Review

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Site-Specific Conjugation of Polymers to Proteins

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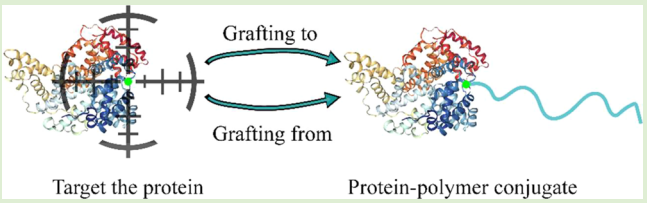
ABSTRACT: Conjugation of polymer to protein has been widely employed in therapeutics, medicine, biotechnology, and enzymatic catalysis. The synergistic effect benefits both counterparts and potentially overcomes their inherent limitations. This article reviews the strategies for the site-specific synthesis of well-defined protein−polymer conjugates, aiming to provide a toolbox for the community. First, it is essential to set a definite reactive site on the protein because the position of the reaction site can directly influence the reaction activity and the bioactivity of the protein after modification. The origins of the specific functional groups on protein include the utilization of the unique natural amino acid, mutagenesis to introduce a sole reactive amino acid, chemical modification, noncanonical amino acid incorporation, and enzyme-mediated introduction of functional groups. Second, the main conjugation methods, i.e., "grafting to" and "grafting from" methods, are summarized and compared with each other. In the "grafting to" method, a comprehensive investigation on the reactions used to attach an end functional polymer chain to a protein is conducted according to the position of the target site and its nature. In the "grafting from" method, a comparison between the commonly used controlled polymerization, i.e., atom transfer radical polymerization (ATRP) and reversible addition−fragmentation transfer (RAFT), is surveyed. Further, a special case where a noncovalent bond is adopted to link the protein and polymer together is investigated due to its high specificity and reversibility, typically biotin-(strept)avidin-based interactions and metal-mediated conjugation. Finally, applications of protein−polymer conjugates in drug delivery, biomedicine, biosensor, and the disease-related protein self-assembly are illustrated. This precise review on the conjugation of polymer chain to protein to form well-defined protein−polymer conjugates summarizes the representative strategies and may provide useful cues in the areas of biotechnology, therapeutic drugs, and biomedicine.



1. INTRODUCTION

Protein has been a representative candidate in biopharmaceuticals. To date, there are over 130 proteins and peptides approved for clinical use by the Food and Drug Administration (FDA).[1] Although proteins have their advantages as therapeutic drugs due to the large size, specific conformation, diverse biological functions, and biocompatibility,[2] shortcomings such as short in vivo half-life, poor stability, low solubility, and immunogenicity limit their applications.[3] The limitation of proteins can be overcome by the linkage of well-defined, suitable polymers to proteins, which has been successfully applied in therapeutic drugs.[2] Since the first covalent linkage of poly(ethylene glycol) (PEG) to bovine serum albumin (BSA) reported by Abuchowski and co-workers in the 1970s,[4] conjugation of polymer chains to proteins has been widely investigated. Protein−polymer conjugates are hybrid molecules that can benefit from the synergistic behavior of both components and should be able to overcome some of their respective intrinsic limitations.[5,6] For instance, attachment of synthetic polymer to protein can improve the solubility, stability, and biocompatibility, which is well-employed in therapeutics, biomedicine, nanotechnology, bioengineering, catalysis, and sensing,[2,7,8] and the protein or peptide can impart (bio)functional properties to the bioconjugate. The conjugation of synthetic polymer to protein can also introduce novel self-assembly and phase-separation behaviors.[9] Specific applications focusing on the protein−polymer therapeutics[10−14] and drug delivery[15−20] have been thoroughly summarized in the literature.

The strategy for the synthesis of protein−polymer conjugates has been extensively reviewed in detail over the past two decades.[2,3,5,7,21−23] Generally, there are three strategies to synthesize protein−polymer conjugates, which are summarized in Scheme 1.[24,25] The "grafting to" method, where the preformed polymer is directly coupled to the target protein, represents the most common and straightforward methodology.[11] Even though the "grafting to" approach has its advantages that the polymer component can be synthesized in a nonaqueous solution prior to the final conjugation step, shortcomings such as low yield of the reaction between two large molecules and difficulty purifying the products from reactants limit its further application.[26] As a consequence, the second, "grafting from" method has emerged due to the highly developed living polymerization techniques focusing on

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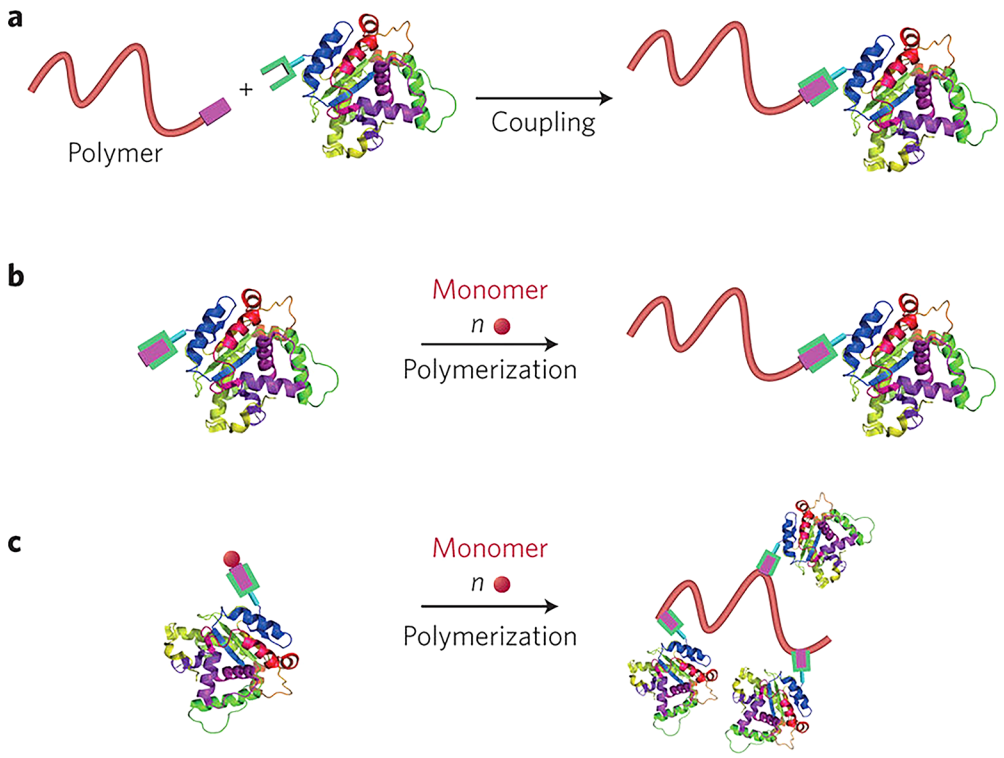
Biomacromolecules XXXX, XXX, XXX−XXX

Scheme 1. General Methods to Prepare Protein−Polymer Conjugates: (a) Grafting to, (b) Grafting from, and (c) Grafting through



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controlled radical polymerization (CRP), such as reversible addition−fragmentation transfer (RAFT) and atom transfer radical polymerization (ATRP). For the "grafting from" approach, the initiator is first linked to a protein, forming a macroinitiator wherefrom the living polymerization is induced to achieve the controlled growth of the polymer chain, leading to a high yield due to the high efficiency between small molecules and proteins. Moreover, the small monomer molecules and additives can be easily separated from the resultant protein−polymer conjugates after polymerization.[3] The third strategy is the "grafting through" method. A macromonomer with protein as the pendant group is first synthesized for further polymerization or by postpolymerization conjugation of multiple protein molecules to a polymer chain, resulting in a high density of protein with a comb-shaped structure. The relatively low polymerization degree of this method and the complexity of the product make this approach less common and will not be covered in this review.[21,26] The above methods are adopted exclusively or in a combination to prepare conjugates with higher functionality.[27,28] Besides the three strategies described above, there is also a method called cofactor reconstitution, where a heterobifunctional spacer is first linked to the protein to create a reactive protein hybrid followed by the attachment of a polymer onto the spacer.[6] Such an approach is cataloged as a general "grafting to" method and will not be discussed separately herein. When designing a specific protein−polymer conjugate, it is essential to consider the appropriate protein, the choice of polymer, the type of conjugation chemistry used, the stoichiometry (ratio of the two components), the attachment site, and the supramolecular architecture of the conjugates.[2]

On the basis of the introduction above, conjugation of a polymer chain to protein benefits both counterparts and has various applications in different areas. However, because of the abundant functional amino acid residues present on the protein, the synthetic polymer chains will be docked onto the protein in a random distribution if the conjugation condition is not well-designed, which usually leads to a significant loss of critical protein properties[23,29] despite the few reported cases in which nonspecific conjugation increases the protein activity.[30,31] It is always desirable to dock the synthetic polymer to the target protein in a controlled way, especially to a predefined site. Such site-specific conjugation will allow us to finely tune the functionality of the protein and is helpful to understand the structure−property relationship of the target protein. In this concise review, we will mainly focus on site-specific polymer conjugation, which produces well-defined bioconjugate materials for further biomedical applications, aiming to provide the community a toolbox for the rational design of protein modifications. The site-specific protein−polymer conjugate here is refined to have (1) a conjugated polymer with a defined chain length and a narrow distribution, (2) a specific linking point between the protein and the polymer, and (3) a definite stoichiometric ratio between the protein and the polymer, presumably to be one. Following this, the origin of functional groups on proteins will be first introduced, followed by a detailed discussion of the conjugation methods, especially the "grating to" and "grafting from" methods with recent research outcomes. Finally, a brief introduction on the application of the site-defined protein−polymer conjugates in protein stabilization and protein−polymer behavior regulation will be emphasized.

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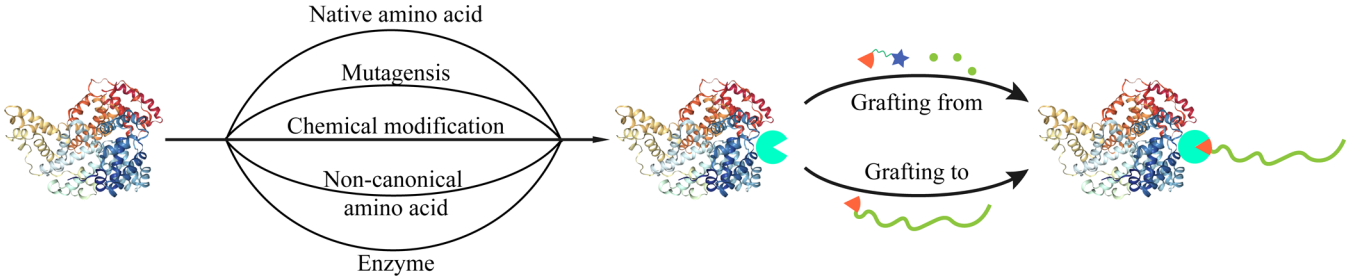
Biomacromolecules XXXX, XXX, XXX−XXX

Scheme 2. Overall Scheme for the Preparation of the Site-Specific Polymer−Protein Conjugates



2. STRATEGIES FOR SITE-SPECIFIC CONJUGATION OF POLYMERS TO PROTEINS

Site-specific conjugation is to link a polymer chain to a defined site on a protein, resulting in a controlled structure of the protein−polymer conjugate.[29,32] However, it is difficult to determine the number and location of the polymer chains when multiple attachment points are accessible. For example, most proteins contain several internal lysine residues in addition to the N-terminal amine, which are reactive to functional groups such as activated ester or aldehyde, leading to a series of protein−polymer mixtures with a heterogeneous distribution of polymers on the protein.[33] In such cases, one needs to have a detailed knowledge of the protein structure and carefully calculate the relative reactivities of the potential sites. Only with this information might one conjugate the synthetic polymer to the predetermined site via fine-tuning the reaction conditions.[34] Such a method is of low yield and too sophisticated to be followed by general practitioners. Without optimization of the reaction strategy, the synthesis of polymer-modified proteins introduces heterogeneity both in the dispersity of the attached polymers and in the attachment site on proteins.[35] For site-specific protein−polymer conjugates with high yield to be achieved, rational design and selection of the substrates and reaction conditions are crucial. Here, a point-by-point introduction, starting from the installation of a functional handle on the protein to the conjugation strategy, will be delivered as shown in Scheme 2.

2.1. Origin of Functional Groups on Proteins. For a site-specific protein−polymer conjugate to be synthesized, the selection of functional groups on proteins is of great importance. One essential prerequisite is using a protein that contains only one potential reaction site. The site may be a lone, unique accessible amino acid in the primary sequence of a protein, the N- or C-terminus, or an engineered noncanonical amino acid.[2] If the modification is directed to a specific site, the protein conformation or function may be better tuned. For instance, the site for polymer conjugation can be far away from the active center to avoid influence on protein activity or be located nearby or even within the active site to control the bioactivity of the protein.[21] Thus, it requires knowledge of the primary structure of the protein and the presence of amino acid residues that include the specific functional groups for modification. Usually, the target amino acid for the conjugation should be presented on the surface of the protein instead of being buried in the interior for reaction accessibility.[5,22] In summary, there are five ways to define the reaction sites on proteins.

2.1.1. Functional Groups from the Natural Amino Acids. Proteins, especially large ones, usually contain multiple copies of some, if not all, 20 standard amino acids so that directing the modification at a specific site is challenging. A comprehensive knowledge of the protein sequence is of great help for the success of site-specific conjugation. When such information is deficient, basic information concerning the natural amino acid abundance, their average distribution within the three-dimensional structure, and their average surface accessibility is essential if one wants to use the functional group from natural amino acids as the reactive site. Table 1 lists the statistical information on the 20 native amino acid abundances and average surface accessibilities based on the database proteins.[5,22] Here, the average surface accessibility indicates whether an amino acid is present more in the core or on the surface. Strategically, a less abundant amino acid is a priority for better control over of the synthesis of site-specific protein−polymer conjugates. For instance, free cysteine is rare in native proteins, and utilizing the thiol group from a free cysteine residue has been a common routine for the synthesis of a specific product.

Table 1. Amino Acid Composition, Location, Functionality, Natural Abundance, and Their Average Surface Accessibility

Amino acid	Location[a]	Functionality[b]	Natural abundance	ASA[b]
Cysteine	C	Thiol	1.36	0.268
Isoleucine	C	Aliphatic	5.97	0.273
Tryptophan	C	Indole	1.08	0.279
Phenylalanine	C	Benzyl	3.86	0.290
Valine	C	Aliphatic	6.87	0.306
Tyrosine	C	Phenol	2.92	0.319
Leucine	C	Aliphatic	9.66	0.321
Methionine	C	Thioether	2.42	0.364
Alanine	C	Aliphatic	8.26	0.405
Histidine	M	Imidazole	2.27	0.425
Threonine	M	Hydroxy	5.34	0.480
Proline	M	Aliphatic	4.69	0.502
Arginine	M	Guanidine	5.53	0.539
Asparagine	M	Carboxamide	4.06	0.568
Serine	S	Hydroxy	6.55	0.568
Glutamine	S	Carboxamide	3.93	0.573
Glutamic Acid	S	Carboxylic acid	6.75	0.586
Glycine	S	-	7.08	0.588
Lysine	S	Primary amine	5.85	0.607
Aspartic Acid	S	Carboxylic acid	5.46	0.615

Reprinted from ref 22. Copyright 2012 with permission from Springer Nature. https://link.springer.com/chapter/10.1007/12_2012_169. [a]Location of the amino acid to their average composition in core (C), intermediate (M), and surface (S). [b]Average surface accessibility.

Previously, Maynard and co-workers linked an ATRP initiator onto the only free cysteine, cys-34 of BSA, through a

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Biomacromolecules XXXX, XXX, XXX−XXX

reversible disulfide linkage. The polymerization induced by the macroinitiator resulted in a thermosensitive BSA-poly(*N*-isopropylacrylamide) (BSA-PNIPAAm).[33] Moreover, a chemoselective modification based on the tryptophan residue with rhodium carbenoids in aqueous solution at pH 6−7 is reported,[36] where the horse heart myoglobin and subtilisin Carlsberg are utilized as model systems by modifying tryptophan residues with final conversions estimated to be 90 and 50%, respectively. Other amino acid residues such as tyrosine, N- and C-terminus are summarized in detail elsewhere.[5]

2.1.2. Mutagenesis. The second approach to increase the selectivity of the conjugation reaction from the protein side is mutagenesis. Two directions can be considered here, as summarized in Figure 1. The first is to introduce a reactive group that the native proteins do not bear. For instance, the cysteine residue can be generically incorporated into proteins for site-specific PEGylation. Interferon α-2 has been genetically mutated at a specific site to introduce a unique cysteine residue that can react with maleimide-terminated PEG, resulting in a well-defined PEGylated interferon α-2 that retained both high in vitro bioactivity and enhanced tumoricidal properties in vivo.[37,38] A more general method is the His-tag approach. The His-tag typically consists of a string of 2−6 consecutive histidine residues and can be selectively recognized by a Ni (II) ion bound to nitrilotriacetic acid (NTA). Originally introduced as a convenient means to facilitate purification of recombinant protein by immobilized metal-affinity chromatography, several groups have recently begun to use His-tag to prepare site-specific polymer conjugates.[12,39,40] The His-tag is generally introduced at the end of the N- or C-terminus of a protein, and polymers with a complementary end group can directly attach to the protein at the His-tag end. A typical example of PEGylation using a His-tag approach is shown in Figure 2.

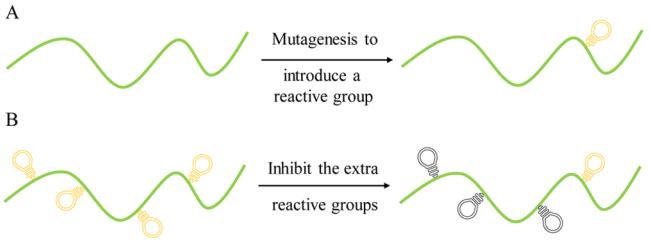

Figure 1. Strategy to introduce a site-specific functional group onto the protein via mutagenesis.

The other method is to limit the availability of the reactive groups on the protein by replacing the reactive amino acids with nonreactive ones through mutagenesis. Yamamoto and co-workers developed a strategy for site-specific mono-PEGylation using tumor necrosis factor-α (TNF-α). By preparing phage libraries expressing TNF-α mutants in which all the lysine residues were replaced with other amino acids, variants with only one available primary amine at the N-terminus can be tethered. The isolated bioactive lysine-deficient mutant was further site-specifically mono-PEGylated at its N terminus, resulting in a mono-PEGylated product with superior molecular uniformity. The conjugate showed higher bioactivity in vitro and greater antitumor therapeutic potency than randomly mono-PEGylated wild-type TNF-α.[41−43] However, care should be taken when applying this strategy because the mutation may lead to loss of the biological function of the protein.[44]

2.1.3. Chemical Modification. Chemical modification is an alternative method to introduce functional groups on proteins for further conjugation of polymers. It should be noted that, when linking polymers to proteins, bifunctional linkers are usually used to avoid the problems associated with low reactivity when the large protein and polymer molecules are brought together. The readers can find a comprehensive summary of the bifunctional linkers in the monograph.[45] A simple example is a heterobifunctional linker of NHS-activated carboxyl acid spacer terminated with a maleimide group. A protein with the ϵ-amine group or the α-N terminus is first reacted with the NHS-activated carboxyl acid on the spacer, resulting in a maleimide functional group on it. Following a second reaction with a thiol-terminated target polymer by the high efficiency thiol−ene click chemistry, the conjugation can be achieved in a higher yield compared with that of the direct linkage between the protein and polymer.[6] Figure 3 shows two typical bifunctional linkers[45] where the DSP is water-insoluble and must be dissolved in organic solvent before addition to a conjugation reaction. The disulfide bond can be cleaved by reducing agent; thus, the conjugation is reversible when this linker is used. The NHS-PEG$_4$-malimide linker is water-soluble with four repeated ethylene glycol units. Researchers can choose the linkers accordingly to coordinate their applications. The second branch of chemical modification is the formation of a protein macroinitiator. In this strategy, an initiator is attached to a specific amino acid on the protein followed by growth of the desired polymer chain from the initiator. This functionalization of protein will be introduced in detail in the following "grafting from" method section.

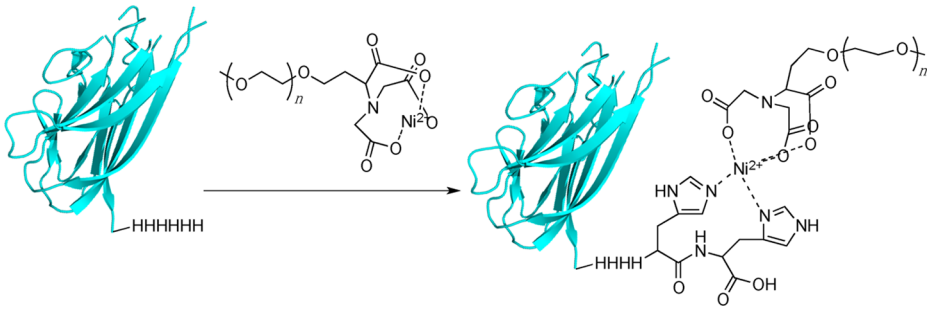

Figure 2. PEGylation using a His-tag approach. A protein with a six-histidine tag is conjugated with Ni-nitrilotriacetic acid (NTA)-PEG reagent. Reprinted from ref 12 under a Creative Commons Attribution 4 International License. https://creativecommons.org/licenses/by/4.0/. Copyright 2015, Dozier et al.

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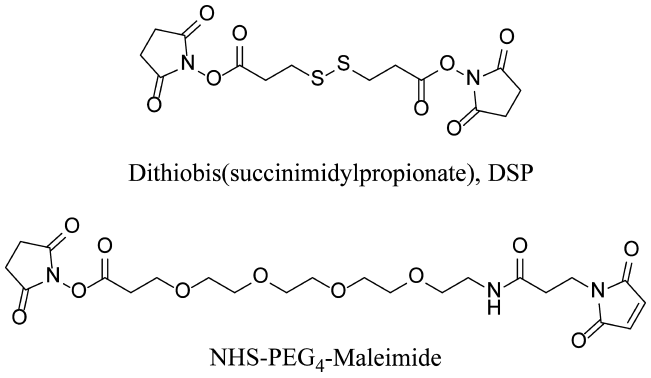

Figure 3. Two typical bifunctional linkers.

2.1.4. Noncanonical Amino Acid Incorporation. Only limited functional groups are covered in the natural amino acids, and the specificity of those groups is relatively poor under the common aqueous reaction conditions. Recent advances in click chemistry, especially the bioorthogonal reaction, provide a new field for highly efficient site-specific protein labeling techniques.[46−48] A variety of unique reactive noncanonical amino acids have been designed and incorporated into proteins at specific points to overcome the poor site-specificity of classical bioconjugation to natural amino acids.[49] The main approaches adopted to introduce noncanonical amino acids are genetic engineering and semisynthetic incorporation with solid-phase peptide synthesis (SPPS). Usually, proteins are synthesized within the ribosomes in cells. The process begins with DNA transcription, which directs the synthesis of mRNA wherein DNA transfers its information to the corresponding mRNA. Then, the mRNA is translated by the ribosomes to create a primary amino acid sequence of the protein.[2] The whole process is simplified as the central dogma in molecular biology and can be interrupted in various ways to incorporate engineered noncanonical amino acids at specific sites on the protein. Generally, the genetic code expansion method, as shown in Figure 4, is utilized to introduce site-specific noncanonical amino acid during the protein translation process in living cells.[46,50,51]

Noncanonical amino acids with orthogonal chemical reactivity enable the direct synthesis of structurally defined protein−polymer conjugates under mild conditions with high efficiency without alternating the biological functionality of the host protein.[52,53] Figure 5 lists typical noncanonical amino acids that have been genetically encoded and can be potentially applied in protein therapeutics.[54] For instance, Cho and co-workers recently incorporated *p*-acetylphenylalanine (pAcF) at residue 35 in human growth hormone (hGH) and site-specifically conjugated a PEG chain onto it via Schiff base formation with an efficiency as high as 97%.[55] The mono-PEGylated hGH produced exhibited favorable pharmacodynamics properties in GH-deficient rats. Further clinical study reveals that the PEGylated hGH showed not only comparable efficacy and safety to native hGH but also increased potency and reduced injection frequency. Similarly, Matyjaszewski et al. site-specifically incorporated *p*-azidophenylalanine (pN_3F) into GFP, and the resulting diN_3-GFP was copolymerized with diacetyl-PEG via the azide alkyne Huisgen cycloaddition.[56]

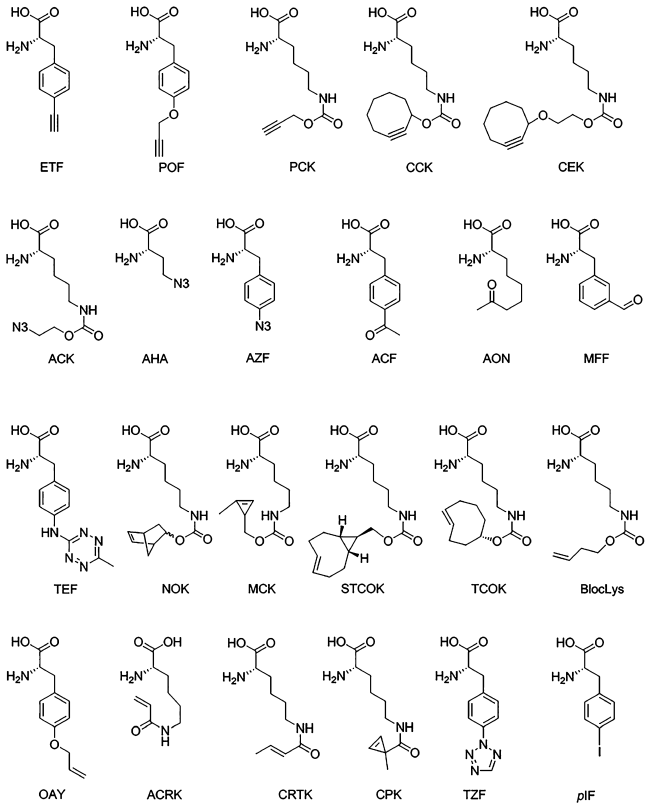

Figure 5. Typical noncanonical amino acids for the potential bioorthogonal reaction with functional polymers. Reproduced from ref 23 with permission of The Royal Society of Chemistry. http://dx.doi.org/10.1039/c6py00856a.

2.1.5. Enzyme-Mediated Introduction of Functional Groups. Alternatively, functional groups can be incorporated into proteins at specific sites via enzymatic reactions,

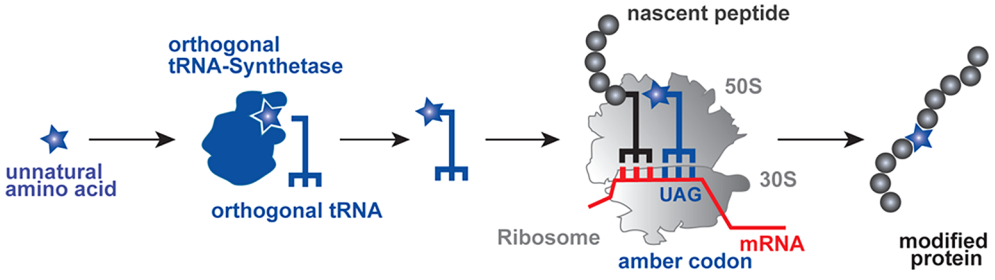

Figure 4. Expanding the genetic code. Noncanonical amino acid (blue star) presented to the cell cultural medium is specifically recognized by the orthogonal aminoacyl tRNA synthetase and attached to an orthogonal amber suppressor tRNA, which is further decoded by the ribosome in response to an amber codon (UAG) introduced into the gene of interest, allowing the synthesis of a protein with a site-specifically introduced noncanonical amino acid. Reproduced from ref 46. Copyright 2014, American Chemical Society.

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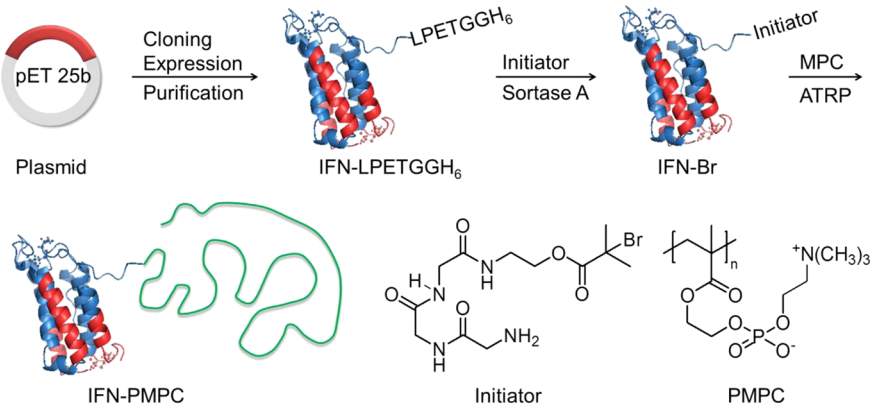

Figure 6. Sortase A-mediated site-specific conjugation of interferon-α via the ATRP process. Reproduced from ref 59. Copyright 2016, with permission from Elsevier.

particularly the moieties that can undergo biorthogonal reactions. Enzymatic-mediated protein−polymer ligation has become an area of great interest due to its high specificity and efficiency. In this method, a special short peptide (tag) is first incorporated into the protein of interest at the site to be modified. The enzyme and the functional molecules bearing the complementary substrate are then introduced to incorporate the functional groups. For instance, Sortase A can catalyze a transpeptidase reaction where it recognizes a specific internal sequence LPXTG of a protein and cleaves the bond between the threonine and glycine, forming a covalent acyl-enzyme intermediate.[57] This thioester intermediate is then attached by the N-terminal amine group of an oligoglycine peptide, resulting in formation of an amide bond between the substrate protein and the incoming nucleophile. One example of synthesizing protein−polymer conjugates by Sortase A-mediated ligation is the site-specific PEGylation of four-helix bundle cytokines, which successfully extends the cytokine half-life with full retention of biological activity.[58] Recently, Gao and co-workers reported the in situ growth of poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC) via the ATRP process from the C-terminal of interferon-α with high yield and activity by Sortase A-catalyzed ligation, as shown in Figure 6.[59] Other generally used enzymes for protein labeling, such as transglutaminase,[60] lipoic acid ligase,[61] and formylglycine generating enzyme[62] can be found in recent reviews along with the recognition tags, substrate, k_M, and modification site.[63−65]

2.2. "Grafting to" Method. In the grafting to method, the target protein and the preformed functionalized polymer react with each other to produce the protein−polymer bioconjugate. The first work was introduced by Abuchowski and co-workers during the 1970s.[4,66] In this initial work, BSA was covalently attached by cyanuric chloride-activated monomethoxypolyethylene glycol (mPEG). The modified BSA with sufficient PEG lost its immunogenicity due to the stealth effects of the incorporated PEG. Since then, numerous protein−polymer conjugates have been prepared via various techniques to fulfill applications in medicine, sensing, enzymatic catalysis, and so forth.[1]

In this strategy, the functional polymer moiety is synthesized separately before the conjugation reaction, potentially broadening the possible polymer spectrum in both the composition and structure because not all of the polymers can be prepared in situ during the conjugation. A typical example is PEG, which is generally synthesized by living anionic polymerization technique. The commercial hydroxyl-terminated PEG with different molecular weights and topological structures can be readily transferred to functional groups that can be used in the grafting to method for protein conjugation.[67−71] This user-friendly modularity is especially attractive for interdisciplinary researchers to prepare functional protein−polymer conjugates. However, this strategy is accompanied by low reaction efficiency between the two macromolecules induced by the steric hindrance and low reactive group concentration. For improving the reaction yield, the polymer is generally fed in an excess molar ratio, leading to a tedious purification process thereafter.

For high quality protein−polymer conjugates with reasonable output to be achieved through the grafting to method, the coupling reaction between the polymer and the protein is required to be (1) specific for the target conjugation site, (2) highly efficient in an ambient environment, particularly under physiological conditions, and (3) nondisruptive toward the structure and functionality of the target protein. In the following sections, a detailed summary of the reaction substrates will be presented.

2.2.1. Natural Amino Acid-Based Reaction. Table 2 lists commonly used complementary reaction group pairs for in chain protein conjugation.[5,72,73] It should be noted that the specificity and efficiency of the listed reaction pairs depend largely on the structure of the protein and the neighboring amino acid sequence. The reaction conditions, especially the catalyst, pH, reaction medium, and temperature, need to be carefully selected for optimum specificity and yield. The readers are encouraged to refer to the comprehensive review by Baran and co-workers.[73] Further, if there are numerous residues in the protein the same as the target one, it is necessary to combine technologies introduced in section 2.1 to direct the reaction specifically to the predefined site.

A special case arises when the reaction targets the terminal amino acid due to the unique microenvironment.[74] The bioconjugation reaction mainly utilizes the α-amino group and the specific residue. The difference in the pKa (∼2 units) of the N-terminal α-amino group and that in lysine discriminates their reactivity and endows a unique reaction type for the N-terminal α-amino group. Table 3 lists typical reactions that target the N-terminal amino acid.

2.2.2. Reactions beyond Natural Amino Acids. Despite the recent advancement in the reaction regime for protein bioconjugation based on the natural amino acid, there is still an increasing demand for a precise and user-friendly conjugation technique with high yield. The marriage of

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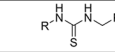
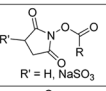
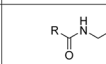
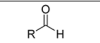
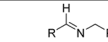
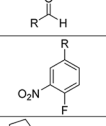
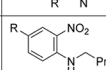
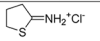
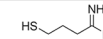
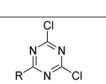
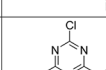
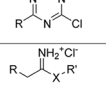
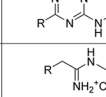
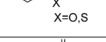
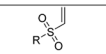
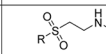
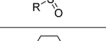
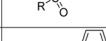
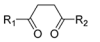
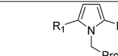
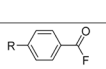
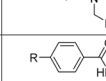
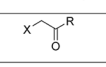
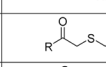
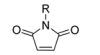
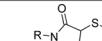
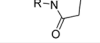
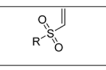
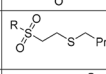
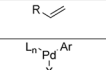
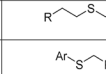
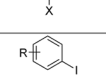
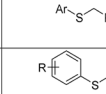
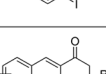
Biomacromolecules XXXX, XXX, XXX−XXX

Table 2. Typical Reactions Toward the Specific Amino Acid Residues[a]

Amino Acid and Chemical Structure	Complementary functional group	Reaction product	Refs
 Lysine			75
			76-79
			80, 81
			82
			83
			4, 66
			84, 85
			86
			87
			88
Cysteine			89
			90, 91
			92
			93
			94
			95, 96
			97
			78, 98
			99
			100
			101
			102
			103
			104

Amino Acid and Chemical Structure	Complementary functional group	Reaction product	Refs
			105
			106
			107
Tryptophan			108
			36
			109
Tyrosine			110
			111
			112
			113-115
			116
			117
Arginine			118
Histidine			119
Aspartic acid Glutamic acid			120, 121
Methionine			122

[a]The reactive moiety of the amino acid is highlighted in red. The chirality in the functional groups is not shown for clarity.

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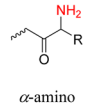
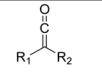
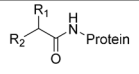
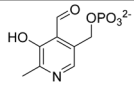
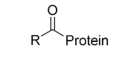
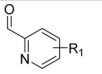
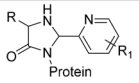
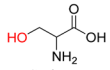
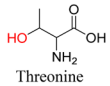
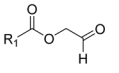
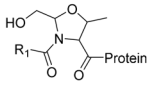
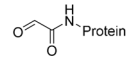
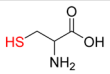
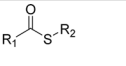
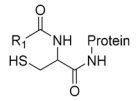
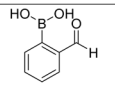
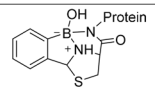
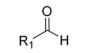
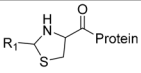
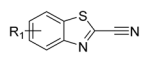
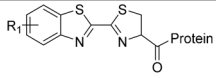
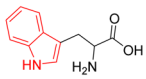
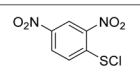
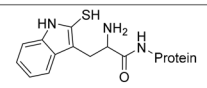
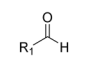
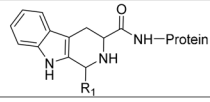
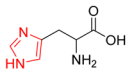
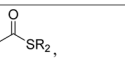
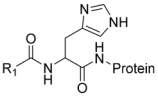
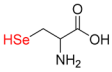
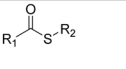
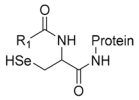
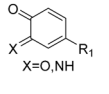
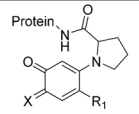
Biomacromolecules XXXX, XXX, XXX−XXX

Table 3. Typical Reactions Toward the Terminal Amino Acid[a]

Amino Acid Chemical Structure	Complementary functional group	reaction product	Refs
 α-amino			123
			124
			125
 Serine  Threonine			126
	IO_4^-		127
 Cysteine	 , with R_2 a good leaving group		128, 129
			130
			131, 132
			133
 Tryptophan			134
			135, 136
 Histidine	 with R_2 a good leaving group		136
 Selenocysteine	 , with R_2 a good leaving group		137
 Proline	 X=O,NH		138

[a]The reactive moiety of the amino acid is highlighted in red. The chirality in the functional groups is not shown for clarity.

incorporating noncanonical amino acids and bioorthogonal reactions offers new opportunities for site-specific protein modification.[23,47,48,139−141] A typical example is the azide alkyne Huisgen cycloaddition. In this strategy, the azide and alkyne group are introduced to the protein and polymer separately, and the cycloaddition reaction can proceed efficiently in PBS in the presence of Cu(I).[142] The reaction is further evolved by using electron-withdrawing fluorine-substituted cyclooctyne, as shown in Figure 7, which eliminates the utilization of toxic Cu(I) with the aid of ring strain and the electron-withdrawing group.[143−146]

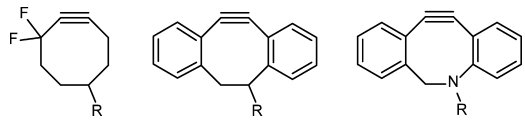


Figure 7. Typical ring-strained cyclooctynes for bioorthogonal cycloaddition reactions with azides.

Recently, Francis and co-workers further simplified the reaction by taking advantage of the supramolecular chemistry.[147] It has been known that the Cucurbit[6]uril (CB6) forms a heteroternary complex with propargylamine and azidoethylamine derivatives, whereby the protonated amine moieties form a hydrogen bonding network with the carbonyl portal of CB6. Such alignment of azido and alkyne groups in the cavity of CB6 facilitates the triazole formation even without the catalysis of Cu(I). This strategy avoids the employment of the substituted ring-strained cyclooctynes, which is generally synthesized in a tedious routine. Table 4 summarizes the typical bioorthogonal reactions for the bioconjugation of polymer and protein.

2.3. "Grafting from" Method. After the pioneering work by Russell et al.[167] and Maynard et al. in 2005,[33] the grafting from method has gained increasing attention due to its excellent yield and ease of purification.[168] Different from the grafting to method, where two macromolecules joint together to form the hybrid molecule, the grafting from method involves the in situ grow-up of a polymer chain from the specific protein/peptide. For this method, a small molecular initiator or chain transfer agent (CTA) is first introduced to the target protein via the well-established bioconjugation reactions as summarized in the "Grafting to" Method section followed by a polymerization process. Compared with the grafting to method, the grafting from method utilizes the newly developed reversible deactivation radical polymerization technique, mainly containing reversible addition−fragmentation chain transfer (RAFT)[169] and atom transfer radical polymerization (ATRP)[170] to control the conjugation site, chain length, polydispersity, and stoichiometric ratio between the host protein and the guest polymer. In such a process, the reaction between two giant macromolecules, where there is a profound steric hindrance during the reaction, is avoided, leading to excellent yield of the target protein−polymer conjugates. Further, the "free" small molecular initiator can access reaction sites that are buried inside the protein conformation, expanding the candidate proteins for further biomedical applications. Moreover, the purification process also becomes straightforward and less time-consuming. It is much easier to separate the conjugates from the unreacted small molecular monomers and catalyst, which is especially important for further medical applications because batch-to-batch variation could be minimized.

However, limitations also come with this method. For the biological function of the protein to be maintained, the reaction conditions have to be compromised to retain the integrity of the protein's structure. During the polymerization process, the reaction center should not undergo side reactions with

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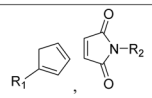
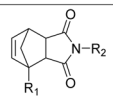
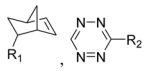
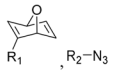
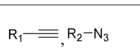
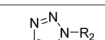
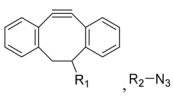
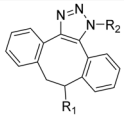
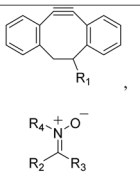
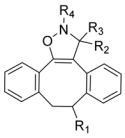
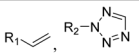
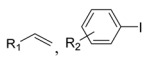
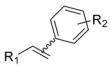
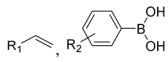
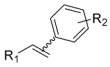
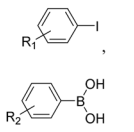
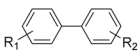
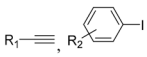
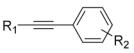
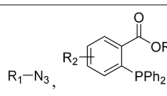
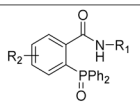
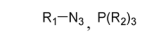
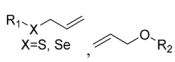
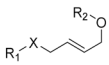
Biomacromolecules XXXX, XXX, XXX−XXX

Table 4. Bioorthogonal Reactions for the Conjugation of Polymer and Protein[a]

Reaction Type	Typical Substrates	Major Product	Refs
Diels-Alder reaction			148
			149, 150
			151
Alkyne-azido reaction			152
			145
Alkyne-nitrone addition			127
Photo-click reaction			153, 154
Mizoroki–Heck reaction			155
			156
Suzuki-Miyaura reaction			157, 158
Sonogashira reaction			159
Staudinger reaction			160-162
			163, 164
Cross-metathesis			165, 166

[a]The reaction conditions are not listed due to the diversity of the substrate. The readers are encouraged to refer to the original articles.

abundant functional groups present on the amino acid residues. Further, special attention should be paid to the reaction solvent and temperature to avoid denaturing of the protein.[24] Currently, only some reversible deactivation radical polymerizations can fulfill this requirement, e.g., ATRP and RAFT. The anionic and cationic living polymerization are forbidden due to their high reactivity and the harsh requirements during the polymerization process. This excludes directly employing the most classical polymer, poly(ethylene oxide) (PEO), in the grafting from method. Moreover, in this method, it is also difficult to characterize the grafted polymer's molecular weight profile and composition in the case of copolymer.

In this section, recent advances in synthesizing protein–polymer conjugates via the grafting from method by using the reversible deactivation radical polymerization, particularly those in the aqueous environment at ambient temperature, will be summarized.

2.3.1. "Grafting from" Method Based on ATRP. The ATRP process, independently developed by Sawamoto and Matyjaszewski,[171,172] is a controlled reversible-deactivation radical polymerization in which the deactivation of the radicals involves reversible atom transfer or reversible group transfer catalyzed usually, though not exclusively, by transition metal complexes.[173] The transition metal generally used is Cu, but other metals have also been documented, e.g., Ru, Fe, Mo, Os.[170] For a typical ATRP reaction, an initiator, mainly alkyl halides, together with a cuprous halide and a proper ligand with proper stoichiometric ratio, which form the transition metal complexes, are introduced into the reaction system besides the monomer and the solvent. The initiating alkyl halides/macromolecular species (P_nX) form an equilibrium during the ATRP process, as shown in Figure 8, which result in the controlled manner of chain propagation.

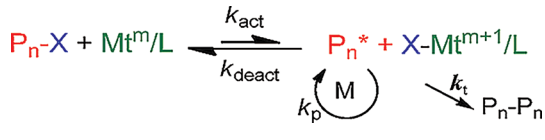


Figure 8. ATRP equilibrium. Reproduced from ref 170. Copyright 2012, American Chemical Society.

However, the primary ATRP process utilizes nearly the same amount of transition metal as the initiator, where a follow-up purification to remove the excess metal salt is inevitable, potentially leading to the possible heavy metal residual problem if further applied in the biomedicine areas. Recent advancement in ATRP, such as activator regenerated by electron transfer (ARGET) ATRP,[174−176] initiators for continuous activator regeneration (ICAR) ATRP,[177] or electrochemical ATRP (eATRP)[178] has lessened the amount of metal halide down to below 50 ppm while the reaction can still be performed in a controlled manner, where the metal salt removal or recycling would be unwarranted for many applications.[170,179]

After being developing over two decades, a library of initiators with various functional groups has been established to meet the tremendous application environments.[180] Lewis's patent first explored the possibility of protein−polymer conjugation via the grafting from method with the ATRP technique.[181] NHS-activated 4-(3-(2-bromo,2-methyl-propionate)phenyl)propionic acid was linked to lysozyme and successfully initiated the ATRP process. This work successfully demonstrated the potential of the grafting from method via the ATRP technique, but the site-specific problem was not addressed then. The following attempt by Russell's group further controlled the ratio between the polymer and the protein using the same strategy and showed that the conjugate

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Biomacromolecules XXXX, XXX, XXX−XXX

could still preserve the biological activity.[167] Later, Maynard's group linked the ATRP initiators to model protein BSA and lysozyme via the thiol−disulfide exchange reaction and thiol−ene reaction, resulting in the protein-based macromolecular initiator. Figure 9 shows the representative synthetic scheme

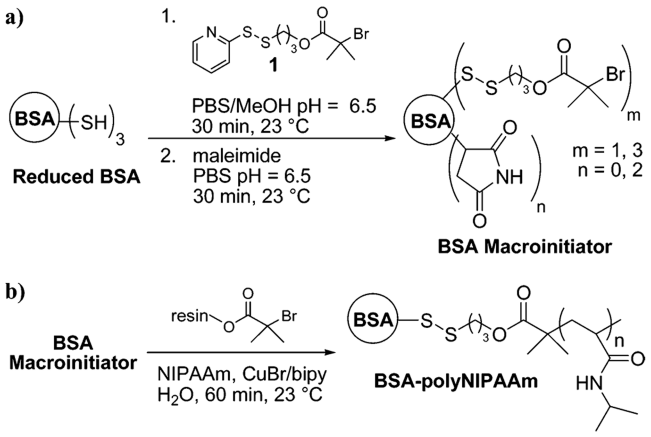

Figure 9. Scheme for synthesis of BSA-polyNIPAAm via the grafting from method. (a) BSA modification with the initiator fragment and "capping" of reduced BSA to form the BSA macroinitiator. (b) ATRP from the BSA macroinitiator in the presence of a 2-bromoisobutyrate-functionalized resin. Reproduced from ref 33. Copyright 2005, American Chemical Society.

based on BSA substrate.[33] Polymerization of *N*-isopropylacrylamide from the protein macroinitiators yielded the thermosensitive BSA-PolyNIPAAm. Further examination of the biological activity showed no distinction between the protein−polymer conjugates and the native protein, indicating preservation of the protein structure during the ATRP process.

Recently, a more straightforward method, as shown in Figure 10, was developed by Mehl and co-workers by combining the

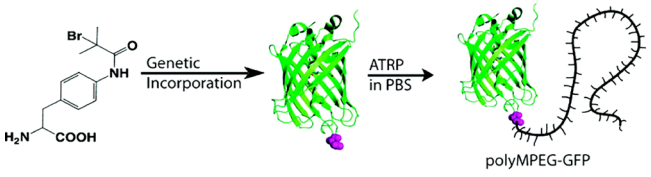

Figure 10. Genetically encoded ATRP initiator for site-specific polymer growth from protein. Reproduced from ref 182. Copyright 2010, American Chemical Society.

genetic engineering and grafting from method.[175,182] 4-(2-Bromoisobutyramido)phenylalanine, acting as both a non-canonical amino acid and ATRP initiator, was genetically encoded on the surface of green fluorescent protein (GFP). Such a protein-based macroinitiator was then used as an initiator for the ATRP to polymerize oligo(ethylene oxide) monomethyl ether methacrylate, efficiently producing a site-specific polymer−GFP bioconjugate.

2.3.2. Grafting from Method Based on RAFT Polymerization. Since first developed by Rizzardo and co-workers at CISRO in the 1990s, the RAFT polymerization technique has been one of the most robust and versatile methods for controlling the radical polymerization process.[183] It is now possible to have precise control over the molecular weight, distribution, and topology structure by carefully choosing the CTA and relevant reaction conditions.[184−186] The overall reaction mechanism of the RAFT polymerization process is shown in Scheme 3.

The key member of the RAFT polymerization is CTA, which includes thiocarbonylthio compounds, dithiocarbamates, trithiocarbonates, and xanthates. A library of CTAs for the RAFT technique has been established over the decades for chemists to choose from for covering various applications.[187,188] For the synthesis of protein−polymer bioconjugates via the grafting from method through RAFT polymerization, the main challenge lies in the appropriate choice of CTA that can (1) react with the protein in high yield and (2) efficiently control the reaction outcome in an aqueous environment without alternating the structure of protein.

Structurally, the RAFT CTA is typically composed of R and Z moieties as shown in Scheme 3. The R group is a free leaving group that must also be able to reinitiate the polymerization, whereas the Z group is responsible for the stabilization of the intermediate radicals that are produced during the polymerization.[189] Approaches based on both moieties have been adopted to prepare protein−polymer conjugates via the RAFT process, as shown in Figure 11.[190] Typical examples will be analyzed in the following context.

The Z group-based strategy yields a biohybrid with a cleavable thiolcarbonylthiol joint between the protein and the polymer. Hence, the resulting bioconjugates can be split to the original protein and the grafted polymer, providing an opportunity to analyze the grafted polymer chain in detail. On the basis of this characteristic, it can be further applied to the drug delivery areas.

The first work on bioconjugation between protein and polymer via the grafting from method by RAFT technique is targeted on the Z group.[191] Boyer and co-workers prepared a water-soluble RAFT agent and linked it to BSA via the thiol−disulfide exchange reaction, as shown in Figure 12. Further, *N*-isopropylacrylamide and hydroxyethyl acrylate were successfully polymerized in a controlled manner in aqueous medium at 25 °C using the prepared BSA-based macroRAFT agent, as determined by the SEC, ^{1}HNMR, MALDI-TOF, PAGE, and kinetic analyses after cleaving the polymer moiety from the conjugates. Most importantly, the structural integrity and the conformation-related esterase activity of BSA were confirmed to not be hampered during the polymerization process.

On the other hand, when CTA is linked to the protein/peptide via the R moiety, the following grafting from method via the RAFT process will have a better control over the molecular weight because the thiolcarbonylthiol group is distal to the protein and is potentially more accessible for chain transfer with propagating chains in aqueous solution.[192] The resulting bioconjugates have the thiolcarbonylthiol moiety in the end, leading to a more stable biohybrid because the thiolcarbonylthiol moiety is prone to cleavage by the amine group. Further, the end group can be transferred to the thiol group for subsequent surface immobilization, labeling, or chain extension.[190]

Right after the appearance of the Z group method, the R group method was introduced by Sumerlin and co-workers using the identical model protein, BSA, and the same chemistry between the thiol group on the protein and the maleimide group on the CTA (as shown in Figure 13).[192] After cleaving the protein, the remaining polymer was found to be in good control over the molecular weight and distribution, as expected for the RAFT process. The secondary structure and biological

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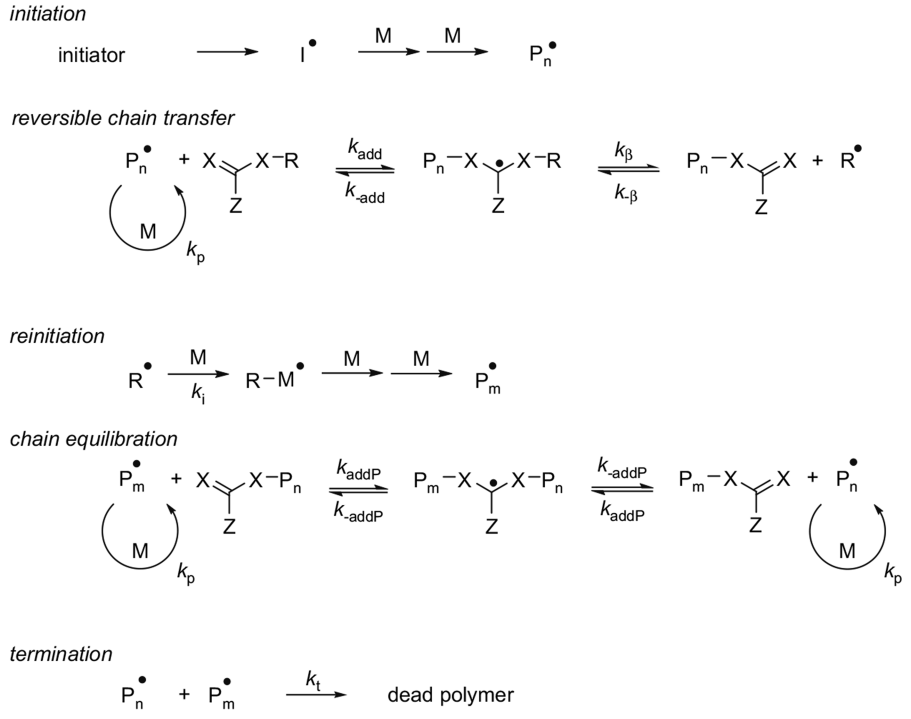
Scheme 3. Mechanism of RAFT Polymerization. Reproduced from ref 187. Copyright 2007, with permission from Elsevier



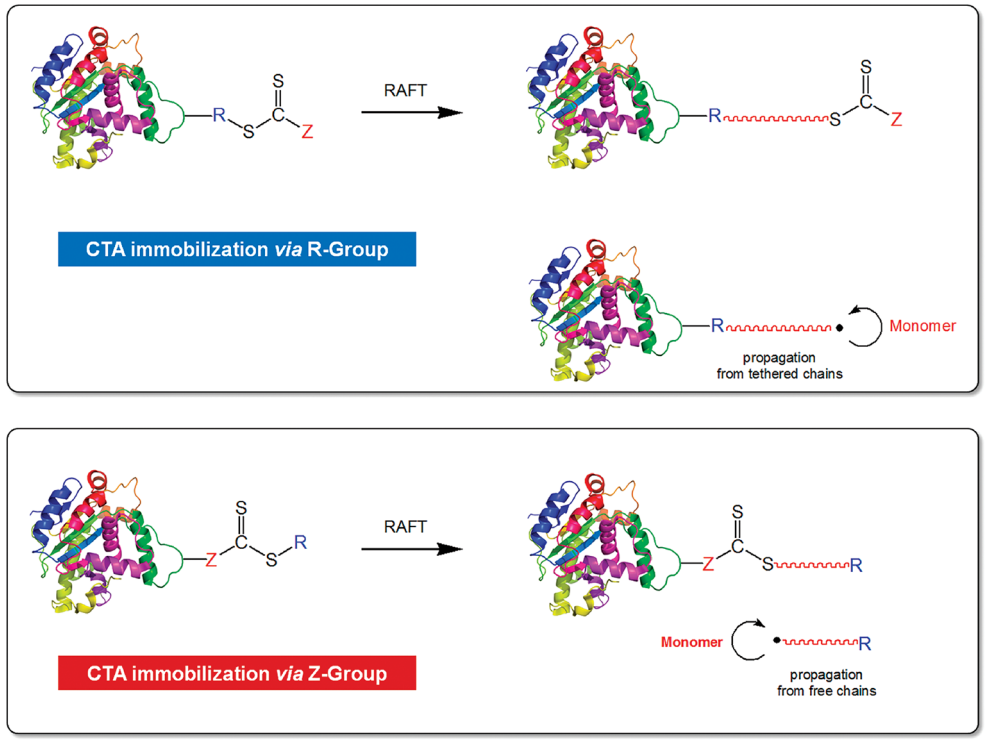


Figure 11. R (top) and Z group (bottom)-based strategies for preparing polymer−protein conjugates by reversible addition−fragmentation chain transfer (RAFT) polymerization. The CTA lies in a different position in the final protein−polymer conjugates. Reproduced from ref 190. Copyright 2012, American Chemical Society.

activity were confirmed to be intact by circular dichroism and the esterase activity assay.

2.4. Noncovalent-Based Conjugation Strategy. A particular field in the synthesis of site-specific protein−polymer conjugates utilizes the noncovalent bond strategy and is introduced here for its high specificity and reversibility.[193] The aforementioned bioconjugation methods are typically based on covalent bonds, where there is a strong and stable linkage between the polymer and the protein. The bond energy typically lies between 100 and 400 kJ/mol.[194] As the structures of macromolecules and functional materials have continued to evolve with higher degrees of complexity and function,

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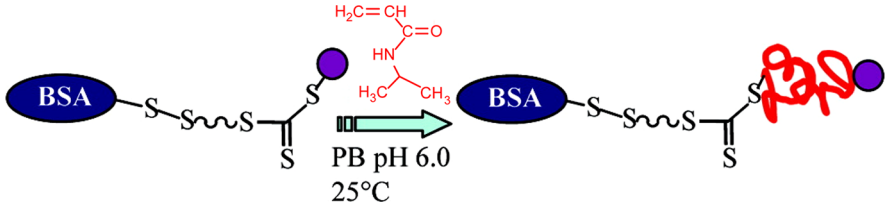

Figure 12. Synthesis of BSA-PNIPAAm via the grafting from method by RAFT polymerization (Z approach). Reproduced from ref 191. Copyright 2007, American Chemical Society.

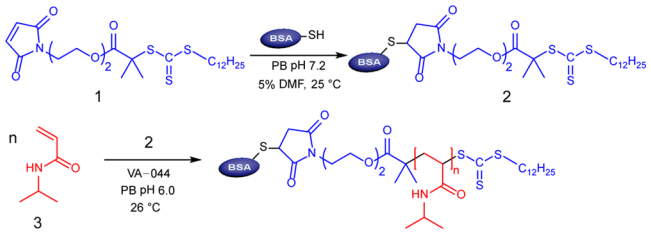

Figure 13. Synthesis of BSA-PNIPAAm via the grafting from method by RAFT polymerization (R approach). Reproduced from ref 192. Copyright 2008, American Chemical Society.

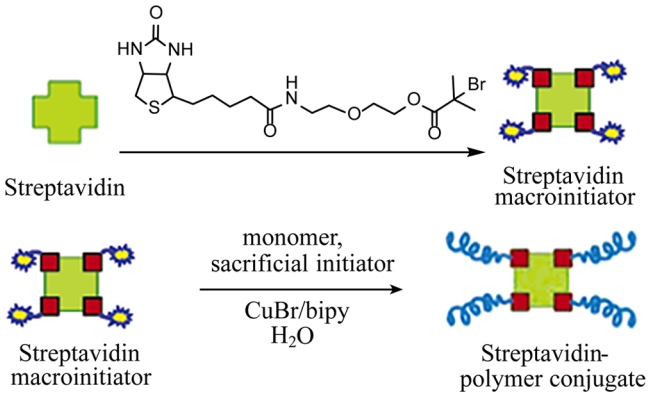

Figure 14. Streptavidin macroinitiator formation through biotin-streptavidin recognition followed by ATRP of NIPAAm to yield a well-defined streptavidin–PNIPAAm conjugate. Reproduced from ref 203. Copyright 2005, American Chemical Society.

traditional covalent-based synthetic strategies have become increasingly difficult to employ. Noncovalent approaches rely on physical interactions with relatively weak intermolecular forces. A stable linkage between the protein and the polymer can be achieved if there is a cooperative effect between the noncovalent bonds. Over the past few years, a noncovalent bond-based strategy has been exploited for advances in the design of sophisticated biomolecules with complex architectures.[9,195] The noncovalent interactions include affinity interactions,[196] electrostatic, metal-mediated coordination,[197] hydrophobic,[198] and hydrogen bonding,[199] but the design of protein–polymer conjugates should be careful for the site specificity.

2.4.1. System of Biotin-(Strept)avidin Interactions. Numerous supramolecular bioconjugation strategies employ the guest–host system, among which the biotin-(strept)avidin interaction is well-studied for its exceptionally high affinity (K_a = 10^{13} and 10^{15} M^{-1} for biotin-streptavidin and biotin-avidin, respectively) and consequent stability of this noncovalent interaction.[200,201] Both avidin and streptavidin possess four binding sites per molecule, and the binding to biotin is specific enough to ensure the binding is directed only to the target of interest. Moreover, biotin is such a small molecule (244 Da) that, when introduced to biomacromolecules, it usually does not affect their biological activity.[202] Thus, the biotin-(strept)avidin system has become a popular method for protein modification, and a number of biotinylated initiators have been designed for the synthesis of bioconjugates. For example, after the streptavidin was modified with a biotinylated initiator, the following site-specific ATRP of *N*-isopropylacrylamide (NIPAAm) resulted in a well-defined streptavidin-PNIPAAm conjugate, where the protein is quantitatively modified with PNIPAAm and the conjugation is at the biotin binding site only (Figure 14).[203] When the resulting conjugate is treated by the dissociation conditions of streptavidin/biotin, the original protein can be recovered as evidenced by SDS-PAGE, indicating a reversible nature of the ligation between the protein and polymer. Similarly, biotin-maleimide difunctional PNIPAAm synthesized by biotinylated CTA via the RAFT polymerization of NIPAAm has also been grafted to fluorescent streptavidin.[204]

In addition to the success in these conceptual designs, the strategy has also found application in the biomedicine area. Pardridge and co-workers used the Trojan horse strategy and avidin–biotin technology to conjugate the human insulin receptor (HIR)-targeted monoclonal antibody-modified avidin (HIRMAb-AV) and biotinylated [^{125}I] Aβ^{1-40}, resulting in a fusion protein that can bypass the blood-brain barrier (BBB) due to transportation through endogenous insulin receptors.[205] The conjugate with biotinylated [^{125}I] Aβ^{1-40} can bind to the amyloid plaque in a brain with Alzheimer's disease (AD) to the same extent when a radiopharmaceutical peptide is either free or conjugated as shown by the film autoradiography and tissue sections of autopsy AD brain, proving its further application in the field of neuro-imaging agents that penetrate the BBB.

2.4.2. Metal-Mediated Noncovalent Conjugation. Metal-mediated interactions could be an alternative noncovalent strategy for the preparation of well-defined protein–polymer conjugates. The interactions are mainly via weak coordination bonding, whereby stability of conjugates is dictated by the equilibrium dissociation constants so that this kind of protein–polymer conjugate is reversible for special design. Because the imidazole rings of the polyhistidine motif are well-known for binding with divalent metal cations (Co^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}),[194] the use of polymers containing Ni^{2+}-complexed nitrilotriacetic acid (Ni-NTA) for the bioconjugation with genetically modified histidine-tagged (His-tag) proteins is straightforward. A typical example is the synthesized nitrilotriacetic acid end-functionalized polystyrene (NTA-PS) for the controlled conjugation with histidine-tagged green fluorescence proteins (His_6-GFP),[206] where Ni-NTA-PS produced well-defined micelles with His_6-GFP in water/DMF system and the size of the micelles can be controlled by the amount of imidazole

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Biomacromolecules XXXX, XXX, XXX–XXX

added. Similarly, Favier and co-workers synthesized an NTA-functionalized RAFT agent, and a fluorescent RAFT polymer bearing a nitrilotriacetic acid (NTA) ligand at the chain end was successfully obtained, as shown in Figure 15. Model His-tagged proteins, including HisTag-TOX4PG, HisTag-INT, and HisTag-NCL, were successfully conjugated by the aforementioned NTA fluorescent polymer as demonstrated by the SDS-PAGE results.[207]

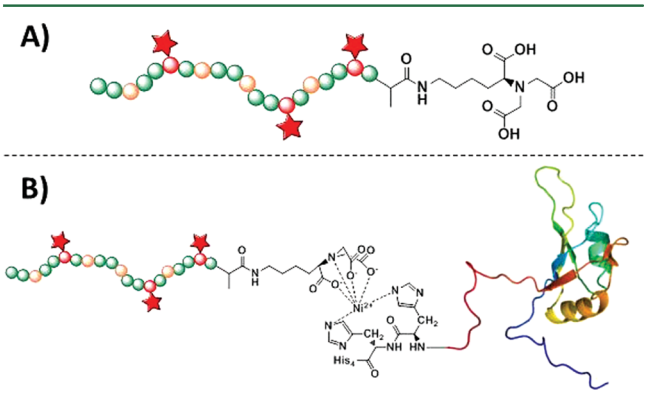

Figure 15. Metal-mediated noncovalent site-specific conjugation of protein and polymer using a combination of nitrilotriacetic acid (NTA), Ni^{2+}, and His-tag. Reproduced from ref 207, with permission of The Royal Society of Chemistry. http://dx.doi.org/10.1039/c6py02222g.

3. APPLICATIONS

Over the past decades, the design and synthesis for protein–polymer conjugates, especially site-specific modifications, have attracted increasing attention from researchers, and new properties and functions are developed for applications. Protein–polymer conjugates are suitable for applications in many fields, and typical cases will be briefly introduced here.

3.1. Protein Stabilization. Proteins are usually unstable and may denature or degrade within days or weeks when exposed to environmental changes, such as temperature and pH. When applied in therapeutics, they possess intrinsic limitations for large-scale applications, e.g., low in vivo stability, short half-life time, and immunogenicity.[208] Consequently, an excessively high protein dose is required to achieve the desired therapeutic effect in vivo, which may be toxic to healthy organs or tissues and is manifested by a number of side effects.[209,210] Conjugation of polymer chains to the protein potentially increases the hydrodynamic volume and decreases the renal excretion rate. Further, the conjugated polymer chains can shield the protein from enzymatic degradation and increase the serum half-life time.[22] Hence, conjugation of a polymer chain, particularly PEGylation, has been an efficient method in improving the pharmacokinetic properties of the therapeutic protein drugs due to its nontoxic and hydrophilic nature. However, the effect of PEGylation is not consistent in every protein as summarized by Price et al.[211] Constructive, destructive, and neutral effects have all been observed experimentally, leading to difficulty in predicting the PEGylation effect. As pointed out by Price, it is partially due to the nonspecific conjugation strategies that generate a heterogeneous mixture of PEGylated proteins that differ in conjugation number and sites. By site-specific PEGylation on the model WW domain of the human protein Pin 1, they clarified the effect of PEG chain length on the conformational stability of the protein. Further, criteria for selecting the conjugation site of PEGylation on proteins for higher thermodynamic and proteolytic stability were established, which successfully predicted the optimal PEGylation site of the chicken Src SH3 domain.[212] Price's work undoubtedly demonstrates the potential of site-specific conjugation in developing optimal protein–polymer drugs with advanced stability in vivo. Recently, Pasut and co-worker reported the synthesis of two site-specific mono-PEGylated forms of human growth hormone (hGH), chemically N-terminal PEGylated hGH (PEG-Nter-hGH) and enzymatically Gln141 PEGylated hGH (PEG-Gln141-hGH) via transglutaminase.[213,214] The results showed that the conjugates have prolonged half-life in vivo and possess a higher thermal stability. Other examples of site-specific preparation of PEGylated protein drugs are well-reviewed eleswhere.[215,216]

Besides the generally used PEG, the PEG-like polymers, particularly the poly(oligo(ethylene glycol) methyl ether methacrylate) [poly(OEGMMA)], are also widely used to improve the pharmacokinetics of the therapeutic proteins. The methacrylate moiety in the monomer makes it possible to synthesize site-specific polymer conjugates via the grafting from approach, which is inaccessible for the PEG conjugates. In Gao and Chilkoti's recent works, poly(OEGMMA) was conjugated to the C/N terminus of the protein (GFP or myoglobin) via ATRP using the grafting from approach, leading to stoichiometric site-specific protein–polymer conjugates.[217,218] Such conjugates successfully improve the pharmacological properties of proteins. Recently, Gao's group further applied this method to modify the protein interferon-α.[219,220] Compared with the commercial PEGASYS (PEGylated interferon-α), the C-terminal-conjugated POEGMMA interferon-α shows a 7.2-fold higher in vitro antiproliferative bioactivity. Further, the site-specific conjugation of poly(OEGMMA) on exendin-4 was found to eliminate the PEG antigenicity without compromising the in vivo drug efficiency when the number of EG repeating units in the side chain equals 3.[221] These advancements in the protein engineering establish a next-generation technique for improving the pharmacological performance of traditional PEGylated drugs.

Further, the stabilization effect of the polymer conjugation has also been tested in the oral therapeutic protein drugs besides the traditional subcutaneous injection. Several protein drugs are now in clinical use or under development for the treatment of pathologies, such as celiac disease and phenylketonuria.[222] However, the orally administered protein drugs meet several challenges due to the harsh environment in the gastrointestinal tract, especially in the stomach. Proteins will be digested or unfolded during the drug delivery process due to the low pH, leading to inactivation of the drugs. The cellular uptake efficiency of the drugs is also compromised due to the presence of barriers.[223] Recently, Leroux et al. realized the stabilization of orally administered therapeutic enzymes (exogenous proline-specific endopeptidase, PEPs) at different locations in the gastrointestinal tract by random covalent conjugation with a polycationic dendronized mucoadhesive polymer.[224] However, the activity of the conjugate was ~4-fold less than that of the wild-type enzyme in this random conjugation. By site-specific conjugation of the polymer (including PEG40 and PAMAM) via the thiol–ene reaction at the cysteine residue, the stability of the conjugates was further achieved both in vitro and in vivo without compromising the catalytic effect.[225] These works lay the

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Biomacromolecules XXXX, XXX, XXX–XXX

foundation for the development of new therapeutic and imaging strategies based on the orally administered proteins and may potentially ease the drug delivery of certain therapeutic proteins.

Another application of the site-specific polymer conjugation to proteins is in the stabilization of some disease-related proteins. The misfolding of prion proteins into insoluble amyloid fibrils is not only useful for biomaterials[226] but is also associated with a range of neurodegenerative-related diseases, such as Alzheimer's disease (AD).[227,228] Therefore, a method that can inhibit the fibril formation could be a potential treatment for such diseases. Previously, Perrier and co-workers demonstrated a water-soluble poly(hydroxyethyl acrylate) (PHEA) conjugation to azide-functionalized β-sheet forming polypeptide (P_{11}-2). The nascent peptide segment has a strong tendency to form fibril structure at acidic pH, but the β-sheet formation and hence fibrillization tendency of P_{11}-2 was severely diminished after PHEA conjugation, as shown in Figure 16. The chimeras were observed to inhibit fibrillization

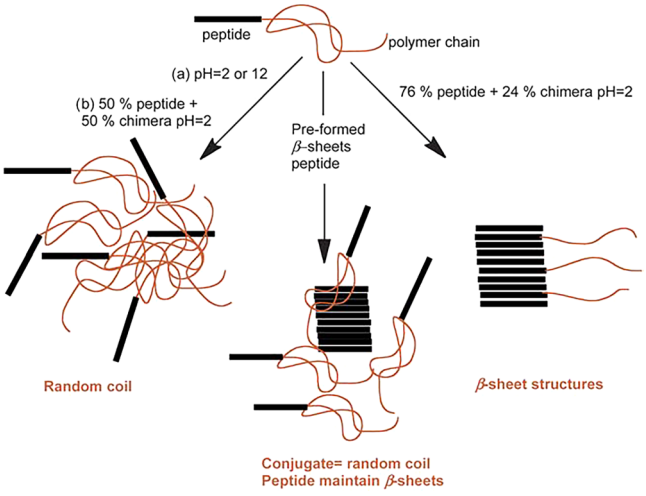

Figure 16. Schematic representation of the self-assembly behavior of peptide P_{11}-2 and its polymer conjugate (chimera), where different amounts of chimeras are mixed with free peptide and pre-self-assembled β-sheets to conduct the fibrillization. Reproduced from ref 229, with permission of The Royal Society of Chemistry. http://dx.doi.org/10.1039/c0sm01237h.

when added with different ratios to a pure β-sheet-forming peptide, providing insight into engineering potential inhibitors for neurodegenerative diseases.[229]

Similarly, our lab recently developed a method to site-specifically link a maleimide-terminated PNIPAAm to a model prion protein Sup35NM at the unique 31st mutated cysteine residue.[91] The results showed that the PNIPAAm linkage decreases the aggregation kinetics both in the oligomerization and fibrillation process, enabling us to establish a controllable and quantitative method to study the oligomerization process of amyloidogenic proteins. Most importantly, it is possible to use this method to further screen out potential drugs to slow the development of neurodegenerative diseases.

3.2. Smart Protein–Polymer Conjugates. If a stimulus-responsive polymer, namely, smart polymer, is included in the protein–polymer conjugate, the property of the polymer can be incorporated into the conjugate so that it can respond to external stimuli such as temperature, light, pH, and so forth.[24,230] Typical proteins such as enzymes have been used to demonstrate the stimuli-responsibility, as shown in Figure 17.[231] The bioactivity of the enzyme can be switched off or on

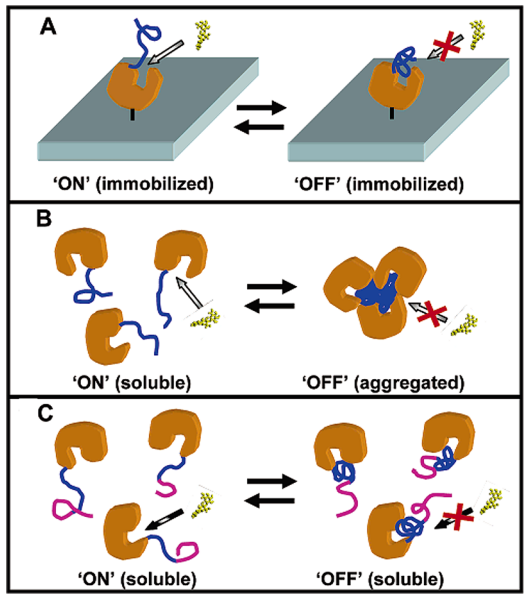

Figure 17. Controlling the ON/OFF state of the enzyme with smart site-specific protein–polymer conjugates. Reproduced from ref 231. Copyright 2006, American Chemical Society.

by molecular collapse or expansion of the smart polymer chain when subjected to the aforementioned stimuli, which cause steric blocking and unblocking of the protein's active site.

The stimuli-responsibility has also been adopted to modulate the interaction between antigen and antibody. Tiwari and co-workers recently reported an on/off-switchable localized surface plasmon resonance (LSPR) nanoimmunoassay for troponin-T (TnT) based on the PNIPAAM-anti-TnT conjugate, as illustrated in Figure 18.[232] By switching the medium temperature, the surface binding mode of TnT can be altered due to the change in the chain conformation of PNIPAAm, leading to a change in the LSPR.

Further, it is possible to separate the protein via the stimuli-responsibility. Previously, thermally responsive PNIPAM was site specifically attached to β-D-glucosidase to form a thermo-reversible, phase-separating polymer–enzyme conjugate[233] that can be used for separation, recovery, and recycling of the enzyme simply by applying small temperature changes to the reaction medium.

Similarly, pH- and light-sensitive polymers have also been documented.[234−236] Stayton and co-workers conjugated an azobenzene-containing copolymer to enzyme endoglucanase 12A at position 55 via the thiol–ene reaction, as shown in Figure 19. Irradiation with ultraviolet light switched the enzyme activity off, whereas under visual light the activity is regained due to the switch between the collapsed and expansion state of the copolymer.

4. CONCLUSIONS AND FUTURE PERSPECTIVES

The conjugation of a polymer chain to protein has been widely exploited in the application of biomedicine, protein self-assembly, and sensing. Site-specific conjugation with polymers offers better control over the bioactivity of the resultant protein–polymer conjugates. Recent advances in genetic engineering, bioorthogonal reactions, and controlled living

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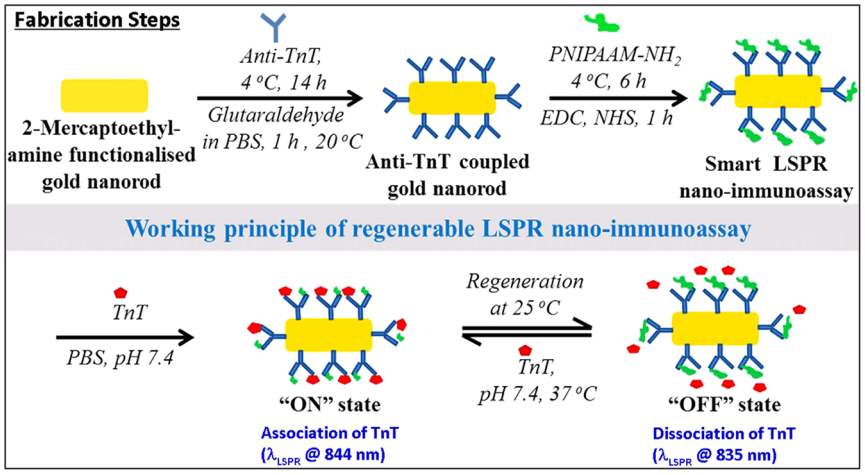

Figure 18. Working principal of regenerable LSPR nanoimmunoassay based on the PNIPAAm–antigen conjugate. The surface binding mode can be altered by the temperature-induced conformational change of PNIPAAm. Reprinted from ref 232 under a Creative Commons Attribution 4 International License. https://creativecommons.org/licenses/by/4.0/. Copyright 2017, Ashaduzzaman et al.

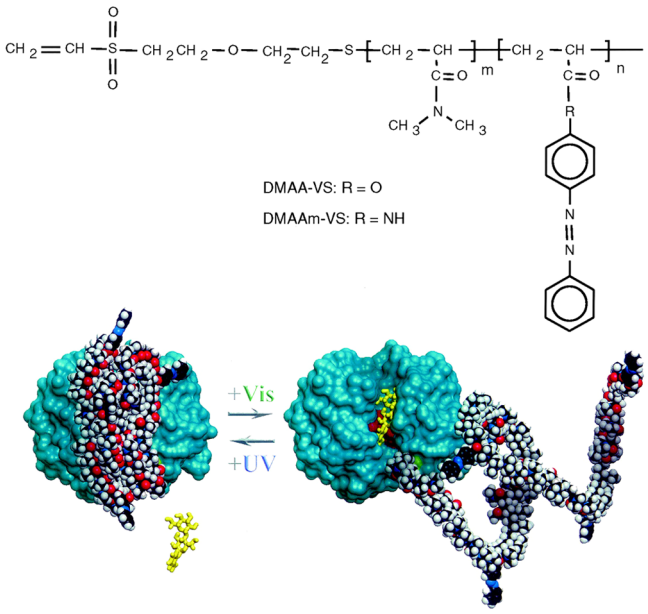

Figure 19. Photoresponsive enzyme switch based on the azobenzene-containing polymer–protein conjugate. The active site is marked in red in the pocket of the enzyme, and the specific conjugation portal is marked in green. Reprinted from ref 236. Copyright (2002), National Academy of Sciences, U.S.A.

polymerization provide new approaches for the synthesis of site-specific protein–polymer conjugates. By careful selection/introduction of the functional group on the protein, the conjugation strategy, and chemistry, it is possible to rationally link a functional polymer to the designated protein in a site-specific manner with high efficiency without hampering the biological activity of the protein. With these widely presented strategies in hand, researchers can choose the one that best meets their demands so that better applications may be achieved.

However, there is still much room for improvement in the area of site-specific protein–polymer conjugation. Major work in this area nowadays is focused on a few model globular proteins, such as BSA, lysozyme, and so forth. Only few works have targeted the therapeutic, functional, or membrane proteins due to the difficulty in accessing these proteins and poor knowledge of their structures. It is essential to conduct conjugation to the proteins described above to further exploit possible applications in new regimes. Despite the examples listed in section 2, bioorthoganol reactions with higher efficiency and specificity are still required. More efficient catalysts with less toxicity will improve the reaction efficiency for attachment of polymer chains to proteins regardless of the reaction medium. Such a user-friendly reaction will permit researchers with diverse backgrounds to enter this area and may offer new insights and applications.

Furthermore, it is also promising to further expand the species of polymers for better tuning the behavior of proteins because most of the currently used polymer-conjugated protein drugs approved by the FDA are PEG derivatives. Despite the success in stabilizing protein, PEGylation is also known to decrease the binding affinity of the protein, hence reducing the bioactivity, especially for the enzymes. One possible candidate to overcome this shortcoming is the zwitterionic polymer, which is a polyelectrolyte containing both positively and negatively charged groups with an overall neutral charge. Recent work by Jiang's group shows that, similar to PEG, poly(carboxybetaine) can also increase the stability of the model α-chymotrypsin protein after conjugation, whereas the new conjugates still retain or even enhance the binding affinity resulting from the improved protein–substrate hydrophobic interactions.[237] Further, in contrast to PEG, a decreased host–antibody response was achieved by the zwitterionic conjugation strategy with the same polymer, indicating that the zwitterionic polymer is promising for solving the possible immunogenic problem induced by PEGylation.[238] These seminal works introduce a new player in the field of polymer-conjugated protein drugs and are hopeful for promoting protein therapeutics.

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Notes

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Biomacromolecules XXXX, XXX, XXX–XXX

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