugates utilizing dextrin, polysialic acid, and other biodegradable polymers have demonstrated improved therapeutic properties in animal studies.[91] A recombinant human epidermal growth factor (rhEGF)−dextrin conjugate demonstrated greater wound healing in vivo than free rhEGF.[92] Studies in rat models have reported that conjugation of polysialic acid to insulin prolonged the reduction of glucose levels.[93] Drug conjugates containing the enzymatically degradable poly(glutamic acid) (PGA) have been prepared and shown to increase tumor exposure to chemotherapeutics,[94] but its conjugation to proteins has not yet been extensively explored.

PEG Alternatives with Varied Architectures. Polymer architecture is an important consideration when designing protein conjugates, with branched[95,96] or dendritic[97] PEG-like polymers generally displaying improved biocirculation and stability. A notable example of branched PEG is the synthetic erythropoiesis protein described above (see Figure 3).[33] Branching can also reduce the solution viscosity at high concentrations.[98] Since PEG-containing protein conjugates must be injected, a decrease in viscosity for PEG alternatives would be especially important in increasing the ease of injection for patients. Furthermore, branched polymers may be able to mimic the glycosylation patterns on native proteins. These covalent glycans can be important for stability and signaling yet are missing from proteins expressed from *Escherichia coli*. Therefore, the exploration of PEG alternatives with disparate architectures will be important. A major recent focus in the field has been on comblike PEG polymers because of their ease of synthesis and potential for low dispersity enabled by CRP techniques. These polymers can also be made degradable by including cyclic ketene acetals (CKAs) in the reaction mixture,[99−101] and the degradable comb PEGs can very likely be conjugated to proteins.[102] Although more intensive studies need to be undertaken to verify the validity of these polymers as potential therapeutics, there are several that have shown promise in vivo; these examples are summarized below.

An early study involved site-specific conjugation of aldehyde pPEGMA to salmon calcitonin (sCT) through reductive amination.[103] In vivo, the conjugate retained 72% of the native bioactivity while displaying an extended half-life in rats.[52] Grafting from recombinant human growth hormone (rh-GH) was shown to produce conjugates with improved properties.[43] The initiator NHS bromoisobutyrate was coupled to free amine groups on rh-GH, and polymerization of PEGMA was conducted. The conjugate showed higher stability toward denaturation and proteolysis compared with unmodified rh-GH. The in vivo efficacy of the hormone also improved, as confirmed by monitoring of weight growth in administrated female rats. Polymerization of PEGMA from the N-terminus of myoglobin and C-terminus of genetically modified green fluorescent protein (GFP) was also shown.[38,41] In mice, these conjugates displayed improved pharmacokinetics compared with unmodified protein. These examples clearly demonstrated that similar to PEG conjugates, pPEGMA conjugates significantly extend the circulation lifetime of the protein and are effective in vivo.

Polyglycerols (PGs) are structurally similar to PEG and have been prepared in both linear form and with tunable branching. While linear PGs have recently been reviewed as alternatives for PEG,[104] polymers are often designed to mimic the typically branched structures of proteoglycans. For example, a library of

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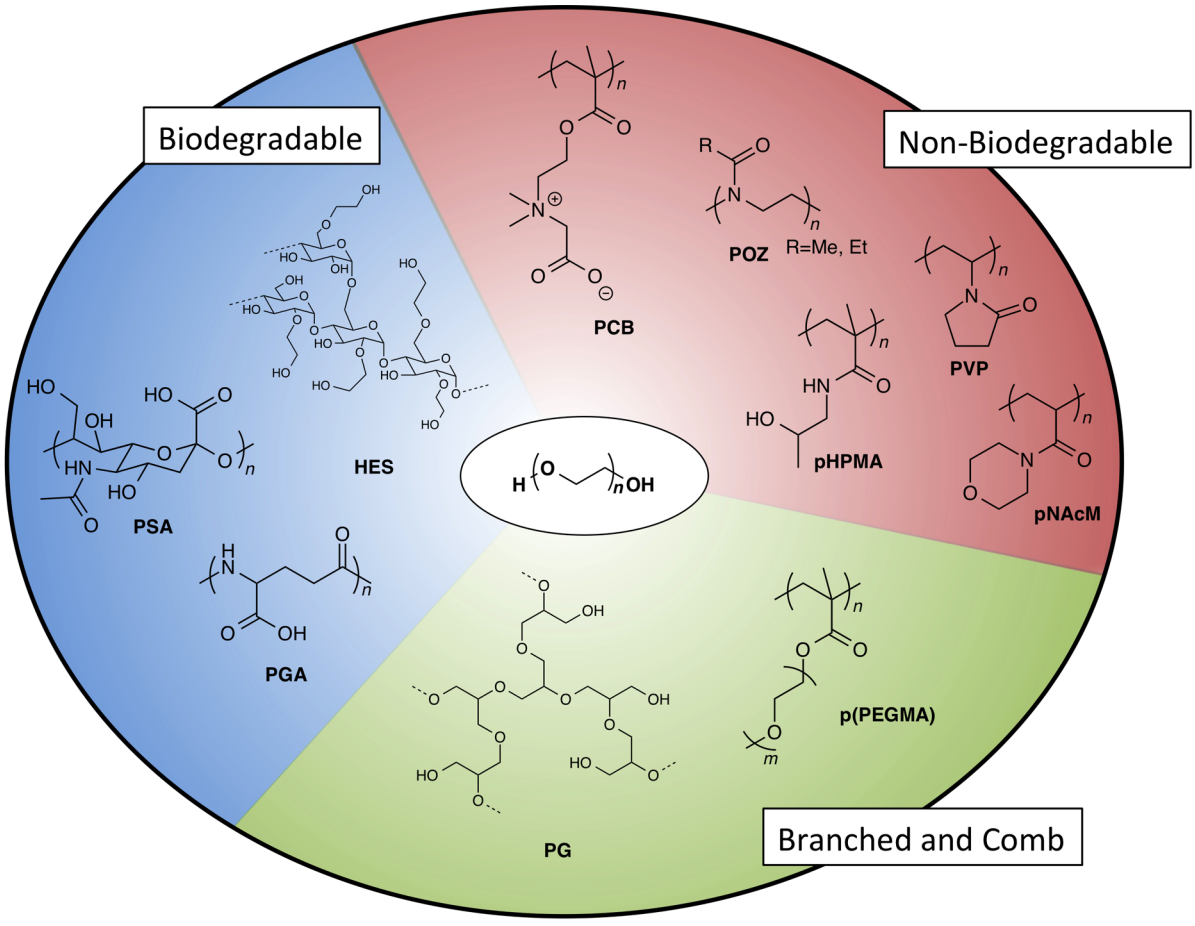

Figure 6. Examples of alternatives to PEG currently in use and development. PCB, poly(carboxybetaine); POZ, poly(2-oxazoline); PVP, poly(vinylpyrrolidone); pHPMA, poly(*N*-hydroxypropyl)methacrylamide); pNAcM, poly(*N*-acryloylmorpholine); pPEGMA, poly(poly(ethylene glycol) methyl ether methacrylate); PG, poly(glycerol); PGA, polyglutamic acid; PSA, polysialic acid; HES, hydroxyethyl starch.

linear, midfunctional, hyperbranched, and linear−hyperbranched PG conjugates were synthesized to assess the effect of structure on conjugate activity.[105] With BSA and lysozyme as model proteins, it was found that in conjugation of high-molecular-weight polymers, midfunctional-PG−lysozyme displayed higher activity than linear-PG−lysozyme while hyperbranched conjugates displayed decreased activity. More recently, PG grafted from BSA under kinetic control to control the degree of branching was reported.[106] Upon ring-opening polymerization with a tin triflate catalyst, short polymers were obtained in situ with relatively low polydispersity index (1.25−1.36) and low dendritic unit composition. These PGs offer promising alternatives to PEGylation.

Significant progress has been made in the synthesis of polymers that mimic natural glycans, especially in the area of ligands that exhibit biological functions,[107,108] and some of these have been conjugated to proteins. For example, an alkyne end-functionalized glycopolymer synthesized via ATRP was conjugated to a cowpea mosaic virus (CPMV) modified with an azide group.[109] This virus−glycopolymer conjugate could potentially be used to target cancer cells for drug delivery. A maleimide end-functionalized glycopolymer with mannose pendant groups was prepared by a combination of ATRP and Cu-catalyzed azide−alkyne cycloaddition (CuAAC) "click" chemistry.[110] The polymer was then site-specially grafted to BSA, a non-glycosylated model protein. Surface plasmon resonance (SPR) studies showed binding of the BSA conjugates to a recombinant rat mannose-binding lectin (MBL). In addition, some groups have synthesized branched glycopolymers in order to better mimic proteoglycans. Highly branched carbohydrate structures are able to display more potent effects. For instance, a galactose dendrimer and subtilisin, a protease, were tethered to construct a glycodendrioprotein.[111] This synthetic glycoprotein demonstrated nanomolar inhibition of Gram-positive bacteria aggregation. This work presents well-defined and synthetically designed glycoprotein mimics that exhibit combined features of the original protein component and the lectin binding properties of the glycopolymer.

Biomimetic Polymer Design. While general PEG substitutes have been discovered and developed, there remains a need for protein−polymer conjugates that exhibit tailored properties. Ideally, the attached polymer should improve existing functions or introduce new properties to the protein. This section details the purposeful synthesis and design of biomimetic, biodegradable, and biocomplementary polymer systems. As with any new entity, these polymers would have to clear a large number of experimental hurdles prior to FDA approval and use as therapeutics, and many of the examples below have not yet been tested in vivo. However, these approaches may significantly enhance the properties of protein therapeutics beyond an enhancement in pharmacokinetics, and thus, innovation in this area should not be deterred.

Polymers with stabilizing properties offer the possibility of increasing not only the circulation lifetime but also the stability

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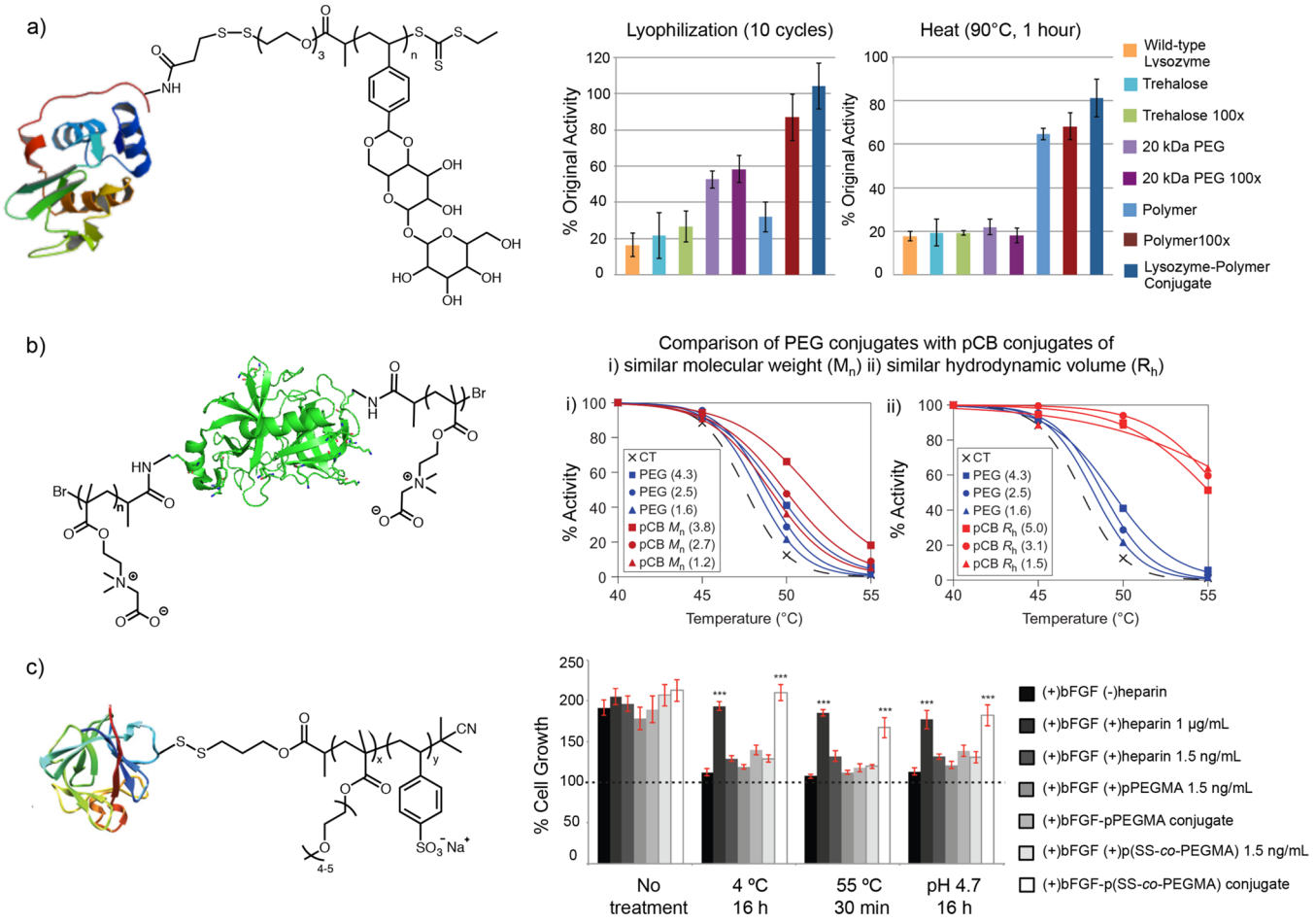

Figure 7. Examples of stabilizing and biomimetic polymers. References: (a) Adapted from ref 113. Copyright 2012 American Chemical Society. (b) Adapted by permission from ref 114. Copyright 2011 Macmillan Publishers Ltd. The protein structure of α-chymotrypsin is from PBD entry 4CHA. (c) Adapted by permission from ref 115. Copyright 2013 Macmillan Publishers Ltd.

of the protein toward storage, transport, and other stresses. Such materials could be superior analogues to PEG if both the pharmacokinetic properties and stability outside of the body are improved, particularly since most proteins need to be refrigerated. To this end, glycopolymers with pendant trehalose side chains were demonstrated as conjugates and excipients to increase protein stability toward common environmental stressors (Figure 7a).[112,113] The polymers significantly enhanced the protein stability toward lyophilization and high temperatures. The polymers combine two important classes of stabilizers, namely, osmolytes and nonionic surfactants, providing superior stabilization compared with PEG and trehalose alone. Poly(zwitterionic) protein conjugates were also recently reported (Figure 7b). Specifically, poly(carboxybetaine) was coupled to α-chymotrypsin, and the protein displayed higher activity than the comparable PEGylated conjugate or native protein at elevated temperatures.[114] Polyionic conjugates that can stabilize proteins in the gut have also been reported; the covalent attachment of a cationic dendronized polymer to a proline-specific endopeptidase (PEP) maintained enzyme activity in the stomach of rats for more than 3 h, while control linear mPEG–PEP conjugates were inactive in the harsh environment.[54] This is important because the conjugate has to pass through the stomach to enter the intestine to be active against celiac disease. In another example, a heparin-mimicking polymer, poly(styrenesulfonate-*co*-PEGMA) [p(SS-*co*-PEGMA)], was conjugated to basic fibroblast growth factor (bFGF).[115] The p(SS-*co*-PEGMA)–bFGF conjugate exhibited superior stability toward heat, acidic and proteolytic conditions, and storage compared with the native protein while maintaining in vitro activity (Figure 7c). This example in particular demonstrates that rationally designing a polymer to mimic a known biological stabilizer can result in conjugates with superior properties.

Precise Sequence Control and Monodispersity. Another area of promise is to precisely control the order of different monomer units within a polymer (sequence control) and to obtain absolutely monodisperse conjugates. The former is important for dialing in properties, and the latter is advantageous for FDA approval as well as precise control over properties. This has recently been accomplished for polymers but has not yet been applied to conjugates. For example, by the use of DNA-templated synthesis, the order of monomer addition can be selectively controlled. Sequence-controlled alkene-linked amino acid oligomers have been synthesized through sequential Wittig reactions.[116] To prepare these oligomers, each amino acid was functionalized with ylide and aldehyde groups and coupled with DNA to yield ylide–aldehyde DNA macromonomers. Strand association brought macromonomers into contact, assisting Wittig reactions between complementary strands. Subsequent strand exchanges introduced new amino acids into the growing oligomer chain. A

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DNA template was also used to synthesize sequence-defined polymers of PEG as well as α- and β-peptides.[117] In a method designed to be analogous to translation, a series of azide- and alkyne-containing monomeric units were functionalized with peptide nucleic acids (PNAs) through cleavable disulfide linkers. The substrates could then site-specifically hybridize to DNA strands, undergo intermonomer CuAAC coupling, and then cleave the PNA-monomer linker to free the sequence-controlled polymer from the DNA template. The polymers produced by this method were up to 26 kD in size.

4. SUMMARY

Outstanding progress has been made in the past four decades on protein−PEG conjugate drugs for use in medicine. Currently there are 10 PEGylated protein therapeutics that are used clinically to treat a range of diseases, with many more currently under investigation. However, room for improvement still remains. Many conjugates, while increasing half-life circulation, exhibit decreased biological activity compared with the native protein. It has been shown that by rational design of the conjugation site and the use of site-selective conjugation reactions, the activity of the protein can be fully retained. Also, the toolbox of protein conjugation techniques is steadily growing with the addition of grafting from methods, genetic engineering, highly efficient conjugation chemistries, and new approaches to synthesize end-functional polymers for conjugation. This has led to PEG alternatives that are degradable and new biomimetic strategies to increase the stability and activity of native proteins. There are many more possibilities in the future, including polymers with precise sequence control and monodispersity and those that increase the activity of the protein by specific orthogonal biological function. Protein−polymer conjugation will continue to be an exciting field requiring scientists with expertise in different disciplines, including protein biologists, polymer, organic, and computational chemists, physicists, and pharmacologists.

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Notes

The authors declare no competing financial interest.

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