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Protein–Polymer Conjugates

Precision Conjugation: An Emerging Tool for Generating Protein–Polymer Conjugates

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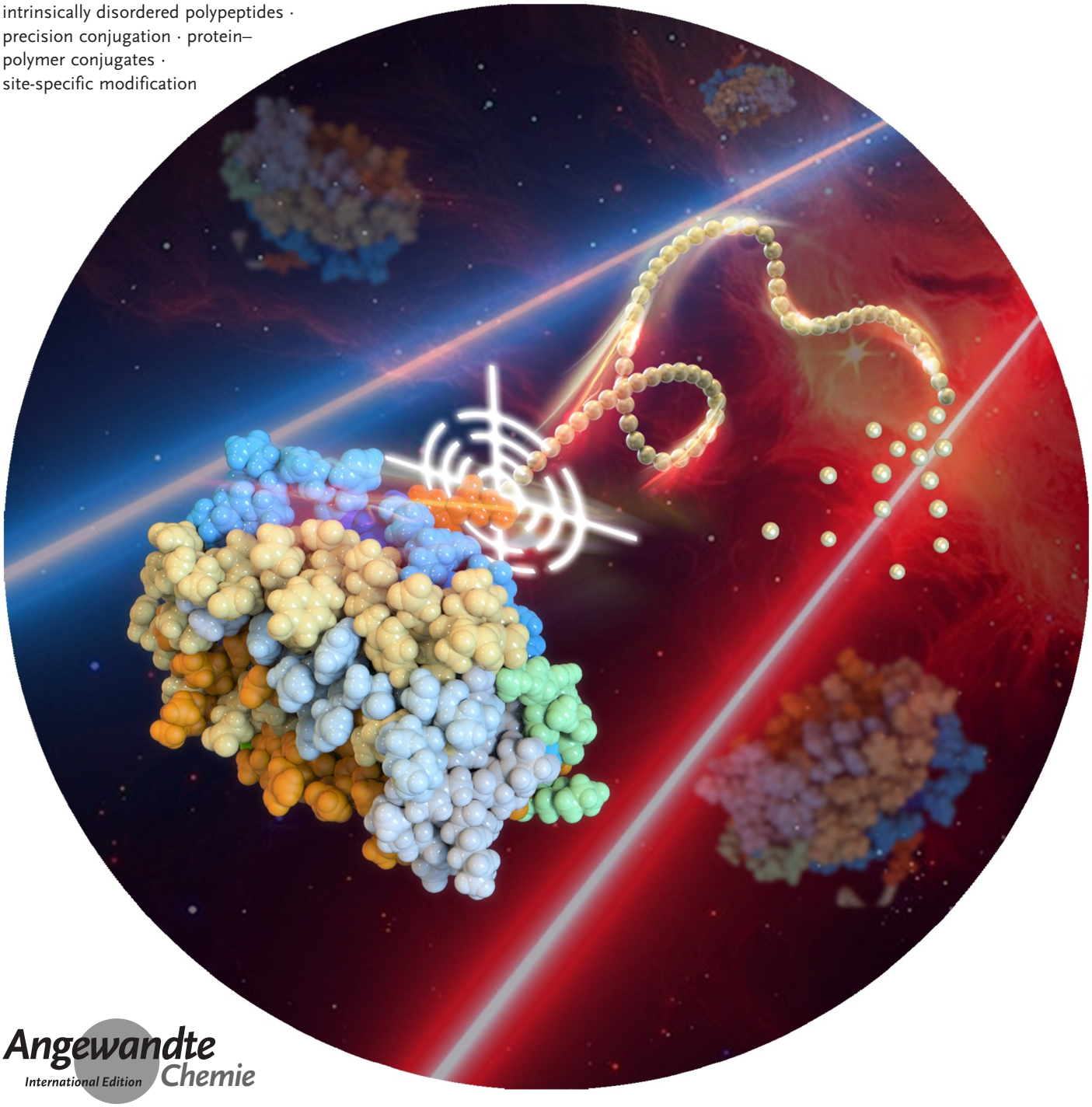

Protein–polymer conjugates are increasingly being applied in biomedicine because of the unique combination of the biological activity of the proteins and the multifunctionality and flexibility of the polymers. However, traditional protein–polymer conjugation techniques suffer from some unavoidable drawbacks, including nonspecificity and low efficiency. In this Minireview, we discuss a new approach based on "precision conjugation" for the construction of the next-generation protein–polymer conjugates in a more controlled, more efficient, and tailorable fashion for a broad range of advanced applications. In illustrating the concept, we highlight two general methods: site-specific in situ growth and intrinsically disordered polypeptide fusion, with a focus on the in situ, efficient, and controllable formation of protein–polymer conjugates. At the end, the challenges associated with this emerging concept are further discussed.

1. Introduction

Protein drugs play an increasingly important role in the treatment of various major diseases, including cancer and diabetes as well as cardiovascular and infectious diseases.[1] Up to now, more than 200 protein and peptide drugs have been approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) for clinical use.[2] Notably, 8 of the top 10 drugs sold in 2018 were protein drugs.[3] However, protein drugs still suffer from some drawbacks.[1] Firstly, proteins are relatively unstable, so they might be denatured and inactivated during the processes of preparation, storage, transportation, and use. Secondly, the blood circulation half-lives of most proteins, except humanized monoclonal antibodies, are short because of clearance by the kidneys and the reticuloendothelial system, as well as thermal and enzymatic degradation. Thirdly, the potential immunogenicity of proteins may reduce their therapeutic efficacy and cause side effects. Finally, the membrane impermeability of most proteins makes them incapable of accessing potential intracellular targets.[4]

Modifying proteins with polymers to form protein–polymer conjugates has been proven to be an efficient way to address the above problems.[5] As a class of bioconjugates, protein–polymer conjugates are typically synthesized by coupling natural and recombinant proteins to synthetic polymers, mainly through covalent bonds. They can be utilized as biopharmaceuticals, biomaterials, and biochemical reagents as a result of their exceptional properties arising from the biological activity of proteins and the multifunctionality typical of manmade polymers.[6] The earliest and most successful technology in the field of protein–polymer conjugates is the attachment of one end of poly(ethylene glycol) (PEG)—a class of hydrophilic, neutral, chemically inert, and protein-resistant polymers—onto the surface of a protein. This process is known as PEGylation, a typical "grafting-to" method. The first example of PEGylation dates back to 1977, when Davis and co-workers for the first time demonstrated that PEGylation could make proteins of non-human origin, such as bovine liver catalase and bovine serum albumin (BSA), less immunogenic.[7] From then on, PEGylation was found to be able to ameliorate the solubility, stability, and in vivo circulation time of many pharmaceutical proteins. After decades of practice and improvement, PEGylation has been fully integrated into the processes of drug research and development as a standard method to develop long-acting protein drugs.[8] To date, more than 16 PEGylated protein drugs have been used clinically around the world for the treatment of various diseases.[5] However, traditional PEGylation is plagued by some unavoidable drawbacks,[8] including: 1) nonspecific modification of proteins with PEGs often results in a mixture composed of positional isomers with unpredictable alterations of the protein activities, pharmacology, biodistribution, and biosafety, as well as difficulties in separation and purification;[9] 2) steric hindrance between the two macromolecules results in a low conjugation efficiency;[10] 3) limited functionalization of PEGs restricts the functional expansion of the final bioconjugates;[11] 4) potential immunogenicity of PEG and preexisting anti-PEG antibodies may compromise the clinical efficacy of PEGylated drugs;[12] and 5) the intrinsic nonbiodegradability of PEG may cause bioaccumulation in vivo.[13]

Much effort has been made to address these issues in the past four decades, but it still remains a great challenge to develop new and general strategies to solve these problems simultaneously.[14] To this end, we and other groups put forward a new concept of precision conjugation, with the aim of constructing the next-generation protein–polymer conjugates in a more controllable, efficient, and tailorable manner for widespread and sophisticated applications (Figure 1). The term "precision conjugation" covers three main points: site-specific protein modification, controlled synthesis of a functional polymer, and efficient protein–polymer conjugation. To illustrate this concept, two major general methods will be discussed in this Minireview, namely, site-specific in situ growth (SIG) and intrinsically disordered polypeptide fusion (IPF). In these methods, a well-defined polymer is grown in situ from a specific site of a protein of interest to obtain a site-specific protein–polymer conjugate with high efficiency and multifunctionality.

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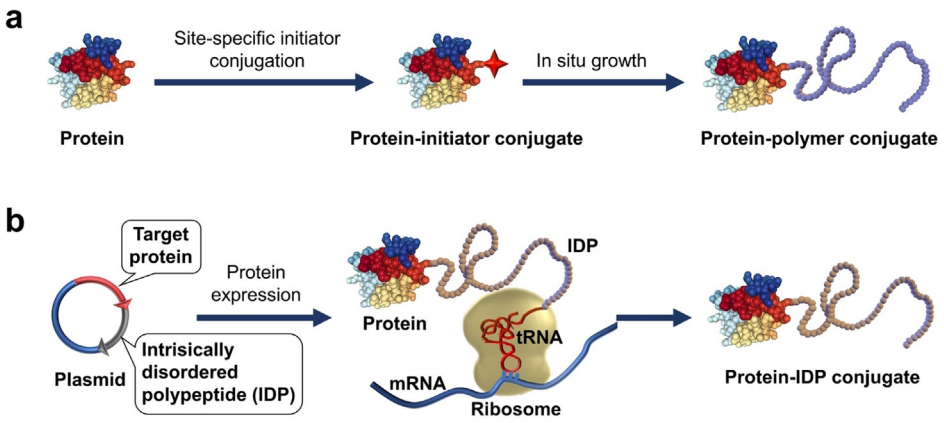

Figure 1. Overview of workflow in precision conjugation: a) site-specific in situ growth (SIG) and b) intrinsically disordered polypeptide fusion (IPF).

2. Site-Specific In Situ Growth (SIG)

SIG is a chemical method in which a controlled polymerization initiator is site-specifically linked to a protein of interest to form a site-specific protein–initiator conjugate, followed by in situ growth of a polymer from the protein–initiator conjugate through a controlled polymerization technique to form site-specific protein–polymer conjugates in situ and efficiently (Figure 1 a).[15] To illustrate the progress made in the realm of SIG, Table 1 summarizes the details of over 40 examples of SIG that have been reported in the literature so far. In this section, we will introduce the strategies of site-specific protein–initiator conjugation and then the in situ growth of site-specific polymer conjugates of proteins.

2.1. Site-Specific Protein–Initiator Conjugation

The first step of SIG is site-specific protein–initiator conjugation. To do so, two prerequisites should be met: 1) the modification site of the protein, whether a reactive group of an amino acid residue or a specific peptide fragment, can be targeted in a highly selective way, and its relationship with the biological activity of the protein fully studied; 2) the bioorthoganal conjugation reaction selected to modify the protein with a controlled polymerization initiator should be performed in aqueous buffered solution at relatively low temperatures to yield a site-specific protein–initiator conjugate with high efficiency. The representative strategies of site-specific protein–initiator conjugation are summarized in Figure 2. The cysteine residue, N-terminus, and C-terminus are the most common sites for efficient site-specific protein–initiator conjugation.

The cysteine residue is a commonly used site for site-specific protein–initiator conjugation because of the higher nucleophilicity of the free thiol group compared to the amino group and its relatively low abundance in proteins.[42] Some proteins, such as human serum albumin (HSA) and BSA, contain a unique single free cysteine residue for the site-specific conjugation of an initiator.[43] However, for most proteins, such a single free cysteine residue is unavailable. In this case, a single free cysteine can be introduced at the surface of the proteins through genetic mutation for site-specific conjugation of an initiator. Typically, the bioorthoganal reactions of Michael addition (thiol-maleimide reactions in the pH range 6.5–7.5)[44] and disulfide bond formation are utilized for attachment of an atom transfer radical polymerization (ATRP)[45] initiator or reversible addition-fragmentation chain transfer (RAFT)[46] agent to the free cysteine residue of the protein with high efficiency and selectivity. Notably, disulfide bond re-bridging could also be utilized to link a functionalized ATRP initiator to the interchain disulfide bonds of Herceptin,[32] a monoclonal antibody approved for cancer therapy, with high efficiency and well-retained structure and function. It is worth noting that

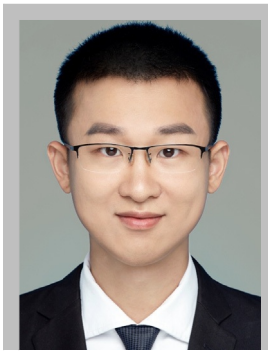

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Table 1: Detailed information about each example of SIG methodology.

Polymerization method	Modification site	Modification chemistry	Protein–polymer conjugate	Research group
ATRP	N-terminus	PLP[a]-mediated transamination	Mb-POEGMA	Chilkoti[15a]
			Mb-PCBMA[b]	Chilkoti[16]
			Mb-PDMAEMA	Chilkoti[16]
			GOX-PHPMA	Gao[17]
			GOX-PDMAEMA	Gao[18]
	C-terminus	intein-mediated ligation	GFP-POEGMA	Chilkoti[15b]
		sortase-mediated ligation	GFP-POEGMA	Chilkoti[19]
			IFN-POEGMA	Gao[15d]
			IFN-PMPC	Gao[15e]
			IFN-POEGMA-PHPMA	Gao[20]
			IFN-PDEGMA	Gao[21]
			exendin-4-POEGMA	Chilkoti[22]
			exendin-4-PTEGMA	Chilkoti[22]
	native cysteine	Michael addition	HSA-PHPMA	Gao[23]
			HSA-PDPA[c]	Gao[24]
			BSA-P(OEGMA-*co*-RMA[d])	Haddleton[25]
			BSA-P(DMAEMA-*co*-RMA)	Haddleton[25]
			BSA-polystyrene	Velonia[26]
			BSA-polyalkyne	Velonia[27]
		disulfide formation	BSA-PHEMA[e]	Liu and Zhao[28]
	mutant cysteine	Michael addition	c-GFP[f]-POEGMA	Gao[15c]
			lysozyme-poly(NIPAAm)	Maynard[29]
			HFBI[g]-POEGA[h]	Milani[30]
		disulfide formation	lysozyme-poly(NIPAAm)	Maynard[29]
			transferrin-PDEGMA	Alexander[31]
	disulfide bonds	disulfide re-bridging	herceptin-P(OEGMA-*co*-RMA)	Gao[32]
	lysine B29	aminolysis of nitrophenyl carbonate	insulin-glycopolymer	Maynard[33]
	UAA at residue 134	genetic incorporation	GFP-POEGMA	Mehl and Matyjaszewski[34]
RAFT	native cysteine	disulfide formation	BSA-POEGA	Bulmus, Davis, and Boyer[35]
			BSA-poly(NIPAAm)	Bulmus and Davis[36]
			BSA-PHEA[i]	Bulmus and Davis[36]
			BSA-PDMAA[j]	Boyer[35b]
			β-glucosidase-poly(NIPAAm)	Datta and De[37]
		Michael addition	BSA-poly(NIPAAm)	Sumerlin[38]
	mutant cysteine	Michael addition	PPase[k]-poly(NIPAAm)	Chen[39]
ROP	native cysteine	Michael addition	BSA-polyglycidols	Harth[40]
		disulfide formation	BSA-polydisulfide	Lu[41]
			sortase A-polydisulfide	Lu[41]
			dihydrofolate reductase-polydisulfide	Lu[41]
			UCHT1[l]-polydisulfide	Lu[41]
	mutant cysteine	disulfide formation	GFP-polydisulfide	Lu[41]
			azoreductase-polydisulfide	Lu[41]

[a] Pyridoxal-5-phosphate. [b] Poly(carboxybetaine methacrylate). [c] Poly(2-(diisopropylamino)ethyl methacrylate). [d] Rhodamine B methacrylate. [e] Poly(2-hydroxyethyl methacrylate). [f] Cyclized GFP. [g] Hydrophobin I. [h] Poly(oligo(ethylene glycol) acrylate). [i] Poly(hydroxyethyl acrylate). [j] Poly(*N*,*N*′-dimethylacrylamide). [k] Escherichia coli inorganic pyrophosphatase. [l] A human anti-CD3 fragment of antigen binding (Fab) antibody.

cysteine modification may be limited by the instability and potential reversibility of the resulting covalent bonds, whether a S–S bond[47] or a S–C bond.[48] On the other hand, these fragile bonds may be desirable for the design of stimulus-responsive protein–polymer conjugates.[49]

The N-terminus of a protein is thought to be a highly selective modification site because of its unique chemical structure.[50] In particular, the Francis group found that PLP can react with the N-terminus to form an imine having an α proton with a much lower pK_a value than other imines.[51] This difference allows tautomerization to occur uniquely at this location. The resulting glyoxylimine hydrolyzes to yield a ketone or aldehyde specifically at the N-terminal site. Subsequently, PLP-mediated N-terminal transamination was utilized to introduce aldehyde and ketone groups solely to the N-termini of myoglobin (Mb)[15a,16] and glucose oxidase (GOX),[17,18] respectively. Treatment of the resulting aldehyde and ketone with an oxyamine-functionalized ATRP initiator yielded N-terminal Mb- and GOX-ATRP initiator conjugates, respectively.

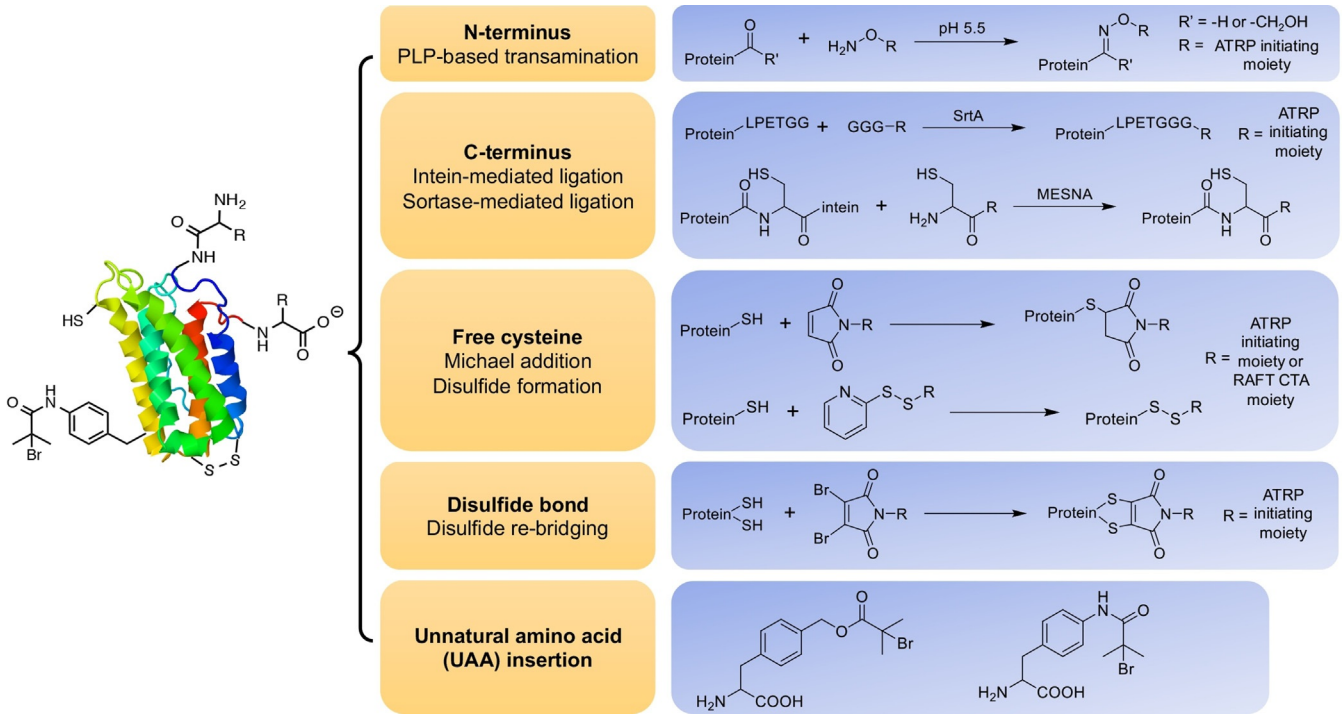

Figure 2. Representative site-specific protein–initiator conjugation methods. SrtA: sortase A; MESNA: 2-mercaptoethane sulfonate sodium.

Unlike the N-terminus, the C-terminus is not chemically different from the carboxy groups of aspartic acid and glutamic acid residues. Therefore, current approaches to C-terminal modification mainly involve the genetic installation of a specific amino acid sequence to the C-terminus for intein-mediated ligation or enzyme-mediated ligation. The Chilkoti group genetically engineered green fluorescent protein (GFP) fused C-terminally with intein for intein-mediated C-terminal modification of GFP with a cysteine-functionalized ATRP initiator.[15b] Later on, the recognition sequence LPXTG of sortase A was genetically fused to the C-terminus of GFP,[19] interferon-α (IFN),[15d,e,20,21] and Exendin-4[22] for sortase A mediated C-terminal modification of these proteins with a triglycine-functionalized ATRP initiator.

In addition, the insertion of unnatural amino acids is promising as a general method for site-specific protein–initiator conjugation, as demonstrated by the insertion of an ATRP initiator into GFP.[34] However, it remains a challenge to insert unnatural amino acids into proteins in an efficient way.

2.2. In Situ Growth of Site-Specific Polymer Conjugates of Proteins

The second step of SIG is the in situ growth of polymer conjugates from the site-specific protein–initiator conjugates by aqueous, controlled polymerization techniques including ATRP, RAFT polymerization, and ring-opening polymerization (ROP; Figure 3). The increasing development of controlled polymerization techniques has made it possible to directly grow well-defined polymers with tailored molecular weights, narrow molecular weight dispersity, varied topologies, and functions at a specific site of a protein of interest to in situ and efficiently form site-specific protein–polymer conjugates in aqueous solutions.

ATRP is the most extensively applied controlled polymerization technique in SIG. Although ATRP had been used in SIG by several research groups, including Maynard,[29,33] Haddleton,[25] Velonia,[26,27] Matyjaszewski,[34] Chilkoti,[15a,b,16,22] and Gao[15c–e,17,18,20,21,23,24,32] since 2005, it only started to be utilized in SIG to generate site-specific protein–polymer conjugates for therapeutic applications in 2016.[15d] As the first and major example, SIG was applied to IFN to construct a series of C-terminal IFN–polymer conjugates including IFN–poly(oligo(ethylene glycol) methyl ether methacrylate) (IFN-POEGMA),[15d] IFN–poly(2-methacryloyloxyethyl phosphorylcholine) (IFN-PMPC),[15e] IFN–poly(di(ethylene glycol) methyl ether methacrylate) (IFN-PDEGMA),[21] and IFN–POEGMA-poly(2-hydroxypropyl methacrylate) (IFN-POEGMA-PHPMA).[20] Notably, the overall yield of IFN-POEGMA synthesized by SIG was found to be more than 60 times higher than that of its analogue prepared by the conventional grafting-to method. The in vitro anti-proliferative bioactivity of IFN-POEGMA was 7.2 times higher than that of PEGASYS,[52] an FDA-approved commercial long-acting PEGylated IFN. The higher bioactivity retention of IFN-POEGMA was mainly attributed to the site-specific conjugation of IFN to POEGMA. In contrast, PEGASYS contained at least 9 positional isomers with much lower bioactivities, typically in the range of 1–7% of the pristine IFN bioactivity.[9] As a result, although the pharmacokinetics of IFN-POEGMA was similar to PEGASYS, IFN-POEGMA cured 75% ovarian tumors in mice,

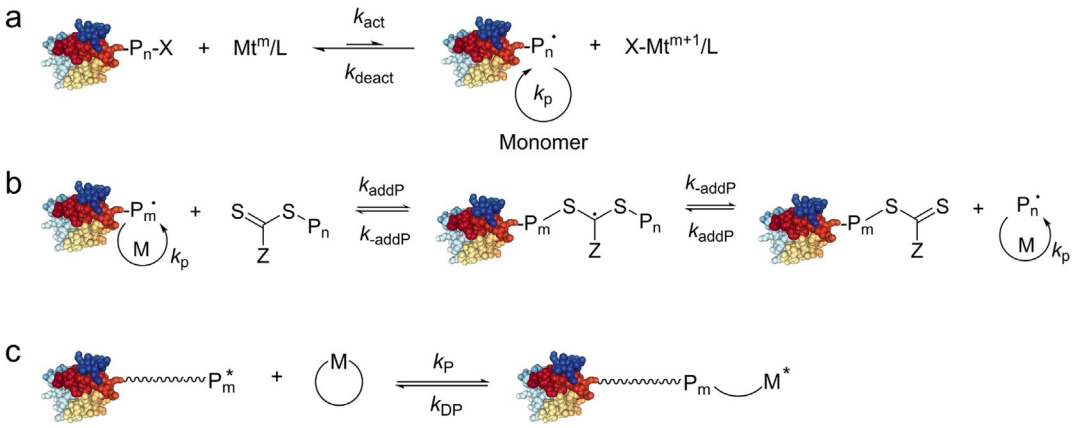

Figure 3. Reaction mechanisms of three controlled polymerization techniques utilized in SIG: a) ATRP, b) RAFT polymerization, and c) ROP.

whereas PEGASYS could not cure the tumors.[15d] In current protein–polymer conjugates, the extension of the half-life is often compromised by a decrease in the bioactivity.[53] To overcome the dilemma, SIG was used to synthesize IFN-POEGMA-PHPMA, which self-assembled into micelles during in situ ATRP (Figure 4).[20] Intriguingly, the cross-linked micelles retained 10% of the pristine IFN bioactivity; however, at concentrations below the critical micelle concentration, the un-cross-linked micelles could dissociate into unimers of the conjugates with an increased bioactivity of 41.9% of that of pristine IFN. These data suggest the absence of multivalent effects. Furthermore, the micelles exhibited an in vivo circulation half-life 1.7 times longer than that of PEGASYS because of its significantly enlarged size. More importantly, it could completely inhibit tumor growth with 100% animal survival, which could not be achieved with PEGASYS. Recently, Gao et al. further developed a series of cationic conjugates of GOX–poly(*N*,*N*′-dimethylamino-2-ethyl methacrylate) (GOX-PDMAEMA) with different molecular weights of PDMAEMA by SIG for tumor starvation and oxidation therapy.[18] An optimized GOX-PDMAEMA conjugate showed 1.5 times enhanced cytotoxicity, 2 times increased tumor retention, and 5 times improved tolerability than GOX itself, mainly because of its electrostatic interaction with tumor cells. As a result, it could completely eradicate tumors without apparent side effects after a single intratumoral injection, whereas GOX itself was ineffective and caused severe side effects. The Chilkoti group reported two C-terminal Exendin-4–polymer conjugates, Exendin-4–POEGMA and Exendin-4–poly(tri(ethylene glycol) methyl ether methacrylate) (PTEGMA), prepared by SIG, and their efficacy in blood glucose control.[22] Interestingly, they found that POEGMA and PTEGMA showed reduced binding affinity towards patient-derived pre-existing anti-PEG antibodies as a result of the reduced number of ethylene glycol (EG) repeat units in the side chains of POEGMA and PTEGMA compared to PEG. Similarly, the Maynard group prepared a lysine 29 B-chain specific insulin–trehalose glycopolymer conjugate by SIG to improve the pharmacology of insulin.[33]

RAFT polymerization was firstly applied in SIG by the Davis and Bulmus groups to prepare cysteine-specific BSA-POEGMA conjugates.[35a,36] The controllability of RAFT polymerization in SIG was subsequently further improved by the Sumerlin group through altering the BSA-conjugating site of the RAFT agent from the "Z" group to the "R" group.[38] Additionally, cysteine-specific pyrophosphatase–poly(*N*-isopropyl acrylamide) (PNIPAAm)[39] and β-glucosidase–PNIPAAm conjugates[37] were constructed using RAFT polymerization mediated SIG by the Chen and the Datta and De research groups, respectively, for thermoresponsive activity modulation or enzyme recycling.

Surprisingly, the Harth group found that a semibranched polyglycidol could directly be grown from the Cys-34 site of BSA in phosphate buffer of pH 6.0 in the absence of a metal catalyst to form cysteine-specific BSA–polyglycidol conjugates.[40] This is the first example of ROP-mediated SIG. However, it is necessary to further study the dynamics of the ROP and perform detailed physicochemical and biological characterization of the bioconjugates. Very recently, the Lu group reported another example of ROP-mediated SIG to form cysteine-specific protein–polydisulfide conjugates in situ with a traceless release profile for potential applications in the adjustment of protein activity, downstream purification, and intracellular delivery of proteins.[41] A variety of cysteine-containing proteins including EGFP, BSA, sortase A, azoreductase, and dihydrofolate reductase have been verified to be compatible with this method. This is the first example of in situ growing backbone-degradable polymers from proteins.

Generally speaking, the molecular weight of the in situ prepared polymer could be controlled by the polymerization time and the ratio of the monomer to macroinitiator, which was found to be close to the theoretical value.[24,38] The dispersity values were typically between 1.2 and 1.4. However, narrower molecular weight dispersities of less than 1.2 could be achieved by exploiting activators regenerated by electron-transfer ATRP,[22] photoinduced electron transfer RAFT,[35b] and cryopolymerization.[41]

3. Intrinsically Disordered Polypeptide Fusion (IPF)

IPF is a biological method in which an IDP as an alternative to PEG is genetically fused to the N- or/and C-

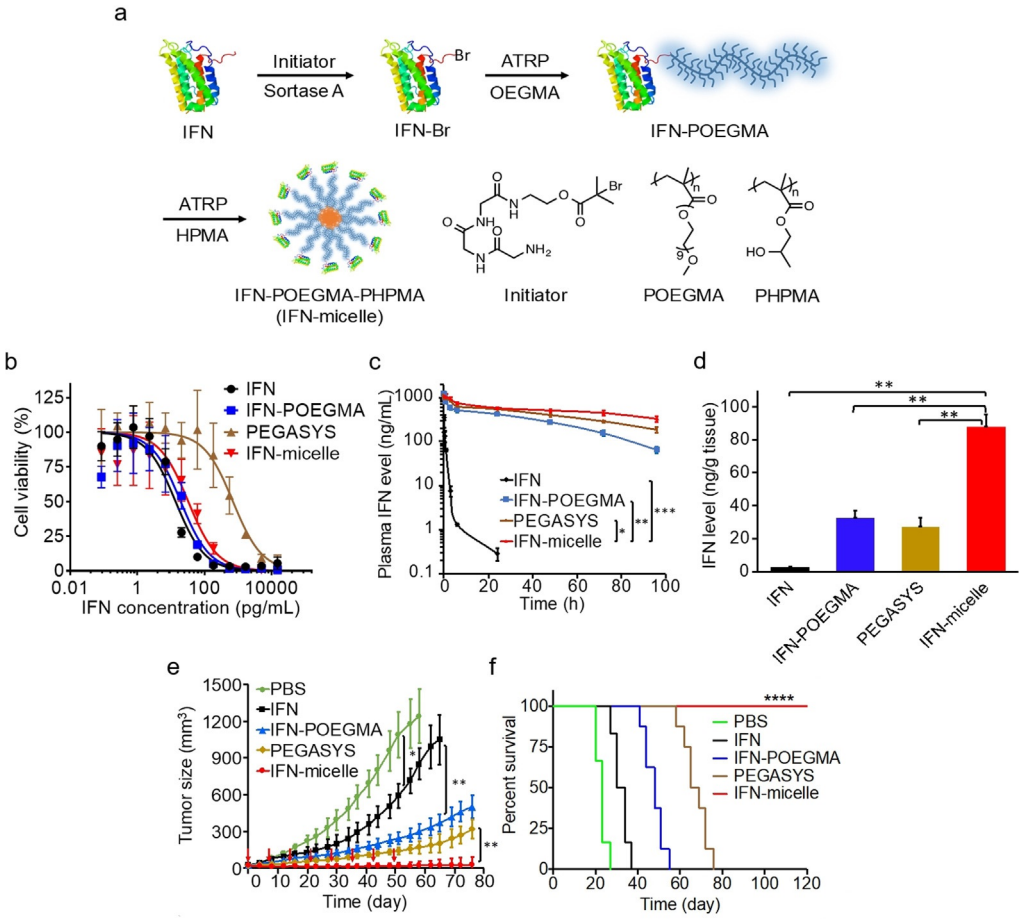

Figure 4. a) Preparation of IFN-POEGMA-PHPMA (IFN-micelle). b) In vitro anti-proliferative bioactivity, c) in vivo pharmacokinetics, d) tumor retention, e) in vivo inhibition of tumor growth, f) cumulative animal survival. Reprinted from Ref. [20] with permission. Copyright (2018) American Chemical Society.

terminal of a protein of interest in a plasmid vector, followed by routine protein expression and purification to yield a precisely defined protein–IDP conjugate with high efficiency and multifunctionality (Figure 1 b). In this section, we will focus on four kinds of IDPs that are being used in the IPF approach, including elastin-like polypeptides (ELPs), XTENs, PAS, and gelatin-like protein (GLK).

3.1. ELPs

ELPs were designed by the Urry group in 1985,[54] and are derived from the repeat sequences of the hydrophobic domains of human tropoelastin.[55] ELPs have repeat sequences of Val-Pro-Gly-Xaa-Gly, in which Xaa, also called a "guest residue", can be any amino acid residue except proline. Interestingly, ELPs have thermally responsive behaviors with tunable inverse transition temperatures (T_ts). When the environmental temperature is below the T_t value, ELPs are highly soluble, whereas when the environmental temperature is above the T_t value, ELPs aggregate into insoluble precipitates. This unique property has been applied to purify ELP-fused proteins without traditional chromatography by a technique termed inverse transition cycling.[56] Notably, the T_t value can be regulated by the hydrophobicity or polarity of the guest residues and the chain length,[57] thus making it possible to rationally design thermoresponsive ELPs tailored for different applications.

Although ELPs have been extensively used for protein purification,[58] their application for protein delivery only began a few years ago. As a prime example, a soluble ELP was genetically fused to the C-terminus of IFN to extend its circulation half-life.[59] The resultant conjugate IFN-ELP retained 41 % of the bioactivity of native IFN, and showed a 27-times longer circulation half-life than native IFN. Consequently, IFN-ELP accumulated in tumor more efficiently than did IFN and significantly extended the median survival time of mice bearing tumors relative to when native IFN was used. Furthermore, thermoresponsive IFNα-ELP-(V)[60] and thermally and enzymatically responsive IFNα-MMPS-ELP(V)[61] were developed to realize one-month zero-order sustained release and super-long circulation half-lives (497 h for IFNα-ELP(V), 422 h for IFNα-MMPS-ELP-(V), 1.9 h for IFNα) after single subcutaneous injection at their maximum tolerated doses in mice (Figure 5). Notably, IFNα-MMPS-ELP(V) exhibited considerably improved tumor penetration in response to matrix metalloproteinases (MMPs) overexpressed in various tumors compared to IFNα-

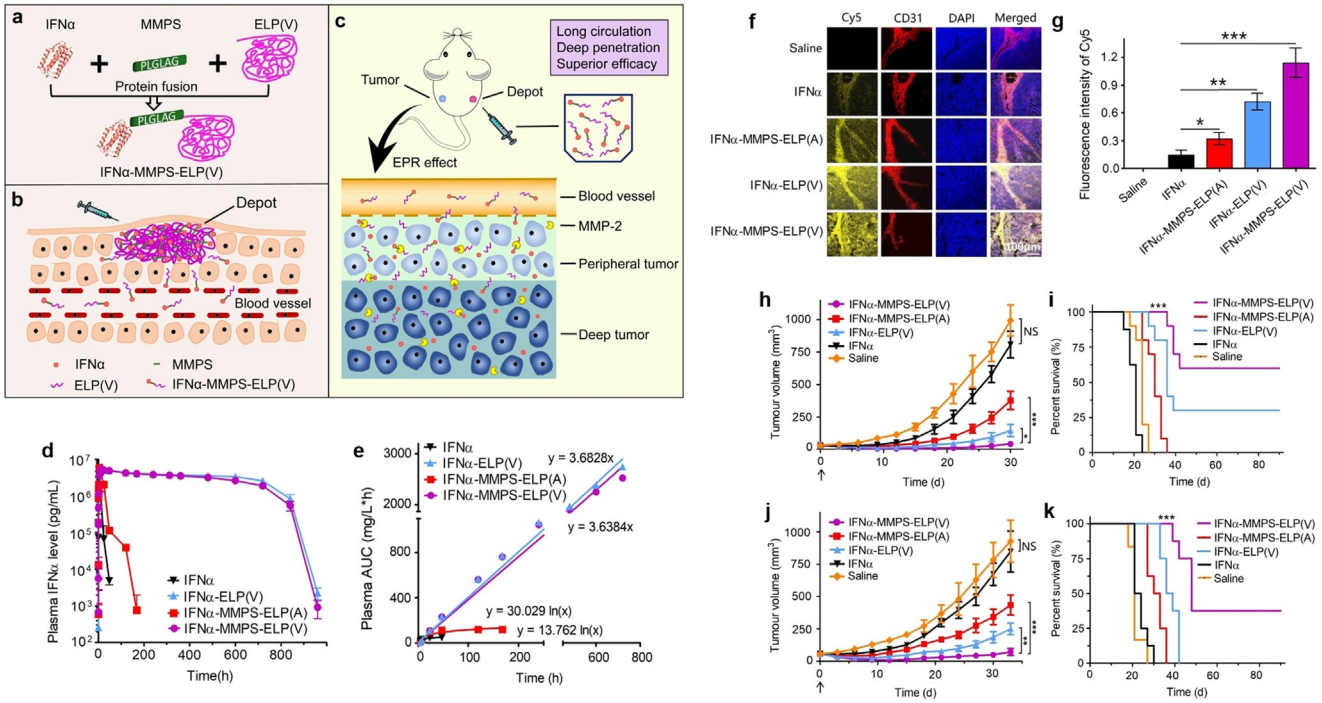

Figure 5. a) Structure of IFNα-MMPS-ELP(V). MMPS: matrix metalloproteinase substrate. b) In situ formation of an IFNα-MMPS-ELP(V) depot post-subcutaneous injection and sustained release of free IFNα-MMPS-ELP(V) from the depot. c) Tumor penetration of free IFNα-MMPS-ELP(V). d) Pharmacokinetic profiles after subcutaneous administration at its maximum tolerated dose. e) Cumulative area under the curves (AUCs). f) Tumor penetration. IFNα and its conjugates were labeled by Cy5 (yellow), vessels were stained with anti-CD31 antibody (red), and cell nuclei were stained with DAPI (blue). g) Quantitative fluorescence intensity of Cy5. h,i) Inhibition of melanoma growth and cumulative survival. j,k) Inhibition of ovarian tumor growth and cumulative survival. Reprinted from Ref. [61].

ELP(V). Consequently, IFNα-MMPS-ELP(V) exhibited remarkably enhanced antitumor efficacy and considerably increased animal survival rates in two mouse models of ovarian tumor and melanoma compared to IFNα-ELP(V). Similarly, the Chilkoti group fused glucagon-like peptide-1 (GLP-1) with thermoresponsive ELPs to achieve glucose control over one week and long circulation times of 10 days in mice and 17 days in monkeys.[62] To date, at least three ELP-fused peptides and proteins have been investigated in clinical trials by PhaseBio Pharmaceuticals Inc.[63]

3.2. XTENs

XTENs were developed by Amunix. On the basis of the hydrophobicity, stability, charge, and reactivity of each amino acid, Pro, Glu, Ser, Thr, Ala, and Gly were chosen as the components of the first XTEN in 2009.[64] Subsequently, more XTENs with different lengths were developed.[65] To date, nine XTEN-fused peptides and proteins are in preclinical or different clinical trial stages.[63] Among them, VRS-317 is a human growth hormone (hGH)-XTEN conjugate that is at the phase III clinical trial stage. hGH is a pharmaceutical protein whose half-life is only 2–3 h. Two different XTENs, XTEN912 and XTEN144, are separately fused to the N-and C-termini of hGH to reduce the kidney clearance and receptor-mediated clearance. The elimination half-lives of VRS-317 in rats and monkeys were 15 and 110 h, respectively.[66]

3.3. PAS

PAS was designed by the Skerra group on the basis of XTEN, whereby negative Glu, β-sheet-preferring Thr, and poorly soluble Gly were further excluded.[67] PAS consists of proline, alanine, and serine, with these amino acid residues distributed randomly within a 20- or 24-amino-acid sequence to further form PAS polypeptides containing over 200 amino acid residues by repetitive ligation. This composition makes PAS polypeptides highly hydrophilic and conformationally disordered, thereby leading to a large hydrodynamic volume that is necessary for PSA to be an ideal alternative to PEG. In the first reported study, three proteins—Fab fragment, IFN-α2b, and hGH—were fused with PAS polypeptides with different lengths.[67] All of them exhibited significantly extended circulation half-lives compared with native proteins in a mouse model. So far, more than ten PASylated peptides and proteins, including PAS-leptin,[68] PAS-IFN-β1b,[69] and PAS-IL-1Ra,[70] have been in preclinical development with XL-Protein GmbH.

3.4. GLK

GLK was constructed by replacing all the hydrophobic residues and reserving the Gly-Xaa-Yaa repeats in gelatin.[71] By introducing more hydrophilic and negatively charged amino acids, GLK has a more open and unfolded conformation than gelatin and can serve as a PEG analogue for protein delivery. For example, the Huang group developed a GLK-G-CSF conjugate which could retain 71.3 % of the bioactivity of G-CSF in vitro.[72] The circulation half-life of the conjugate was up to 9.96 h, which is 5.7-times longer than that of G-CSF (1.76 h) and similar to that of PEGylated G-CSF (7.05 h).[72] More studies need to be performed to unveil the potential of GLK for constructing precise protein–polymer conjugates.

4. Comparison between SIG and IPF

4.1. Mutual Advantages

Compared with conventional PEGylation, SIG and IPF have several mutual advantages. Firstly, precise controlling of the conjugation site on a protein can be achieved by SIG and IPF, which not only enables maximum retention of the activity or a decline on demand but also benefits subsequent purification, characterization, and even the entire quality control process. Secondly, a high conjugation efficiency can be reached by SIG (typically $>50\,\%$) and IPF (100 %), which is particularly advantageous to the production of site-specific protein–polymer conjugates with high molecular weights. For example, a conjugated synthetic polymer of over 600 kDa and an IDP of nearly 200 kDa were synthesized by SIG and IPF, respectively[16,66] Thirdly, multifunctionality such as self-assembly and responsibility can be introduced into site-specific protein–polymer conjugates by SIG and IPF,[17,20,21,23,24,26,27,29,31,36–39,41,60–62] which extremely expands the scopes of protein–polymer conjugates in terms of the structure, function, and application.

4.2. Pros and Cons

Although SIG and IPF share these mutual advantages, they are different in many aspects and have their own superiorities (Table 2). Firstly, the conjugation site of a protein in IPF is limited to the N-/C-terminus, while any site on a protein can, in principle, be the conjugation site in SIG through protein engineering technologies such as cysteine mutation and unnatural amino acid insertion. For example, Alexander and co-workers genetically engineered five transferrin variants whereby mutant cysteine was distributed at five distinct sites for the construction of five different site-specific transferrin–polymer conjugates.[31] Secondly, the dispersity of polymers synthesized by SIG is inevitable because of the intrinsic mechanisms of controlled polymerization techniques,[73] while the polypeptides synthesized by IPF have precisely defined molecular weights and sequences. Thirdly, multistep operations related to genetic engineering, protein chemistry, and polymer chemistry technologies are involved in SIG, which is unfavorable for technology transfer, but, on the other hand, makes SIG a modular and flexible method to generate multifunctional protein–polymer conjugates. In contrast, only a one-step operation of genetic engineering is required in IPF, which makes IPF simpler and easier to realize technology transfer to the biopharmaceutical industry, as evidenced by several ELP- and XTEN-fused proteins undergoing clinical trials.[63] Fourthly, the intrinsic biodegradability of the polypeptides synthesized by IPF is beneficial to in vivo applications, but leads to short circulation half-lives for their protein conjugates, whereas the polymers synthesized by SIG are typically non-biodegradable, but their protein conjugates have long circulation half-lives. In the cases of IFN-ELP and IFN-POEGMA, which have similar polymer molecular weights of about 40 kDa, their in vivo half-lives vary greatly, being 8.6 and 31 h, respectively.[59,20]

Table 2: Pros and cons of SIG and IPF methods.

Strategy	Pros	Cons
SIG	· specific modification potentially at any site on the surface of protein · modular and flexible method favorable to generating multifunctional protein-polymer conjugates · long circulation half-life	· polymers with dispersity · multistep reactions unfavorable to technology transfer · non-biodegradable polymers unfavorable to in vivo metabolism
IPF	· polypeptides with precisely-defined molecular weights and sequences · simple and easy method favorable to technology transfer · biodegradable polypeptides favorable to in vivo metabolism	· specific modification limited to the N- or C-terminus · relatively short circulation half-life

4.3. Guidance for Choosing a Method

Figure 6 shows a flowchart for choosing the most appropriate method based on SIG or IPF when precision conjugation is required. Generally speaking, the modification site needs to be determined by the comprehensive consideration of both the structure and function of the protein of interest and the purpose of the precision conjugation. If N- and C-terminal modifications are unacceptable, IPF cannot be adopted and SIG should be considered. Next, if biodegradability is not desirable, SIG should be the choice. Last, if the fusion protein cannot be expressed or is expressed at a very low amount, SIG should be adopted. It should be noted that this is not a comprehensive guide suitable for all cases, since other factors may affect the final choice in reality.

5. Summary and Perspectives

As shown in this Minireview, SIG is a modular and flexible chemical method, through which a variety of synthetic polymers including hydrophobic and stimulus-respon-

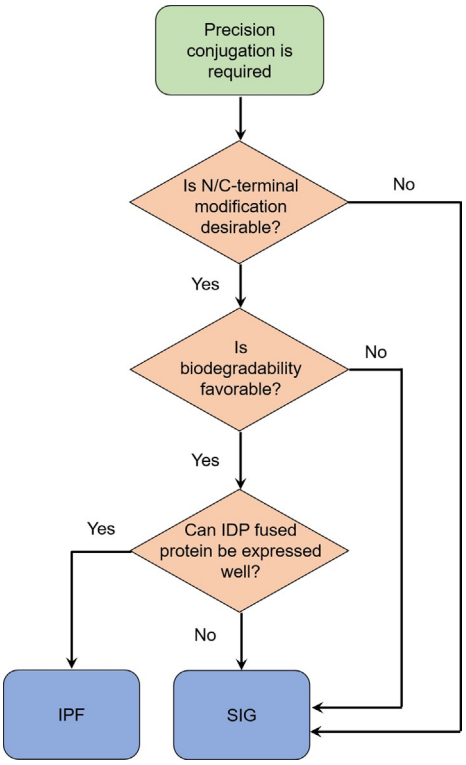

Figure 6. A guide for choosing the correct method between IPF and SIG for precision conjugation.

sive polymers can be grown at a specific site of a protein of interest to form site-specific protein–polymer conjugates with multifunctionality in situ and efficiently. However, although several strategies of site-specific protein modification such as cysteine modification, N- or C-terminal modification, and unnatural amino acid insertion have been developed for SIG, they have their own limitations in terms of efficiency, specificity, or generality.[50,74,75] Other bioorthogonal reactions at specific sites of proteins, such as methionine[76] and tyrosine[77] residues, can be exploited in SIG. Therefore, it still remains a great challenge to develop new, general, and efficient strategies for site-specific protein modification for SIG. Additionally, current controlled polymerization techniques are underdeveloped for SIG, which should further be optimized to improve the controllability and biocompatibility, especially when used to site-specifically grow functional polymers from proteins in aqueous buffer solutions. Notably, photoinitiated,[78] enzyme-catalyzed,[79] oxygen-tolerant,[80] and electrochemically mediated[81] controlled polymerization techniques are being developed and expected to be applied in SIG to overcome these problems. Therefore, SIG is still in its infancy and more effort is required to improve SIG for precision conjugation.

Unlike SIG, IPF is a simple and easy biological method, through which various IDPs can, at the gene level, be conjugated to the N- or C-terminus of protein to form genetically encoded protein–IDP conjugates in situ with high efficiency and multifunctionality. These features make IPF interesting and useful as the first kind of precision conjugation method for the efficient development of precise and multifunctional protein–polymer conjugates. However, the intrinsic biodegradability of IDPs leads to short circulation half-lives (typically < 10 h) of protein–IDP conjugates synthesized by IPF. In contrast, the circulation half-lives of protein–polymer conjugates synthesized by SIG are typically longer than 30 h because of the non-biodegradability of synthetic polymers. Therefore, it is a considerable challenge to overcome the drawback of IDPs to extend the in vivo half-life of protein–IDP conjugates made by IPF. The self-assembly of protein–IDP conjugates into micelles would be a promising way to extend the half-life of proteins considerably, inspired by the above-mentioned IFN–micelle example.[20]

For translation to the clinic, more attention needs to be paid to the potential immunogenicity of the polymers in the protein–polymer conjugates prepared by SIG and IPF. Notably, the multifunctionality of the protein–polymer conjugates prepared by SIG and IPF has not been well-exploited to meet the tough demands of biomedicine. However, the increasing development and cross-fusion of chemistry, materials, and biology will lead to the challenges faced by SIG and IPF being overcome in the future. We speculate that SIG and IPF as representative grafting-from methods will hold great promise as precision conjugation methods for the development of next-generation protein–polymer conjugates for widespread applications even beyond biomedicine.

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Conflict of interest

The authors declare no conflict of interest.

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