Polymer 66 (2015) A1–A10

Contents lists available at ScienceDirect

Polymer

journal homepage: www.elsevier.com/locate/polymer

Feature article

Synthesis of well-defined protein–polymer conjugates for biomedicine

Wenguo Zhao [1], Fei Liu [1], Yue Chen, Jing Bai, Weiping Gao*

Department of Biomedical Engineering, School of Medicine, Tsinghua University, Beijing 100084, PR China

ARTICLE INFO

Article history:
Received 31 December 2014
Received in revised form
17 March 2015
Accepted 22 March 2015
Available online 31 March 2015

Keywords:
Protein–polymer conjugate
Bioconjugate chemistry
Controlled radical polymerization

ABSTRACT

Covalently conjugating proteins with synthetic polymers, particularly poly(ethylene glycol) (PEG) is widely used as a means to improve protein solubility and stability, prolong their circulating half-lives, and lower their immunogenicity. Conventionally, these polymers are attached to random locations on the protein surfaces through the modification of the reactive side chains of amino acid residues such as lysine and cysteine. The "grafting to" polymer conjugation usually leads to heterogeneous products with reduced activity and low yield, which may not be compatible with the intended applications. Therefore, it is highly desirable to synthesize well-defined protein–polymer conjugates by site-specific polymer conjugation. Recently, in situ growth of polymer conjugates from proteins ("grafting from") has emerged as an alternative to the "grafting to" method. Particularly, site-specific in situ growth of polymer bioconjugates (SIP) is promising in overcoming the limitations of the "grafting to" method. In this review, we introduce the chemistry for synthesis of well-defined protein–polymer conjugates, and emphasize the SIP method as the next-generation platform for synthesis of well-defined protein–polymer conjugates. Furthermore, we exemplify biomedical applications of well-defined protein–polymer conjugates. In the end, we come up potential directions in this research field.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

Proteins and peptides play important roles as drugs in the pharmaceutical industry and as reagents in biomedical research. To date, more than one hundred different proteins or peptides have been approved for clinical use by the US Food and Drug Administration (FDA), and many more are in development. Therapeutic proteins have frequently been reviewed in the past decade [1–22]. While therapeutic proteins have advantages in the treatment of diseases due to their high activity and specificity, they suffer some shortcomings such as short *in vivo* half-life, poor stability, low solubility [23–25], and immunogenicity. One solution to these shortcomings is conjugating therapeutic proteins with polymers. Conjugation of a protein with a polymer results in a new macromolecule with significantly changed physicochemical characteristics. These changes are typically reflected in alterations of solubility, stability, *in vitro* activity, biodistribution, pharmacokinetic and pharmacodynamic profiles, as well as reduced immunogenicity and toxicity.

The first protein–polymer conjugate was reported in 1970's, where poly(ethylene glycol) (PEG) was conjugated to bovine serum albumin (BSA) [26,27] (Fig. 1a). This polymer conjugation represented a typical "grafting to" methodology and opened a new area in protein post-translation modification, which has led to an explosion of protein–polymer conjugate population. Conjugating with PEG is often named PEGylation. The clinical success of PEGylation has led to a number of FDA approved PEGylated drugs on the market. As an alternative to the "grafting to" method, in situ growth of polymer conjugates from proteins ("grafting from") has recently emerged, in which polymerization initiators are attached to proteins to form macroinitiators, followed by growing polymer conjugates from the macroinitiators through controlled radical polymerization technologies (Fig. 1b) [28]. In brief, "grafting to" is to attach a pre-prepared polymer to a protein, while "grafting from" is to in situ grow a polymer from a protein. Typically, "grafting from" does have two major benefits over "grafting to". First, "grafting from" usually results in high yield due to the high efficiency of the two-step reactions between small molecules (initiator and monomer) and a protein, while "grafting to" often leads to low yield because of the low efficiency of the reaction between two

* Corresponding author.
E-mail address: gaoweiping@tsinghua.edu.cn (W. Gao).
[1] Equal contribution as co-first authors.

http://dx.doi.org/10.1016/j.polymer.2015.03.054
0032-3861/© 2015 Elsevier Ltd. All rights reserved.

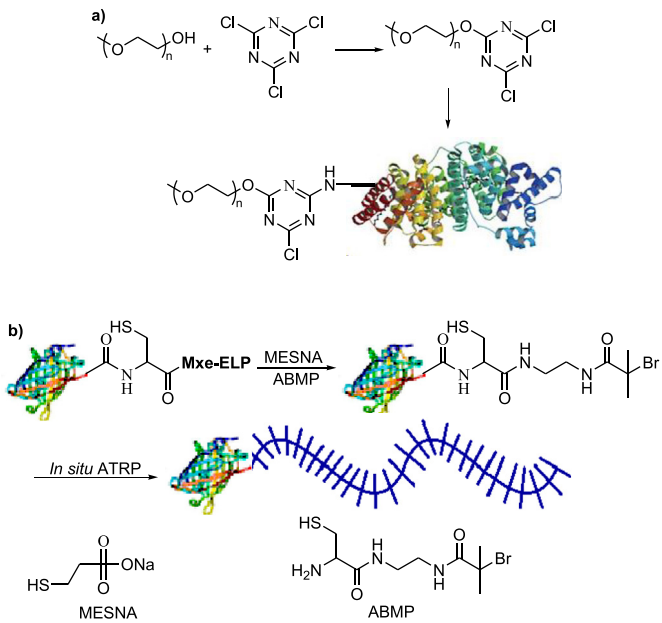

Fig. 1. a) Conventional conjugation of PEG to a protein. Ref. [26]. Reprinted with permission; Copyright© 1977, by the American Society for Biochemistry and Molecular Biology. b) In situ growth of a PEG-like polymer from a protein-based macroinitiator. Ref. [28].

large macromolecules (a polymer and a protein). Second, "grafting from" usually yields products with ease of purification because of the absence of free polymers and high yield.

Conventionally, polymers are conjugated to proteins typically at the sites of lysine or cysteine residues that are ubiquitously present on the protein surfaces, which makes it difficult to control the site of conjugation and the stoichiometry of the conjugates. The non-specific nature of the conventional polymer conjugation methods, including "grafting to" and "grafting from", often leads to a heterogeneous product mixture of positional isomers with significantly reduced biological activity [29–31]. Furthermore, it is difficult to isolate and purify the conjugate mixture, especially positional isomers. These limitations of the conventional polymer conjugation methods generally complicate the development process of protein–polymer conjugates, limiting their wide-spread applications. Therefore, there is a need to synthesize well-defined protein–polymer conjugates in which both the site of conjugation and the stoichiometry of the conjugates should be controlled [32].

In our opinion, a well-defined protein–polymer conjugate consists of at least two components, a functional protein, and an end-functionalized polymer with designed molecular weight and narrow polydispersity, in which the protein and the polymer are site-specifically conjugated together to form a site-specific and stoichiometric protein–polymer conjugate by "grafting to" or "grafting from" method. Therefore, the structure and properties of a well-defined protein–polymer conjugate can be precisely designed, which is particularly important for advanced applications [28,33–35]. Usually, the protein acts as an active component in biomedicine; meanwhile, the polymer plays a role as drug delivery carrier, targeting moiety, or a co-functional group. Up to date, there are many review papers on synthesis of well-defined protein–polymer conjugates by the "grafting to" method [32,36–45], in which a well-defined end-functionalized polymer is synthesized via a controlled polymerization process, and then attached to a protein at a specific site. In this review, we focus on synthesis of well-defined protein–polymer conjugates by site-specific in situ polymerization (SIP) due to the advantages of the "grafting from" method, such as high yield and simplified purification, over the "grafting to" method. Particularly, we emphasize the chemistry of site-specific protein modifications and biomedical applications of well-defined protein–polymer conjugates.

2. Controlled polymerization

The past decades have witnessed a huge development in controlled polymerization where the polymerization process is controllable to precisely synthesize well-defined polymers with desired structure and properties [46–56]. Particularly, controlled radical polymerization (CRP) techniques, such as atom transfer radical polymerization (ATRP) and reversible addition fragmentation chain transfer (RAFT) polymerization, are powerful to synthesize well-defined polymers, especially under mild aqueous conditions, which is highly desirable for the "grafting from" method. Here we focus on ATRP and RAFT polymerization as both techniques have been used to grow polymer conjugates from proteins.

2.1. Atom transfer radical polymerization

ATRP is one of the most powerful and versatile CRP techniques [57]. It was discovered independently by Sawamoto in 1994 [58] and Matyjaszewski in 1995 [59–61]. In ATRP, a transition-metal complex (generally copper-, ruthenium-, iron-, or nickel-based) is used as a catalyst for initiation and chain growth, which is an extension of the Kharasch reaction [62]. The polymer growth is controlled by a redox equilibrium between macroradicals and dormant species end-capped by a halogen atom (Fig. 2). There are several advantages in ATRP. It enables precise control on molecular weight, polydispersity, and functionality. It can be carried out in a variety of different solvents and conditions, including water at room temperature, and is tolerant of most functional groups. The polymerization conditions and parameters can be tuned, providing control over reaction kinetics. In addition to homogeneous and heterogeneous solution polymerization, polymers can be grown from nearly any surface or material that is attached with ATRP initiators, including proteins, organic materials, and inorganic materials like nanoparticles (NPs). Particularly, ATRP is well suitable for the synthesis of polymer bioconjugates. Peptide sequences, biotin, and proteins such as chymotrypsin, streptavidin and bovine serum albumin (BSA) have been successfully modified to become ATRP initiators [28,39,63–68]. Polymerization from ATRP initiators containing proteins and short peptide sequences offers an attractive route to produce polymer–protein conjugates.

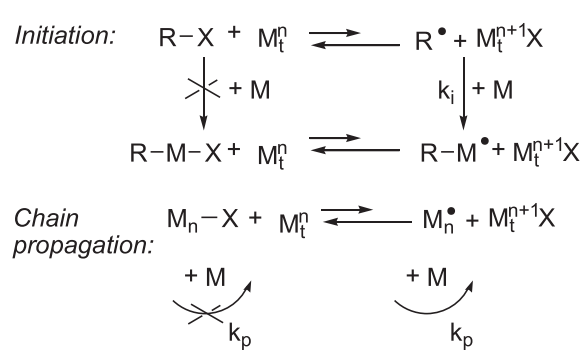

Fig. 2. Proposed mechanism of ATRP.

2.2. Reversible addition-fragmentation chain transfer polymerization

The RAFT polymerization was developed by Rizzardo, E. and coworkers in 1990s, and it has been marked as a versatile CRP tool [69,70], especially in terms of its tolerance of many functional groups with the exception of nucleophilic groups such as amines or thiols. The RAFT technique does not require any metal catalyst which must be used in ATRP. Instead, thiocarbonylthio compounds such as dithioesters, dithiocarbamates, trithiocarbonates, and xanthates are used (named chain-transfer agents (CTAs)) in order to coordinate the polymerization via a reversible chain-transfer process. The process of RAFT polymerization is based on two chain-transfer and two chain-propagation equilibria that establish control over the radical polymerization (Fig. 3). In this process, a growing polymer chain reacts with the CTA, yielding a new chain-transfer agent and a free radical for propagation. Thus, the propagation probability is distributed equally over all polymer chains, which is the reason for the narrow size distribution of the polymers produced. RAFT polymerization is potentially universal and can be applied to a wide range of functional monomers (styrenics, alkyl(meth)acrylates, acrylic acid, vinyl acetate, etc.), allowing the preparation of polymers with precisely controlled structural parameters such as random, block, gradient, grafted, and star copolymers. RAFT polymerization can be performed in homogeneous media (bulk and solution) as well as in ionic liquids and aqueous dispersed systems. So far, RAFT polymerization has been applied for in situ synthesis of protein–polymer conjugates [71–74], such as bovine serum albumin-poly(oligo(ethylene glycol) acrylate) (BSA-POEGA) [71], BSA-poly(N-isopropylacrylamide) (BSA-PNIPAM) and BSA-poly(hydroxyethyl acrylate) (BSA-PHEA) [72].

3. Site-specific protein modifications

Conventional protein modification technologies generally result in the non-specific modification of the target proteins, yielding heterogeneous populations often with compromised biological activity, and large batch to batch variations in their production. Therefore, site-specific protein modification is of great importance for synthesis of well-defined protein–polymer conjugates. Herein, we introduce site-specific protein modification technologies that have been exploited to synthesize well-defined protein–polymer conjugates by us or other research groups.

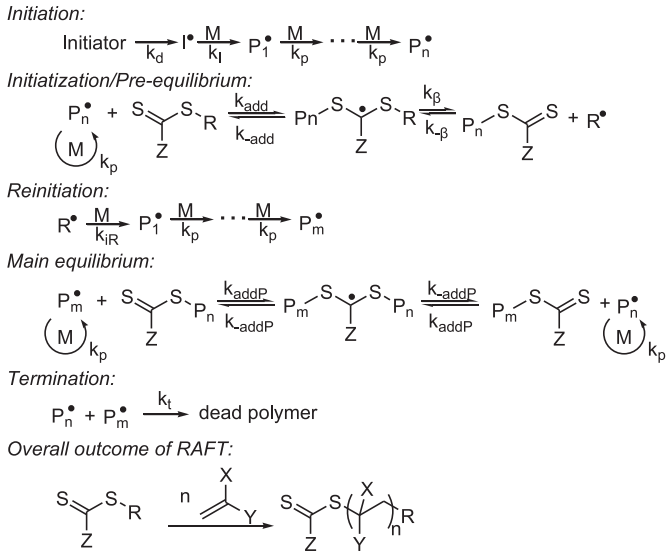

Fig. 3. Proposed mechanism of RAFT polymerization.

3.1. N-terminal modification

The alpha-amino group of the N-terminal amino acid residue has a sufficiently different p*K*a (7.6–8.0) from the amino groups of the lysine residues (p*K*a 10–12) that are typically distributed on the protein surface, which enables the N-terminal protein modification. Kinstler and coworkers for the first time exploited the p*K*a difference to prepare N-terminal PEGylated proteins like human granulocyte colony-stimulating factor (G-CSF) and human megakaryocyte growth and development factor (MGDF) by reductive alkylation of the proteins with linear monomethoxyPEG aldehydes [36]. These well-defined PEGylated proteins have been approved for clinical use.

Recently, Francis and coworkers reported a biomimetic transamination reaction for N-terminal protein modification [75,76]. They screened out pyridoxal-5-phosphate (PLP) as the most effective aldehyde. Typically, the aldehyde group of PLP condenses with amino groups on a protein to form imines. However, the alpha proton of the imine formed with the N-terminus has a much lower pKa value, allowing tautomerization to occur uniquely at this site (Fig. 4a). Hydrolysis of the resulting glyoxyl imine yields an aldehyde or ketone site-specifically at the N-terminus. Furthermore, they identified a highly reactive sequence AKT for PLP-mediated N-terminal protein modification [77] and a highly effective transamination reagent N-methylpyridinium-4-carboxaldehyde benzenesulfonate salt (Rapoport's salt, RS) for N-terminal antibody modification [78]. They demonstrated that PEG-alkoxyamine was coupled to N-terminal carbonyl groups introduced using PLP/RS-mediated transamination to form well-defined protein–PEG conjugates. Additionally, Chilkoti and coworkers exploited the PLP-mediated transamination to selectively attach an ATRP initiator to the N-terminus of myoglobin to form a macroinitiator, followed by in situ growth of a PEG-like polymer, poly(oligo(ethylene glycol) methyl ether methacrylate) [POEGMA)] by ATRP from the macroinitiator (Fig. 4b) [35]. The site-specific (N-terminal) and stoichiometric (1:1) conjugate had low polydispersity, high yield, and highly retained activity.

Conceptually, these methods are powerful, but they can be hampered by harsh reaction conditions such as long reaction times, large excess of reagent, and at least two-steps for the attachment of functional molecules. More recently, Francis and coworkers reported the oxidative coupling of *o*-aminophenols to N-terminal proline, where *o*-aminophenols is oxidized by potassium ferricyanide to yield *o*-iminoquinones for the selective reaction with N-terminal proline residues (Fig. 5) [79]. They further demonstrated that PEG could be attached to the N-terminus of a protein by the oxidative coupling reaction to form well-defined protein–PEG conjugates. As compared to other N-terminal protein modification methods, the oxidative coupling method has two distinct advantages as follows: 1) a single step reaction that does not require initial oxidation of the N-terminus; 2) the fast second-order kinetics that allow for low concentrations of the coupling reagents to be used. However, it is limited to the N-terminal proline residue for the sake of high yield.

3.2. C-terminal modification

Unlike the case of the N-terminus, the C-terminal carboxylic acid group has a similar p*K*a (~3.0) to the carboxylic acid group on the side chains of aspartic acid (p*K*a ~ 3.7) and glutamic acid (p*K*a ~ 4.0), consequently cannot be chemoselectively modified. Instead, biological technologies are used to modify the C-terminus

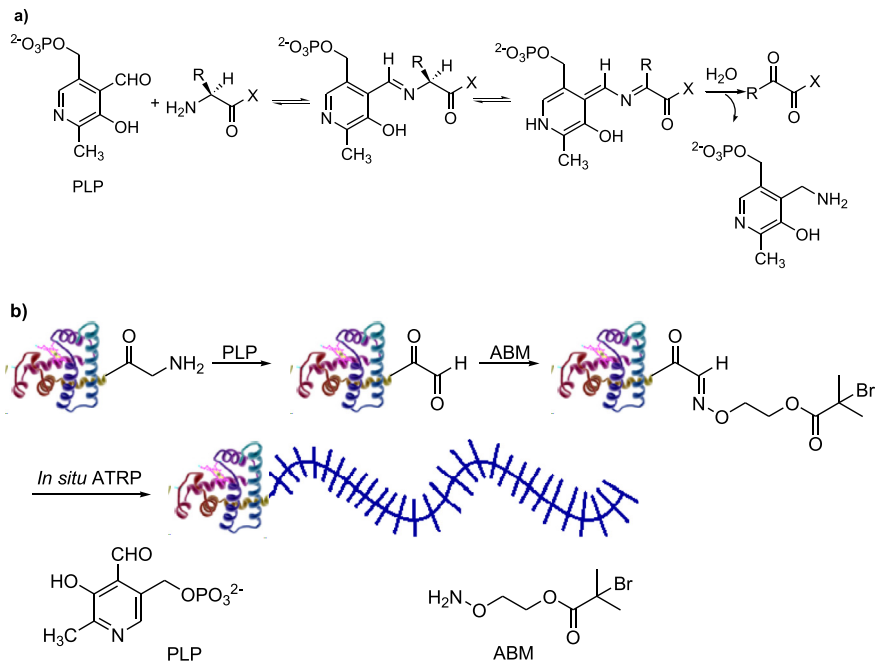

Fig. 4. a) Proposed mechanism of PLP-mediated N-terminal transamination. Ref. [75]. Reprinted with permission; Copyright© 2006 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. b) In situ growth of a PEG-like polymer from the N-terminus of myoglobin to form a site-specific and stoichiometric protein–polymer conjugate using N-terminal transamination and in situ ATRP. Ref. [35].

of a protein. Intein-mediated protein ligation (IPL) is a very useful tool for the C-terminal modification of the protein. An intein is a protein sequence embedded inframe within a precursor protein sequence, which autocatalytically excises itself out to ligate the flanking sequences together through a peptide bond [80–82] (Fig. 6a). Mutation of inteins at their C-termini allows controllable cleavage of peptide bonds at the N-terminus of the inteins, which has been used to isolate recombinant proteins with a C-terminal thioester moiety for a variety of applications in biotechnology and chemical biology [83–85]. Chilkoti and coworkers utilized IPL and native chemical ligation (NCL) to attach a cysteine-functionalized ATRP initiator to the C-terminus of a model protein, green fluorescence protein (GFP), to form a macroinitiator (Fig. 1b) [28]. Subsequent ATRP of OEGMA from the macroinitiator yielded a site-specific (C-terminal) and stoichiometric (1:1) conjugate with high yield and highly retained activity. Although the strategy is applicable to a large subset of recombinant proteins to form well-defined protein–polymer conjugates, it may not be compatible with folded disulfide bond-containing proteins because of the presence of thiol additives. To solve the problem, Cotton and coworkers exploited chemical cleavage of precursor intein-fusion proteins with hydrazine to produce recombinant protein hydrazides for C-terminal PEGylation by hydrazone-forming ligation reactions (Fig. 6b) [86]. This approach was used to create folded, C-terminal PEGylated interferon alpha with good retention of antiviral activity. However, given the relatively large molecular weight and protein property of inteins, their incorporation may adversely affect the expression level of the fusion protein. Another potential drawback of IPL is that some fraction of intein-fusion proteins is prematurely cleaved within the cell, which may decrease the overall yield after purification.

To address these limitations of IPL, sortase-mediated protein ligation (SPL) has been used for C-terminal protein modification [87–89]. Sortase A is a thiol-containing transpeptidase that

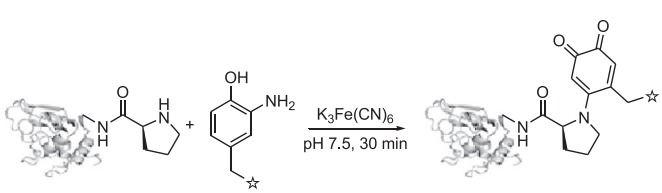

Fig. 5. Oxidative coupling of N-terminal proline residue with *o*-aminophenols using potassium ferricyanide as the oxidant. Ref. [79]. Reprinted with permission; Copyright© 2014, American Chemical Society.

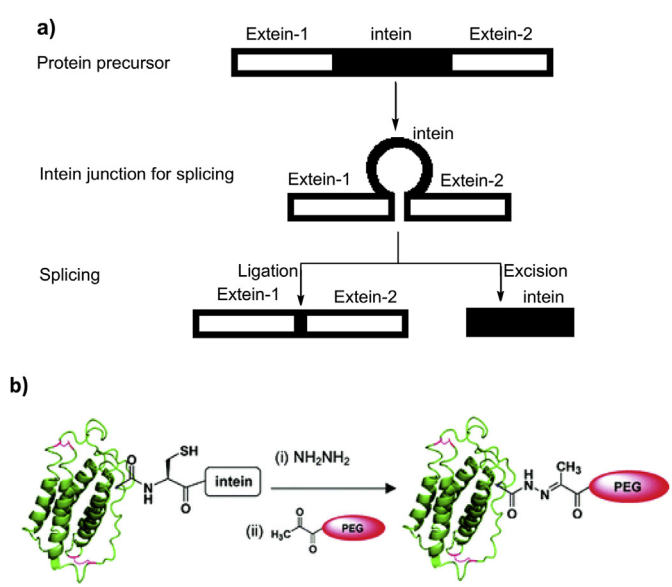

Fig. 6. a) Intein-mediated protein ligation (IPL) undergoes an intein junction and self-excision procedure to ligate the flanking sequences together. Ref. [82]. Reprinted with permission; Copyright© 1994, Oxford University Press. b) PEGylation through a protein hydrazide intermediate. Ref. [86]. Reprinted with permission; Copyright© 2011, American Chemical.

recognizes an LPXTG motif in substrates. It cleaves the peptide bond between the threonine and glycine residues to form a thioacyl enzyme intermediate. This intermediate is nucleophilically attached by the N-terminus of an oligoglycine peptide to generate an amide bond between the substrate protein and the incoming nucleophile (Fig. 7a). Ploegh and coworkers used SPL to attach PEG to the C-terminus of cytokines to form C-terminal PEGylated cytokines with low yield and reduced activity [90]. More recently, Chilkoti and coworkers employed SPL to install an ATRP initiator solely at the C-terminus of GFP to yield a macroinitiator, followed by in situ growth of POEGMA from the macroinitiator to form C-terminal and stoichiometric (1:1) GFP-POEGMA conjugates with high yield and highly retained activity (Fig. 7b) [34].

3.3. Cysteine modification

Cysteine residue is rare in proteins and can be introduced to proteins genetically. The thiol group on the side chain of cysteine is a mild nucleophile, thus, it can be modified selectively, rapidly, and quantitatively under the appropriate conditions, which is the fact that cysteine has been chosen as the specific chemical modification site of proteins.

In general, the cysteine modification reactions can be classified as alkylation and disulfide formation (Fig. 8) [32]. Alkylation of cysteine can be achieved using either α-halo (usually iodine)

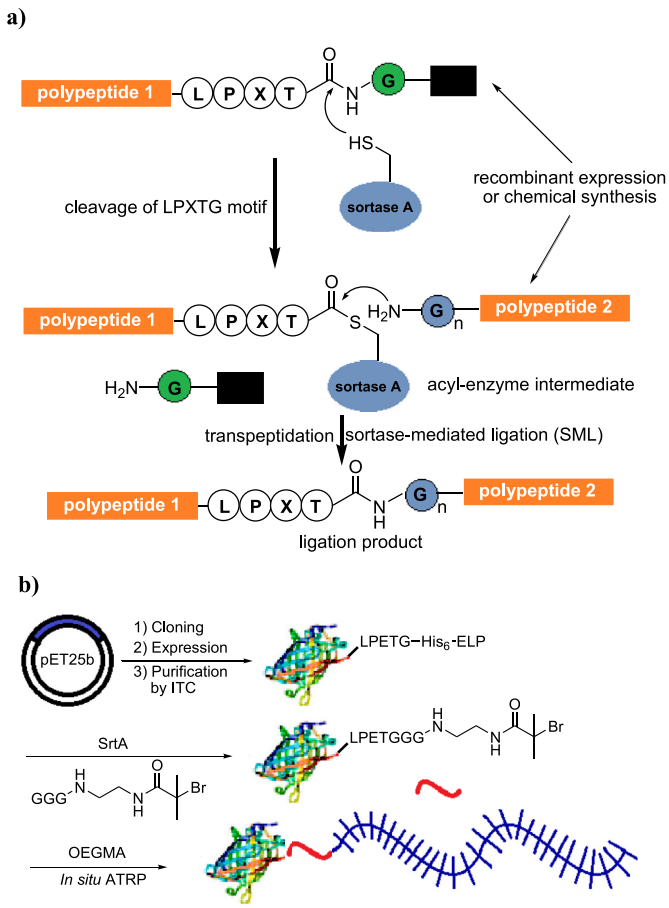

Fig. 7. a) Sortase-mediated protein ligation (SPL) of a LPXTG motif and a G_n-polypeptide motif to form the ligation product. Ref. [89]. Reprinted with permission; Copyright© 2009 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. b) In situ growth of a PEG-like polymer from the C-terminus of GFP to yield site-specific and stoichiometric GFP-POEGMA conjugates using SPL followed by in situ ATRP. Ref. [34].

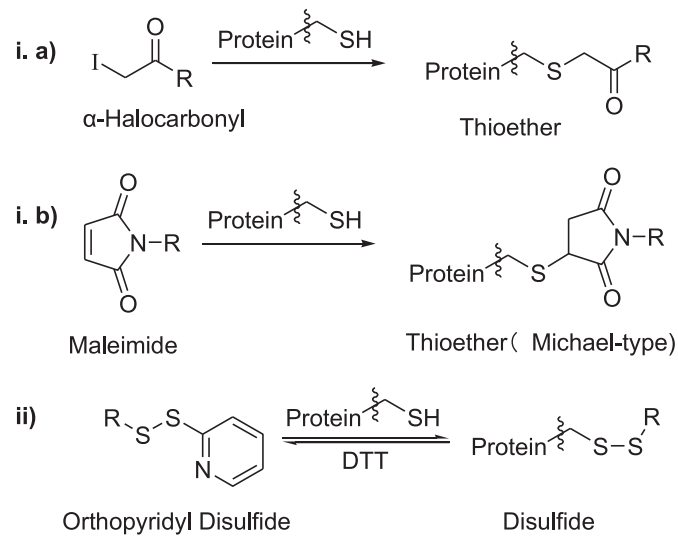

Fig. 8. The two general ways of cysteine modification: i) alkylation via dehydrohalogenation (a) or Michael addition reaction (b), and ii) disulfide linkage via group exchange. Ref. [32]. Reprinted with permission; Copyright© 2006, Springer-Verlag.

carbonyl compounds or maleimides. Thiols react with α-halo carbonyl compounds at pH 9–10 via dehydrohalogenation to form thioethers, while they can also be modified using maleimide-functionalized groups through Michael addition at neutral pH (6.8–7.0) [91]. The latter of the two methods is much better than the former one, for the milder conditions, greater specificity, and the better reactivity of maleimide [45]. Disulfide formation, especially with o-pyridyldisulfide terminated reagents, is the most specific method for cysteine modification. The overall reaction process can be regarded as a reversible group exchange procedure [92,93].

Cysteine can be genetically incorporated into proteins for site-specific PEGylation. For example, interferon alpha has been genetically mutated to introduce a unique free cysteine residue on the protein surface to form well-defined PEGylated interferon alpha [94,95]. Maynard and coworkers modified free cysteines, Cys-34 of bovine serum albumin (BSA) and Cys-131 of T4 lysozyme (LYS) V131C with ATRP initiators either through a reversible disulfide linkage or irreversible bond by reaction with pyridyl disulfide- and maleimide-functionalized initiators, respectively [93] (Fig. 9). Polymerization of NIPAM from the protein macroinitiators resulted in site-specific BSA–PNIPAM and lysozyme–PNIPAM in high yield and with highly retained activity. However, proteins containing multiple cysteine residues are prone to form disulfide bond linkages or cause thiol-disulfide exchanges between two or more proteins, which may have negative effects on the solubility and stability of the proteins.

3.4. Histidine tag modification

Histidine tag, particularly six-histidine tag acts as a receptor for protein crosslinking reagents [96]. Histidine tag can be tagged both on the C- and N- termini of proteins [97–99]. Genetically engineered histidine tag on proteins can be regarded as an important modification site [100–102], which has been exploited as a methodology of site-specific protein modification. Cong, Y.H. et al. demonstrated site-specific PEGylation of a domain antibody and interferon alpha at a His-tag by bis-alkylation with monosulfone-functionalized PEG (Fig. 10) [103]. However, the stoichiometry of the PEGylation is not well-defined.

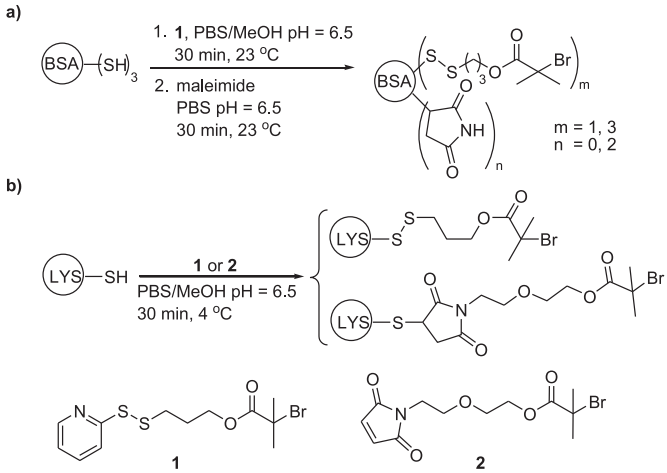

Fig. 9. a) BSA-macroinitiators formed via disulfide linkage. b) LYS-macroinitiators formed via disulfide linkage or Michael addition. Ref. [93]. Reprinted with permission; Copyright© 2005, American Chemical.

3.5. Glutamine tag modification

Glutamine (Gln) residue (Q-tag) is a unique type of target for selective functionalization of proteins, in which transglutaminase (TGase) is used to catalyze an acyl-transfer reaction between the γ-carboxyamide group of glutamine residues and the ε-amino group of lysine residues, resulting in the formation of an isopeptide bond (Fig. 11) [104–106]. This biotechnological approach have been used to prepare well-defined PEGylated proteins [107,108]. However, the site-selectivity of this method is poor in that many proteins contain two or more Gln as TGase substrates.

3.6. Aldehyde tag modification

Bertozzi et al. for the first time reported the site-specific introduction of aldehyde groups into recombinant proteins using the 6-amino-acid consensus sequence recognized by the formylglycine-generating enzyme (FGE) [109]. The recognition sequence is called "aldehyde tag". FGE oxidizes cysteine to formylglycine that can be used for site-specific PEGylation by reaction with aminooxy-PEG (Fig. 12). One disadvantage of this method is that the cysteine containing "aldehyde tag" might not be compatible with disulfide bond stabilized proteins due to the potential thiol-disulfide exchange.

3.7. Disulfide bond modification

Some therapeutic proteins have no free cysteine residue but have paired disulfides [110]. Some of the disulfides can be reduced

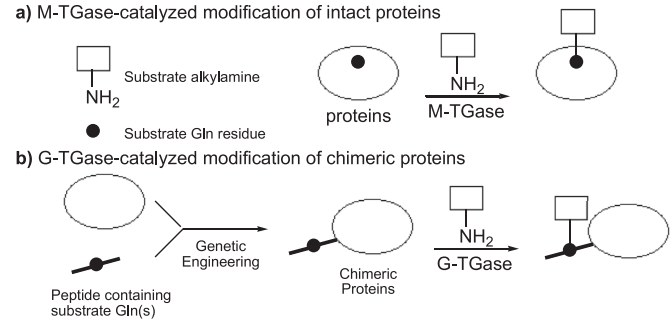

Fig. 11. TGase catalyzed modification of glutamine. Ref. [106]. Reprinted with permission; Copyright© 2001, American Chemical.

into thiols without any damage of the protein structure and activity under mild conditions. The thiols can be further modified site-specifically using thiol-specific reagents such as three-carbon bridge sulfone compounds [111–114] and maleimide derivatives [115]. Shaunak and Brocchini et al. exploited the reactivity of the two sulfur atoms of a native disulfide for site-specific PEGylation using a thiol-specific, cross-functionalized PEG monosulfone [111]. After addition of thiol to the conjugated double bond in the PEG or poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC) monosulfone, elimination of sulfinic acid yields another conjugated double bond for the second thiol, which results in the formation of a three-carbon bridge between two sulfur atoms (Fig. 13) [112,114,116].

Caddick and Baker et al. for the first time reported dibromomaleimide-based disulfide bridging for protein modification [117]. Haddleton et al. took advantage of the dibromomaleimide-based disulfide bridging for site-specific PEGylation by reaction of reduced salmon calcitonin with a dibromomaleimide-functionalized PEG chain (Fig. 14) [118]. Furthermore, they prepared a well-defined salmon calcitonin-POEGMA conjugate using dibromomaleimide-functionalized POEGMA that was synthesized by ATRP followed by click chemistry. The disulfide bond modification methods are only applicable to proteins containing disulfide bonds which are not at or near the active sites of the proteins.

3.8. Biotin-streptavidin recognition based modification

Streptavidin is a well-studied protein that consists of four subunits, each of which is capable of binding one molecule of biotin with very high affinity [68,119]. Maynard and coworkers exploited the biotin-streptavidin recognition for installation of ATRP initiators to streptavidin to form a macroinitiator, followed by in situ ATRP of OEGMA or NIPAM to yield well-defined streptavidin-POEGMA/PNIPAM conjugates (Fig. 15) [68]. Unfortunately, this

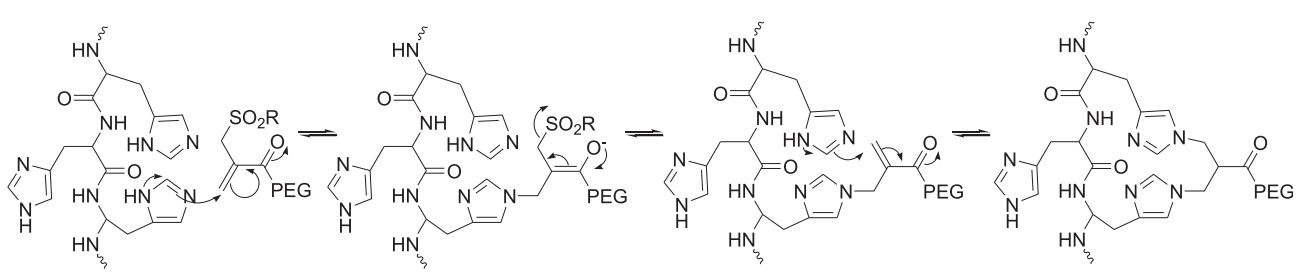

Fig. 10. Possible mechanism for site-specific PEGylation at a His-tag by bis-alkylation with PEG-mono-sulfones. Ref. [103]. Reprinted with permission; Copyright© 2012, American Chemical.

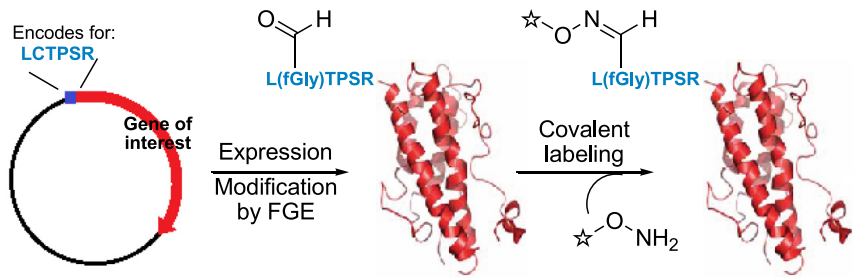

Fig. 12. Generation of aldehyde tagged proteins for site-specific PEGylation. Ref. [109]. Reprinted with permission; Copyright© 2007, Rights Managed by Nature Publishing Group.

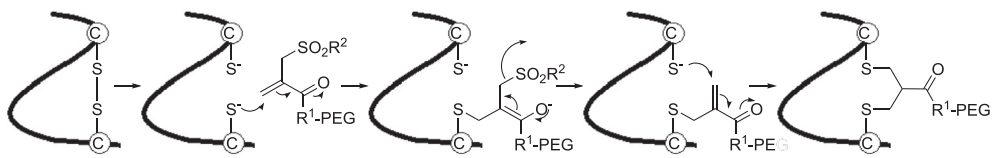

Fig. 13. Cleavage and re-bridging of protein-disulfide through a three-carbon bridge way to form a protein–PEG conjugate. Ref. [114]. Reprinted with permission; Copyright© 2007 Elsevier B.V.

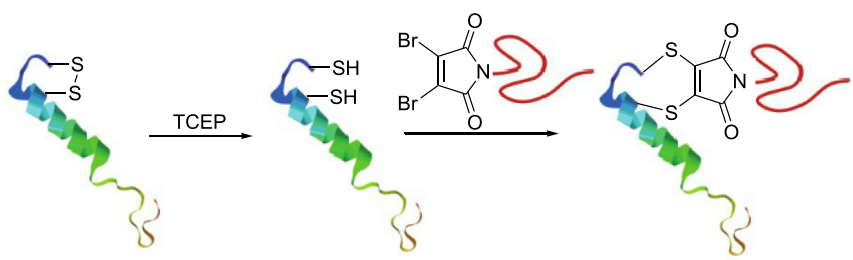

Fig. 14. Cleavage and re-bridging of protein disulfide to form a protein–PEG conjugate using dibromomaleimide-based disulfide re-bridging. Ref. [118]. Reprinted with permission; Copyright© 2012, American Chemical.

strategy is so special that it cannot be applied to other proteins to generate well-defined protein–polymer conjugates.

3.9. Unnatural amino acid modification

Unnatural amino acids (UAAs) with orthogonal chemical reactivity can be genetically introduced into native proteins, enabling the synthesis of structurally defined protein conjugates [120].

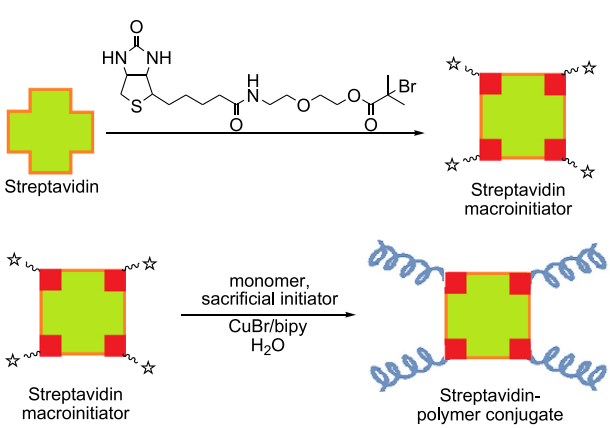

Fig. 15. Streptavidin macroinitiators formed via biotin-streptavidin recognition, followed by in situ ATRP to yield streptavidin polymer conjugates. Ref. [68]. Reprinted with permission; Copyright© 2005, American Chemical.

Particularly, Schultz et al. expanded the genetic code beyond the canonical amino acids by evolving additional tRNA/aminoacyl-tRNA synthetase (aaRS) pairs that site-specifically incorporate UAAs in response to either nonsense or 4-base frameshift condons [121–124] (Fig. 16). Cho and coworkers incorporated p-acetylphenylalanine in human growth hormone (hGH), allowing site-specific conjugation with PEG to produce homogeneous PEGylated hGH [125]. Pinkstaff and coworkers used the same strategy to prepare site-specific PEGylated fibroblast growth factor 21 (FGF21) [126]. This method is potentially general, but it needs complicated chemical and biological knowledge and techniques.

4. Biomedical applications

4.1. Drug delivery

Covalent conjugation with polymers such as PEGylation can improve the pharmacokinetics and biodistribution, and reduce the immunogenicity of therapeutic proteins and peptides [59]. There exists more than ten FDA-approved PEG–protein conjugate drugs and many others are currently being studied in clinical trials [44,127]. Recently, site-specific PEGylation has attracted more attention for the sake of preparation of well-defined protein–polymer conjugates. For example, N-terminal PEGylated G-CSF and MGDF have been proven for clinical use [37]. C-terminal PEGylated interferon alpha has been showed to have good retention of bioactivity and improved pharmacokinetics [94]. Interferon alpha was mutated with cysteine to make well-defined PEGylated

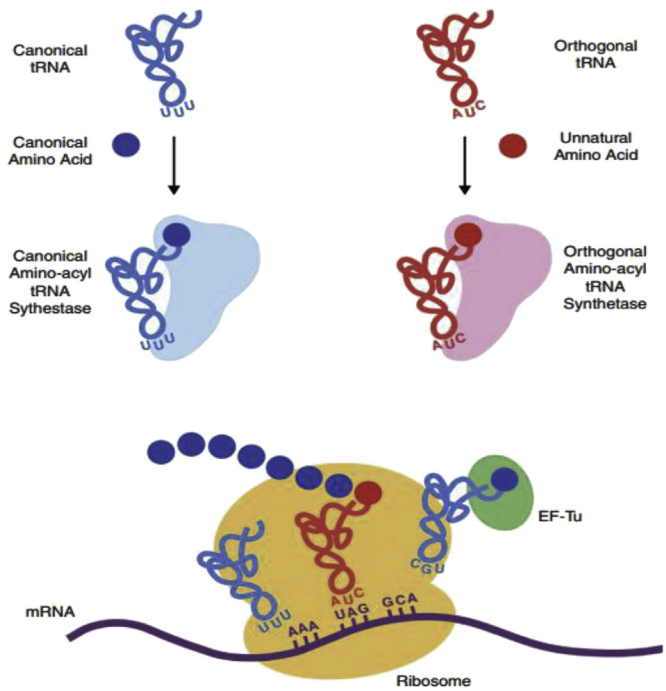

Fig. 16. Scheme for the genetic incorporation of unnatural amino acids. Ref. [121].

interferon alpha with highly retained bioactivity and enhanced pharmacokinetics [95]. hGH was genetically incorporated with p-acetylphenylalanine for site-specific PEGylated hGH with increased potency and reduced injection frequency [125]. Similarly, site-specific PEGylated FGF21 showed improved pharmacokinetics and antidiabetic pharmacology in rodents [126]. Recently, Gao et al. demonstrated that N/C-terminal protein-POEGMA conjugates showed significantly improved pharmacokinetics and tumor accumulation over the native proteins [28,35]. More recently, Schultz and coworkers exploited the unnatural amino acid strategy to synthesize well-defined antibody-polymer conjugates for siRNA delivery [128].

4.2. Biotechnology

Conjugation with "smart" polymers can also impart versatile and useful behaviours such as externally triggered activity switching of the biomolecule [129]. Chilkoti et al. created a mutant cytochrome-b5 with a cysteine located far from the active binding site, and conjugated a thermoresponsive polymer PNIPAM to the thiol group using maleimide-based chemistry [130]. PNIPAM has a characteristic lower critical solution temperature (LCST) in water at 32 °C [41,131–133], above which PNIPAM undergoes a hydrophobic collapse and phase separates from solution [41]. The site-specific PNIPAM conjugation did not significantly reduce the activity of the protein. They showed that the protein was reversibly precipitated and redissolved by thermal cycling through the LCST of PNIAPM. Stayton et al. conjugated PNIPAM to a genetically engineered streptavidin at a specific cysteine site that was specifically designed to be near the biotin-binding site of streptavidin. They showed that alternations in temperature caused the polymer coil to collapse, simultaneously also caused blockage of biotin binding to streptavidin [133]. They further found that the thermally induced collapse of the conjugated PNIPAM can cause the release of a significant fraction of already-bound biotin from the streptavidin-binding pocket [132]. Additionally, they demonstrated that replacing the PNIPAM with a pH-sensitive copolymer of acrylic acid and PNIPAM enabled pH control of biotin binding and release [134]. These stimuli-responsive properties of well-defined protein–polymer conjugates may be relevant to affinity separations, biosensors, diagnostics, enzyme process, and targeted drug delivery.

5. Conclusions and perspectives

Typically, conjugating proteins with polymers can improve stability, increase solubility, enhance systemic circulation of the proteins, and also potentially reduce the immunogenicity and antigenicity, which has many interesting applications in diagnostics, affinity separations, drug delivery, tissue engineering, and life science. In the past decades, much effort has been made to synthesize well-defined protein–polymer conjugates for biomedicine, but the challenges, particularly in control of the site of conjugation and the stoichiometry of the conjugate, still remain. The "grafting to" polymer conjugation usually leads to heterogeneous products with reduced activity and low yield, which may not be compatible with the intended applications. SIP, on the other hand, as a powerful "grafting from" methodology, still has its own limitations. In situ growth of polymers from proteins and peptides typically requires that the reaction be carried out in aqueous conditions at low temperatures to maintain solubility and stability of the biomolecules. This requirement challenges the capacity of controlled radical polymerization techniques. ATRP conducted in aqueous buffers typically suffers from a variety of complications, including decreased stability of catalyst/ligand complex, disproportionation of activator, dissociation of halide from deactivator, very high activation rate, and hydrolysis of carbon–halogen bond. RAFT polymerization usually requires higher temperature and organic medium, which may not be compatible with the requirement. In summary, the major challenges in synthesis of well-defined protein–polymer conjugates are: (i) site-specificity, (ii) stoichiometry, (iii) controllable molecular weight with narrow polydispersity, (iv) high yield, (v) ease of separation and purification, (vi) excellent retention of protein activity, (vii) significantly improved pharmacological properties over the native proteins.

Acknowledgements

This work was supported by the National Natural Science Foundation of China under Grants No. 21274043 and 81227901 and the National Basic Research Program of China (973) under Grant No. 2011CB707701.

References

[1] Kochendoerfer GG. Curr Opin Chem Biol 2005;9(6):555–60.
[2] Carter PJ. Nat Rev Immunol 2006;6(5):343–57.
[3] Walsh G, Jefferis R. Nat Biotechnol 2006;24(10):1241–52.
[4] Brain JD. Diabetes Technol Ther 2007;9(Suppl. 1):S4–15.
[5] Dingermann T. Biotechnol J 2008;3(1):90–7.
[6] Singh R, Singh S, Lillard Jr JW. J Pharm Sci 2008;97(7):2497–523.
[7] Antosova Z, Mackova M, Kral V, Macek T. Trends Biotechnol 2009;27(11):628–35.
[8] Grant SK. Cell Mol Life Sci 2009;66(7):1163–77.
[9] Li H, d'Anjou M. Curr Opin Biotechnol 2009;20(6):678–84.
[10] Czabotar PE, Lessene G. Curr Pharm Des 2010;16(28):3132–48.
[11] Jevsevar S, Kunstelj M, Porekar VG. Biotechnol J 2010;5(1):113–28.
[12] Lofblom J, Feldwisch J, Tolmachev V, Carlsson J, Stahl S, Frejd FY. FEBS Lett 2010;584(12):2670–80.
[13] Pisal DS, Kosloski MP, Balu-Iyer SV. J Pharm Sci 2010;99(6):2557–75.
[14] Argiris K, Panethymitaki C, Tavassoli M. Exp Biol Med 2011;236(5):524–36.
[15] Grimm D, Bauer J, Pietsch J, Infanger M, Eucker J, Eilles C, et al. Curr Med Chem 2011;18(2):176–90.
[16] Wurch T, Pierre A, Depil S. Trends Biotechnol 2012;30(11):575–82.
[17] Gupta S, Jain A, Chakraborty M, Sahni JK, Ali J, Dang S. Drug Deliv 2013;20(6):237–46.

[18] Ivens IA, Baumann A, McDonald TA, Humphries TJ, Michaels LA, Mathew P. Haemophilia 2013;19(1):11–20.
[19] Kesikli SA, Kilickap S. Protein Pept Lett 2013;20(12):1345–59.
[20] Kimchi-Sarfaty C, Schiller T, Hamasaki-Katagiri N, Khan MA, Yanover C, Sauna ZE. Trends Pharmacol Sci 2013;34(10):534–48.
[21] Ozgur A, Tutar Y. Protein Pept Lett 2013;20(12):1365–72.
[22] Zhang F, Liu MR, Wan HT. Biol Pharm Bull 2014;37(3):335–9.
[23] Ruschak AM, Rose JD, Coughlin MP, Religa TL. Protein Sci 2013;22(11):1646–54.
[24] Kramer RM, Shende VR, Motl N, Pace CN, Scholtz JM. Biophys J 2012;102(8):1907–15.
[25] Schein CH. Nat Biotechnol 1990;8(4):308–17.
[26] Abuchowski A, van Es T, Palczuk NC, Davis FF. J Biol Chem 1977;252(11):3578–81.
[27] Abuchowski A, McCoy JR, Palczuk NC, van Es T, Davis FF. J Biol Chem 1977;252(11):3582–6.
[28] Gao W, Liu W, Christensen T, Zalutsky MR, Chilkoti A. Proc Natl Acad Sci U S A 2010;107(38):16432–7.
[29] Veronese FM. Biomaterials 2001;22(5):405–17.
[30] Foser S, Schacher A, Weyer KA, Brugger D, Dietel E, Marti S, et al. Protein Expr Purif 2003;30(1):78–87.
[31] Wang YS, Youngster S, Grace M, Bausch J, Bordens R, Wyss DF. Adv Drug Deliv Rev 2002;54(4):547–70.
[32] Thordarson P, Le Droumaguet B, Velonia K. Appl Microbiol Biotechnol 2006;73(2):243–54.
[33] Hu J, Zhao W, Gao Y, Sun M, Wei Y, Deng H, et al. Biomaterials 2015;47:13–9.
[34] Qi Y, Amiram M, Gao W, McCafferty DG, Chilkoti A. Macromol Rapid Commun 2013;34(15):1256–60.
[35] Gao W, Liu W, Mackay JA, Zalutsky MR, Toone EJ, Chilkoti A. Proc Natl Acad Sci U S A 2009;106(36):15231–6.
[36] Kinstler O, Molineux G, Treuheit M, Ladd D, Gegg C. Adv Drug Deliv Rev 2002;54(4):477–85.
[37] Pasut G, Veronese FM. Adv Drug Deliv Rev 2009;61(13):1177–88.
[38] Gauthier MA, Klok HA. Polym Chem 2010;1(9):1352–73.
[39] Salmaso S, Bersani S, Scomparin A, Mastrotto F, Caliceti P. Isr J Chem 2010;50(2):160–74.
[40] Mero A, Campisi M. Polymers 2014;6(2):346–69.
[41] de Las Heras Alarcon C, Pennadam S, Alexander C. Chem Soc Rev 2005;34(3):276–85.
[42] Agrawal D, Hackenberger CPR. Indian J Chem A 2013;52(8–9):973–91.
[43] Polyak D, Eldar-Boock A, Baabur-Cohen H, Satchi-Fainaro R. Polym Adv Technol 2013;24(9):777–90.
[44] Knop K, Hoogenboom R, Fischer D, Schubert US. Angew Chem Int Ed 2010;49(36):6288–308.
[45] Veronese FM, Pasut G. Drug Discov Today 2005;10(21):1451–8.
[46] Davis TP, Haddleton DM, Richards SN. J Macromol Sci Rev Macromol Chem Phys 1994;C34(2):243–324.
[47] Gaynor S, Greszta D, Mardare D, Teodorescu M, Matyjaszewski K. J Macromol Sci Pure Appl Chem 1994;A31(11):1561–78.
[48] Matyjaszewski K. Curr Opin Solid State Mater Sci 1996;1(6):769–76.
[49] Braunecker WA, Matyjaszewski K. Prog Polym Sci 2007;32(1):93–146.
[50] Sivaram S. J Sci Ind Res 1997;56(1):1–16.
[51] Stridsberg KM, Ryner M, Albertsson AC. Degradable aliphatic polyesters, vol. 157; 2002. p. 41–65.
[52] Cianga I, Yagci Y. Prog Polym Sci 2004;29(5):387–99.
[53] Yokozawa T, Ajioka N, Yokoyama A. New frontiers in polymer synthesis, vol. 217; 2008. p. 1–77.
[54] Roghani-Mamaqani H, Haddadi-Asl V, Salami-Kalajahi M. Polym Rev 2012;52(2):142–88.
[55] Nomura K, Takamizu K, Fujio T. J Syn Org Chem Jpn 2013;71(1):2–13.
[56] Ohta Y, Yokozawa T. Hierarchical macromolecular structures: 60 years after the Staudinger Nobel Prize II, vol. 262; 2013. p. 191–238.
[57] Lucon J, Qazi S, Uchida M, Bedwell GJ, LaFrance B, Prevelige Jr PE, et al. Nat Chem 2012;4(10):781–8.
[58] Kato M, Kamigaito M, Sawamoto M, Higashimura T. Macromolecules 1995;28(5):1721–3.
[59] Wang JS, Matyjaszewski K. J Am Chem Soc 1995;117(20):5614–5.
[60] Iovu MC, Matyjaszewski K. Macromolecules 2003;36(25):9346–54.
[61] Matyjaszewski K. Macromolecules 1998;31(15):4710–7.
[62] Andrus MB, Lashley JC. Tetrahedron 2002;58(5):845–66.
[63] Siegwart DJ, Oh JK, Gao HF, Bencherif SA, Perineau F, Bohaty AK, et al. Macromol Chem Phys 2008;209(21):2180–93.
[64] Trzebicka B, Robak B, Trzcinska R, Szweda D, Suder P, Silberring J, et al. Eur Polym J 2013;49(2):499–509.
[65] Paira TK, Banerjee S, Raula M, Kotal A, Si S, Mandal TK. Macromolecules 2010;43(9):4050–61.
[66] Le Droumaguet B, Velonia K. Angew Chem Int Ed 2008;47(33):6263–6.
[67] Peeler JC, Woodman BF, Averick S, Miyake-Stoner SJ, Stokes AL, Hess KR, et al. J Am Chem Soc 2010;132(39):13575–7.
[68] Bontempo D, Maynard HD. J Am Chem Soc 2005;127(18):6508–9.
[69] Chiefari J, Chong YK, Ercole F, Krstina J, Jeffery J, Le TPT, et al. Macromolecules 1998;31:5559–62.
[70] Moad G, Rizzardo E, Thang SH. Chem Asian J 2013;8(8):1634–44.
[71] Liu JQ, Bulmus V, Herlambang DL, Barner-Kowollik C, Stenzel MH, Davis TP. Angew Chem Int Ed 2007;46(17):3099–103.
[72] Boyer C, Bulmus V, Liu JQ, Davis TP, Stenzel MH, Barner-Kowollik C. J Am Chem Soc 2007;129(22):7145–54.
[73] De P, Li M, Gondi SR, Sumerlin BS. J Am Chem Soc 2008;130(34):11288–9.
[74] Heredia KL, Nguyen TH, Chang CW, Bulmus V, Davis TP, Maynard HD. Chem Commun 2008;28:3245–7.
[75] Gilmore JM, Scheck RA, Esser-Kahn AP, Joshi NS, Francis MB. Angew Chem Int Ed 2006;45(32):5307–11.
[76] Scheck RA, Dedeo MT, Iavarone AT, Francis MB. J Am Chem Soc 2008;130(35):11762–70.
[77] Witus LS, Moore T, Thuronyi BW, Esser-Kahn AP, Scheck RA, Iavarone AT, et al. J Am Chem Soc 2010;132(47):16812–7.
[78] Witus LS, Netirojjanakul C, Palla KS, Muehl EM, Weng CH, Iavarone AT, et al. J Am Chem Soc 2013;135(45):17223–9.
[79] Obermeyer AC, Jarman JB, Francis MB. J Am Chem Soc 2014;136(27):9572–9.
[80] Kane PM, Yamashiro CT, Wolczyk DF, Neff N, Goebl M, Stevens TH. Science 1990;250(4981):651–7.
[81] Hirata R, Ohsumk Y, Nakano A, Kawasaki H, Suzuki K, Anraku Y. J Biol Chem 1990;265(12):6726–33.
[82] Perler FB, Davis EO, Dean GE, Gimble FS, Jack WE, Neff N, et al. Nucleic Acid Res 1994;22(7):1125–7.
[83] Muir TW, Sondhi D, Cole PA. Proc Natl Acad Sci U S A 1998;95(12):6705–10.
[84] Muralidharan V, Muir TW. Nat Methods 2006;3(6):429–38.
[85] Esser-Kahn AP, Francis MB. Angew Chem Int Ed 2008;47(20):3751–4.
[86] Thom J, Anderson D, McGregor J, Cotton G. Bioconjug Chem 2011;22(6):1017–20.
[87] Popp MW, Antos JM, Ploegh HL. Curr Protoc Protein Sci 2009 [Chapter 15: Unit 15 13].
[88] Leung MK, Hagemeyer CE, Johnston AP, Gonzales C, Kamphuis MM, Ardipradja K, Such GK, Peter K, Caruso F. Angew Chem Int Ed 2012;51(29):7132–6.
[89] Tsukiji S, Nagamune T. Chembiochem 2009;10(5):787–98.
[90] Popp MW, Dougan SK, Chuang TY, Spooner E, Ploegh HL. Proc Natl Acad Sci U S A 2011;108(8):3169–74.
[91] Hodgson DR, Sanderson JM. Chem Soc Rev 2004;33(7):422–30.
[92] Velonia K, Rowan AE, Nolte RJ. J Am Chem Soc 2002;124(16):4224–5.
[93] Heredia KL, Bontempo D, Ly T, Byers JT, Halstenberg S, Maynard HD. J Am Chem Soc 2005;127(48):16955–60.
[94] Rosendahl MS, Doherty DH, Smith DJ, Carlson SJ, Chlipala EA, Cox GN. Bioconjug Chem 2005;16(1):200–7.
[95] Bell SJ, Fam CM, Chlipala EA, Carlson SJ, Lee JI, Rosendahl MS, et al. Bioconjug Chem 2008;19(1):299–305.
[96] Fancy DA, Melcher K, Johnston SA, Kodadek T. Chem Biol 1996;3(7):551–9.
[97] Chopra A. [99mTc(CO)3]+-Labeled anti-epidermal growth factor receptor (HER2) affibody ZHER2:342 with a hexa-histidine tag (H6) on the C-terminal. Bethesda (MD): Molecular Imaging and Contrast Agent Database (MICAD); 2004.
[98] Chopra A. [99mTc(CO)3]+-Labeled anti-epidermal growth factor receptor (HER2) affibody ZHER2:342 with a tri-(histidine-glutamate) peptide tag (HE) 3 on the N-terminal. Bethesda (MD): Molecular Imaging and Contrast Agent Database (MICAD); 2004.
[99] Strugnell SA, Wiefling BA, Deluca HF. Anal Biochem 1997;254(1):147–9.
[100] Meredith GD, Wu HY, Allbritton NL. Bioconjug Chem 2004;15(5):969–82.
[101] Chen G, Heim A, Riether D, Yee D, Milgrom Y, Gawinowicz MA, et al. J Am Chem Soc 2003;125(27):8130–3.
[102] Gauthier MA, Klok HA. Chem Commun 2008;23:2591–611.
[103] Cong Y, Pawlisz E, Bryant P, Balan S, Laurine E, Tommasi R, et al. Bioconjug Chem 2012;23(2):248–63.
[104] Aeschlimann D, Paulsson M. Thromb Haemost 1994;71(4):402–15.
[105] Rachel NM, Pelletier JN. Biomolecules 2013;3(4):870–88.
[106] Sato H, Hayashi E, Yamada N, Yatagai M, Takahara Y. Bioconjug Chem 2001;12(5):701–10.
[107] Mero A, Spolaore B, Veronese FM, Fontana A. Bioconjug Chem 2009;20(2):384–9.
[108] Mero A, Schiavon M, Veronese FM, Pasut G. J Control Release 2011;154(1):27–34.
[109] Carrico IS, Carlson BL, Bertozzi CR. Nat Chem Biol 2007;3(6):321–2.
[110] Pavlou AK, Reichert JM. Nat Biotechnol 2004;22(12):1513–9.
[111] Shaunak S, Godwin A, Choi JW, Balan S, Pedone E, Vijayarangam D, et al. Nat Chem Biol 2006;2(6):312–3.
[112] Balan S, Choi JW, Godwin A, Teo I, Laborde CM, Heidelberger S, et al. Bioconjug Chem 2007;18(1):61–76.
[113] Brocchini S, Balan S, Godwin A, Choi JW, Zloh M, Shaunak S. Nat Protoc 2006;1(5):2241–52.
[114] Brocchini S, Godwin A, Balan S, Choi JW, Zloh M, Shaunak S. Adv Drug Deliv Rev 2008;60(1):3–12.
[115] Schumacher FF, Nobles M, Ryan CP, Smith ME, Tinker A, Caddick S, et al. Bioconjug Chem 2011;22(2):132–6.
[116] Lewis A, Tang Y, Brocchini S, Choi JW, Godwin A. Bioconjug Chem 2008;19(11):2144–55.
[117] Smith ME, Schumacher FF, Ryan CP, Tedaldi LM, Papaioannou D, Waksman G, et al. J Am Chem Soc 2010;132(6):1960–5.
[118] Jones MW, Strickland RA, Schumacher FF, Caddick S, Baker JR, Gibson MI, et al. J Am Chem Soc 2012;134(3):1847–52.

[119] Weber PC, Ohlendorf DH, Wendoloski JJ, Salemme FR. Science 1989;243(4887):85–8.
[120] Kim CH, Axup JY, Schultz PG. Curr Opin Chem Biol 2013;17(3):412–9.
[121] Chin JW, Martin AB, King DS, Wang L, Schultz PG. Proc Natl Acad Sci U S A 2002;99(17):11020–4.
[122] Anderson JC, Wu N, Santoro SW, Lakshman V, King DS, Schultz PG. Proc Natl Acad Sci U S A 2004;101(20):7566–71.
[123] Chin JW, Cropp TA, Anderson JC, Mukherji M, Zhang Z, Schultz PG. Science 2003;301(5635):964–7.
[124] Wang L, Xie J, Schultz PG. Annu Rev Biophys Biomol Struct 2006;35:225–49.
[125] Cho H, Daniel T, Buechler YJ, Litzinger DC, Maio Z, Putnam AM, et al. Proc Natl Acad Sci U S A 2011;108(22):9060–5.
[126] Mu J, Pinkstaff J, Li Z, Skidmore L, Li N, Myler H, et al. Diabetes 2012;61(2):505–12.
[127] Alconcel SNS, Baas AS, Maynard HD. Polym Chem 2011;2(7):1442.
[128] Lu H, Wang D, Kazane S, Javahishvili T, Tian F, Song F, et al. J Am Chem Soc 2013;135(37):13885–91.
[129] Hoffman AS. Clin Chem 2000;46(9):1478–86.
[130] Chilkoti A, Chen G, Stayton PS, Hoffman AS. Bioconjug Chem 1994;5(6):504–7.
[131] Kanazawa H, Sunamoto T, Ayano E, Matsushima Y, Kikuchi A, Okano T. Anal Sci 2002;18(1):45–8.
[132] Ding Z, Long CJ, Hayashi Y, Bulmus EV, Hoffman AS, Stayton PS. Bioconjug Chem 1999;10(3):395–400.
[133] Stayton PS, Shimoboji T, Long C, Chilkoti A, Chen G, Harris JM, et al. Nature 1995;378(6556):472–4.
[134] Bulmus V, Ding Z, Long CJ, Stayton PS, Hoffman AS. Bioconjug Chem 2000;11(1):78–83.

Weiping Gao, Ph.D. Professor of Biomaterials and Nanomedicines, Department of Biomedical Engineering, School of Medicine, Tsinghua University, Beijing 100084. Phone: +86 10 62789472 (office), Email: gaoweiping@tsinghua.edu.cn, Lab web: http://openwetware.org/wiki/Gao_lab.Dr. Weiping Gao is Professor of Biomaterials and Nanomedicines in the Department of Biomedical Engineering, School of Medicine, Tsinghua University, Beijing. Dr. Gao obtained his PhD degree in Polymer Chemistry and Physics from Peking University in 2004. He received his Bachelor and Master degrees in Chemical Engineering from Qingdao Institute of Chemical Technology in 1997 and 2000, respectively. From 2005 to 2007, he worked as a COE (Center of Excellence) investigator in Institute for Chemical Research, Kyoto University, Japan. From 2007 to 2011, he was a research associate in Department of Biomedical Engineering and Center for Biologically Inspired Materials and Material Systems, Duke University, USA. He joined as a principal investigator in the Department of Biomedical Engineering, School of Medicine, Tsinghua University in 2011. Dr. Gao's research Interests include, but not limited to polymer/biomolecular engineering, drug/gene delivery, molecular imaging/diagnosis, and regenerative medicine.