Review

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Strategies for Making Multimeric and Polymeric Bifunctional Protein Conjugates and Their Applications as Bioanalytical Tools

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Abstract

Enzymes play a central role in the detection of target molecules in biotechnological fields. Most probes used in detection are bifunctional proteins comprising enzymes and binding proteins conjugated by chemical reactions. To create a highly sensitive detection probe, it is essential to increase the enzyme-to-binding protein ratio in the probe. However, if the chemical reactions required to prepare the probe are insufficiently site-specific, the detection probe may lose functionality. Genetic modifications and enzyme-mediated post-translational modifications (PTMs) can ensure the site-specific conjugation of proteins. They are therefore promising strategies for the production of detection probes with high enzyme contents, i.e., polymeric bifunctional proteins. Herein, we review recent advances in the preparation of bifunctional protein conjugates and polymeric bifunctional protein conjugates for detection. We have summarized research on genetically fused proteins and enzymatically prepared polymeric bifunctional proteins, and will discuss the potential use of protein polymers in various detection applications.

Keywords: detection probes, bifunctional proteins, site-specific conjugation, polymeric bifunctional proteins, protein polymers.
1. Introduction

Proteins are well-defined biopolymers with specific biological functions. Certain proteins, particularly enzymes, are especially useful as probe molecules in diagnostics.\textsuperscript{1} The ability of enzymes to catalytically degrade or crosslink intrinsic substrates can be exploited for the detection of specific target molecules. This makes them promising molecular probes for such applications as enzyme immunoassays (EIAs)\textsuperscript{2} and enzyme-based biosensors.\textsuperscript{1,3} Alkaline phosphatase (AP),\textsuperscript{4,5} β-galactosidase,\textsuperscript{6,7} and horseradish peroxidase (HRP)\textsuperscript{8–11} are often used as probes or reporter enzymes in EIAs. The enzymes must be conjugated with binding modules such as biotin, antigens, and binding proteins, e.g., antibodies, immunoglobulin-binding proteins (IBPs), and streptavidin (SA).\textsuperscript{12} Enzyme-linked molecular probes are generally produced by chemical conjugation reactions; such reactions often deactivate the enzymes or binding modules by random modifications, resulting in low yields and a loss of functionality. Because the signal outputs of detection probes are derived from enzymes, the conjugation of multiple proteins is a promising strategy for the creation of highly sensitive detection probes.\textsuperscript{13} However, joining multiple proteins in a site-specific manner whilst retaining the functionality of each protein unit is challenging.\textsuperscript{14,15}

Genetically fusing proteins enables their stoichiometric coupling with precisely controlled conjugation sites.\textsuperscript{14–16} Bifunctional proteins that contain multimeric enzyme units can be obtained by genetic modification strategies that utilize multimeric binding proteins—such as streptavidin—as fusion partners. Enzymatic reactions provide an alternative strategy for the creation of multimeric or polymeric probes.\textsuperscript{13,17,18} By
genetically introducing specific conjugation points to the target proteins for post-translational modifications (PTMs), enzyme-mediated PTMs can be used to create detection probes with a high degree of conjugation—i.e., polymeric bifunctional proteins.\textsuperscript{17,19,20} Herein, we review promising genetic and catalytic approaches to producing bifunctional protein conjugates with high enzyme-to-binding protein ratios, and the use of such multimeric or polymeric bifunctional proteins as detection probes.

2.1 Detection probes created by genetic modification strategies

Genetic modification via the tandem fusion of proteins is a widely used strategy for the design of artificial protein conjugates, including bifunctional proteins and protein polymers.\textsuperscript{14,15,21} The genes encoding the proteins of interest can be expressed in one expression cassette, and the proteins can be joined by adding flexible or rigid linkers.\textsuperscript{16,21–25} Although helical linkers can improve expression and bioactivity of fusion proteins in several cases, this strategy only works if the proteins of interest can be folded appropriately. Furthermore, only a limited number of protein units can be fused or conjugated. This strategy may produce some unexpected results, such as deactivation, misfolding, and problems with protein expression and purification.\textsuperscript{14,15,26} The most commonly used labeling enzyme for detection probes is HRP. However, the recombinant production of active HRP has hardly ever been achieved. Likewise, there have been only a limited number of reports of the recombinant production of HRP fusion proteins.\textsuperscript{10,27,28} Kang et al. reported one of the few examples of the expression of soluble HRP in \textit{Escherichia coli}—i.e., the expression of HRP fused with \textit{E. coli} phosphoglycerate kinase.
(PGK), which acts as a solubility enhancement module. The PGK-HRP hybrid was expressed with a high yield of up to 72 mg per liter of culture. However, heme, Ca$^{2+}$, and glutathione disulfide were required to recover the activity of the HRP, demonstrating the difficulty of producing active recombinant HRP molecules. In this chapter, we will introduce recent developments in the production of recombinant HRP fusion proteins, in particular using the silkworm-baculovirus expression vector system (silkworm-BEVS).

### 2.1.1 Expression of HRP-protein A/G fusion protein in silkworms

Immunoglobulin-binding proteins (IBPs)—such as protein A (pA) from *Staphylococcus aureus*, and protein G (pG) from *Streptococcus* sp.—are useful biotools for the detection and purification of immunoglobulin G antibodies (IgGs), because they can bind strongly to IgGs of different subtypes from a wide range of animals. HRP chemically conjugated with IBPs for use as detection probes in EIAs are commercially available from various manufacturers. The production of recombinant fusion proteins of HRP and IBPs is advantageous because it directly creates HRP-based detection probes for IgGs, without the need for additional conjugation reactions or purification steps.

Our group reported the production of a recombinant chimera protein of HRP fused with a pA unit and two pG units (HRP-pAG), with a peptide tag containing lysine (i.e., a K-tag) using silkworm-BEVS (Figure 1A). The K-tag in the fusion protein—i.e., HRP-pAG—is a peptidyl substrate for microbial transglutaminase (MTG), which can be used to introduce functional molecules. We demonstrated the functionalization of the K-tag...
with biotin by conjugating a biotinylated dipeptide QG—i.e., biotin-QG—via an MTG reaction to yield HPR-pAG(biotin) (Figure 1B); subsequently, the HRP-pAG(biotin) was conjugated with SA to form a multimeric conjugate via biotin-SA interaction (Figure 1C). SA-conjugated HRP-pAG(biotin) can accumulate multiple HRP units to form a complex of pAG and IgG, resulting in strong signals in EIAs (Figure 1D).

A soluble form of HRP-pAG was successfully expressed in silkworm larvae, with a yield of approximately 2.2 mg per 10 mL of hemolymph obtained from approximately 25 larvae. Most of the HRP-pAG was in the apo form, but simple incubation with heme spontaneously produced the heme-incorporated holo-form, which exhibited comparable activity to the plant-derived HRP. A model enzyme-linked immunosorbent assay (ELISA) system was set up to detect ovalbumin (OVA), and the functionality of the HRP-pAG as an ELISA probe was evaluated by comparing it with that of a commercially available HRP-pG conjugate produced by chemical conjugation. The monomeric HRP-pAG produced signals that were comparable to those produced by the commercial HRP-pG, whereas the SA-conjugated HRP-pAG produced signals that were approximately twice as strong (Figure 1E). This demonstrated the benefit of the multivalency of the enzyme units in detection probes. HRP derived from natural resources does not have purification tags or specific conjugation points for PTM. Therefore, the purification of HRP, its conjugation with IBPs, and the subsequent purification steps require extra care and effort. The robust silkworm-BEVs protein expression system enabled the production of recombinant HRP-pAG conjugates with purification tags and specific conjugation points (K-tags), thereby demonstrating easier purification and greater functionalization than is
possible using HRP from natural sources.

Figure 1 Gene construct and activity of HRP-pAG in an OVA-detecting ELISA. (A) Gene construct of recombinant HRP-pAG using pENTR11 as a vector. L21: the 21 base leader sequence derived from a lobster tropomyosin cDNA at the 57 untranslated regions; 30K: a secretion signal peptide of 30 kDa protein from silkworm (*B. mori*); H: an octahistidine-tag; StrepTag II: a peptide tag that binds to Streptactin for purification with a sequence of WSHPQFEK; TEV: the recognition sequence of tobacco etch virus protease, with a sequence of ENLYFQG; HRP: the gene encoding HRP; pA: the gene encoding protein A (pA); pG: the gene encoding protein G (pG); K-tag: a peptide tag containing a lysine with a sequence of MRHKGS. (B) Schematic illustration of the conjugation reaction of HRP-pAG with biotin-QG to form HRP-pAG(biotin) conjugate catalyzed by a microbial transglutaminase (MTG). (C) Schematic illustration of the conjugation reaction of HRP-pAG(biotin) with streptavidin (SA) to form HRP-pAG(biotin)-SA conjugate. (D) Schematic illustration of OVA detection using HRP-pAG(biotin)-SA conjugate. (E) OVA-detecting ELISA using HRP-pAG(biotin)-SA and commercial HRP-pG. The concentrations of all HRP were fixed at 0.05 U/mL. The error bars represent standard errors of absorbance value obtained from four individual wells of 96 well plates. Reproduced from ref.27 Copyright with permission from the Willey.
2.1.2 Expression of HRP-streptavidin fusion protein in silkworms

As an alternative to IBPs, streptavidin (Stav or SA) has often been used as an enzyme conjugation partner for the detection of biotinylated molecules. Stav forms a tetramer. Therefore, genetically fusing an enzyme to Stav automatically yields a detection probe with four enzyme units. Patmawati et al. reported the production of HRP-Stav chimeras using the silkworm-BEVS expression system. They constructed two differently ordered chimeras: HRP-Stav and Stav-HRP (Figure 2A). Both proteins were expressed in the soluble fraction of the silkworm larvae hemolymph as apo-forms. After activation by the addition of heme, both (hHRP)$_4$-Stav and Stav-(hHRP)$_4$ regained their catalytic activity in the same manner as HRP-pAG. The functionalities of (hHRP)$_4$-Stav and Stav-(hHRP)$_4$ as detection probes were evaluated in an OVA-detecting ELISA (Figure 2B and C). Both chimeric HRPs successfully exhibited signals on the ELISA plate, demonstrating that both the HRP and Stav units in the probes were active. The order of HRP and Stav conjugation was critical to the functionality of the hybrids as detection probes, because (hHRP)$_4$-Stav exhibited stronger signals than Stav-(hHRP)$_4$. This implied that the structure of HRP in Stav-(hHRP)$_4$ was partially disordered during its expression and the folding of its tertiary and quaternary structures. Nevertheless, both chimeric HRPs produced stronger signals in the ELISA than commercial HRP-Stav conjugates (Figure 2C). The conjugation ratio of Stav to HRP in the commercial HRP-Stav conjugates is unclear. However, considering the molecular weights of HRP and the Stav tetramer—which are 44 and 53 kDa, respectively—the average number of HRP units
in one Stav tetramer should not exceed three. In contrast, the conjugation ratio of HRP to
the Stav tetramer in the HRP and Stav chimeras in the research by Patmawati et al. was
strictly limited to four. Consequently, the functionalities of those HRP chimeras were
superior to that of the chemically conjugated HRP-Stav. The recombinant expression of
HRPs using the silkworm-BEVS system enables the direct and facile production of
detection probes with high functionalities. Together with the functionalization strategy
using enzyme-mediated PTMs, the functionality of recombinant HRPs could be further
improved. We anticipate the further application of this straightforward strategy for
producing recombinant detection probes to the labeling of other enzymes such as calf
intestinal alkaline phosphatase (CIAP).
Figure 2 Production of genetically fused HRP and Stav using silkworm-BEVs. (A) Gene constructs of recombinant HRP-Stav and Stav-HRP using pENTR11 as a vector. L21: a 21 base leader sequence derived from a lobster tropomyosin cDNA at the 57 untranslated regions; 30K: a secretion signal peptide of 30 kDa protein from silkworm (B. mori); H_H is a hexahistidine-tag; HRP: the gene encoding HRP; Stav: the gene encoding streptavidin (Stav) (B) Schematic illustration of (hHRP)_4-Stav and Stav-(hHRP)_4 in an OVA-detecting ELISA. hHRP refers to holo-HRP. (C) OVA-detecting ELISA using (hHRP)_4-Stav and Stav-(hHRP)_4. Commercial HRP-Stav conjugate was applied as a control for comparison. The concentrations of all HRP were fixed at 0.5 U/mL. Reproduced from ref.28 Copyright with permission from the Willey.
2.2 Enzymatic crosslinking reaction of proteins

The enzymatic crosslinking reactions of proteins have attracted huge interest from researchers owing to their high site specificity. Table 1 lists some enzymes that have been used to crosslink proteins. Enzymes have potential as powerful biotools for crosslinking proteins if they are used in combination with the genetic modification of target proteins, whereby specific substrate peptide sequences or peptide tags for enzymes are introduced into the target proteins. For example, HRP, laccase, transglutaminase (TGase), sortase A and butelase require additional peptide tags to site-specifically catalyze protein crosslinking. However, the site-specific crosslinking of multiple proteins—i.e., protein polymerization—is still challenging, even using those enzymes. For example, TGase and sortase A must form a tripartite complex with two substrates during their catalytic cycle. However, as the degree of protein conjugation increases, the recognition of crosslinking sites on the protein conjugates is hampered by steric hindrance, resulting in low crosslinking efficiency. Moreover, the introduction sites and peptide tag sequences of target proteins must be carefully designed to prevent intramolecular crosslinking and promote intermolecular crosslinking. In contrast, HRP and laccase are highly effective protein polymerization enzymes, because they do not require the formation of a tripartite complex for crosslinking. The crosslinking occurs through the non-enzymatic radical coupling reactions of tyrosyl radicals. Therefore, as long as, there are tyrosine residues on the protein conjugates that can be recognized by HRP or laccase, the conjugation reaction proceeds further to yield “protein polymers”. Furthermore, the number of intrinsic tyrosine residues exposed on the surface
of the protein is limited, making the HRP- and laccase-mediated protein polymerization reactions highly site-specific in spite of the high degree of conjugation. Tyrosinase- and lysyl oxidase-mediated protein crosslinking involves the nonenzymatic coupling of amino groups. As a result, the site-specific crosslinking of proteins using those enzymes is difficult. In this chapter, we summarize recent research into the creation of site-specifically crosslinked protein probes with high multivalency using enzymes.
Table 1 List of enzymes utilized for protein-protein crosslinking.

| Enzyme                   | Cross-linking target                                                                 | Mode of reactions                                                                 |
|--------------------------|--------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------|
| Transglutaminase         | Side chain of Gln and primary amino groups, typically the ε-amino group of side chain of Lys. | Cross-linking reaction of the side chain of Gln and primary amino group to yield an isopeptide bond. |
| Sortase A                | C-terminal LPXTG motif and N-terminal triglycine (GGG).                               | After cleaving the amide bond between T-G in the LPXTG motif and cross-linking the C-terminus of T with the N-terminal amino group of G3 motif to ligate polypeptides LXPTG motif and N-terminal triglycine. |
| Butelase                 | C-terminal Asx(Asp or Asn)-His-Val motif and N-terminal Xaa1-Xaa2 motif. Xaa1 is any amino acid except Pro, and Xaa2 is Cys, Ile, Leu or Val. | After cleaving the amide bond between Asx and His in the C-terminal Asx-His-Val motif and ligating the C-terminus of Asx with N-terminal amino group of Xaa1-Xaa2 motif. |
| Horseradish peroxidase   | Tyrosine                                                                             | Upon activation by H2O2, HRP catalyzes oxidation of phenolic moiety of tyrosine side chain to form tyrosyl radicals. The tyrosyl radicals then cross-link with each other non-enzymatic manner. |
| Laccase                  | Tyrosine                                                                             | Laccase catalyzes oxidation of phenolic moiety of tyrosine side chain to form tyrosyl radicals by using O2. The tyrosyl radicals then cross-link with each other non-enzymatic manner. |
| Tyrosinase               | Tyrosine                                                                             | Converting the phenolic moiety of tyrosine residue to quinone and subsequently cross-linking with primary amino group or thiol groups in proteins through 1,4-addition manner. |
| Lysyl oxidase            | Lysine                                                                               | Oxidizing the primary amino group of the side chain of Lys to form an aldehyde. Subsequently, the aldehyde cross-link with primary amine to form Schiff base. Alternatively, the aldehyde undergoes aldol condensation reaction with another aldehyde group. |
2.2.1 Transglutaminase-catalyzed formation of polymeric probes

TGases are acyltransferases that catalyze the transfer of the γ-carboxyamine group of glutamines to the ε-amine group of lysines to crosslink proteins.\textsuperscript{43} \textit{Streptomyces mobaraensis} TGase (MTG) is a highly stable cofactor-free enzyme that has strong ligation capacity for a wide range of substrates. Owing to its favorable properties, MTG has become an important catalyst in the food industry,\textsuperscript{44} and in biotechnological research.\textsuperscript{18,45,46}

The utility of MTG in the design of polymeric probes has been demonstrated by generating a protein-grafted synthetic polymer (\textbf{Figure 3A}). Synthetic polymers containing the $N$-benzyloxycarbonyl-$L$-glutaminylglycine (Z-QG) motif have been used as scaffolds to conjugate numerous enzyme units via MTG-catalyzed conjugation reactions. A chimeric molecule comprising alkaline phosphatase (IPP),\textsuperscript{47} pG (IPP-PG), and a K-tag (MRHKGS) was grafted onto the Z-QG polymer using MTG (\textbf{Figure 3A}).\textsuperscript{13} The successful formation of large IPP-PG-Z-QG polymer conjugates was confirmed by size-exclusion chromatography (SEC) (\textbf{Figure 3B}) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis (\textbf{Figure 3C}). The functionality of the IPP-PG-Z-QG polymer conjugates was subsequently evaluated by using them as protein probes in an OVA-detecting ELISA (\textbf{Figure 3D}). The ELISA revealed the presence of OVA, anti-OVA-IgG, and the IPP-PG-Z-QG polymer conjugates. The IPP-PG-Z-QG polymer conjugates produced stronger signals than the monomeric IPP-PG, demonstrating the benefits of assembling enzyme units onto the Z-QG polymer to form multivalent probes. Based on the promising ELISA results achieved by the novel IPP-
PG-Z-QG polymer conjugates with regard to OVA detection, the MTG-mediated assembly of proteins onto a scaffold has been accepted as a promising and versatile strategy for preparing multiple or multimeric protein assemblies.
Figure 3 Schematic illustration of the grafting reaction of a synthetic polymer to protein. (A) Z-QG monomer is used for preparing synthetic polymer. It contains MTG-recognizable glutamine (Q) residue. The Z-QG monomer is then used in the polymerization reaction mediated by acrylamide. The synthesized Z-QG polymer was then applied in a conjugation reaction catalyzed by microbial transglutaminase (MTG) to form IPP-PG-Z-QG polymer conjugates. (B) Chromatogram of size exclusion chromatography (SEC) of IPP-PG-Z-QG polymer conjugates and free Ktag-IPP-PG. (C) SDS-PAGE analysis of conjugation reaction of Z-QG polymer and Ktag-IPP-PG catalyzed by MTG. Lane 1: SA with the addition of CY1-BAP, HRP, and H₂O₂; lane 2: thrombin was added to the samples of lane 1 and incubated for 12 h at 37 °C SA was visualized by biotin-4-fluorescein (in the native-PAGE analysis). (D) Schematic illustration of the expected binding mechanism of free Ktag-IPP-PG and form IPP-PG-Z-QG polymer conjugates in an OVA-detecting ELISA. (E) OVA-detecting ELISA using free IPP-PG and IPP-PG-Z-QG polymer conjugates with some various conditions; the presence and absence of OVA, Anti-OVA-IgG, and IPP-PG. Reproduced from ref. Copyright with permission from the American Chemical Society.
Another interesting study relating to the highly controlled assembly of protein conjugates using MTG was recently reported by Sato et al. (Figure 4). They prepared an enhanced green fluorescent protein (EGFP) with a unique peptide tag containing both of the MTG substrates—i.e., lysine (Lys) and glutamine (Gln) residues (K and Q, respectively)—called “PolyTags” (Figure 4B). MTG treatment of the EGFP with PolyTags resulted in the formation of protein conjugates with ladder-like bands. However, an EGFP with a K-tag at the N-terminus and a StrepTag, which is a Gln-containing peptide tag, at the C-terminus (KTag-EGFP-StrepTag) resulted in the formation of monomeric circular EGFP owing to intramolecular crosslinking between the K-tag and the StrepTag. The adjacent Lys and Gln residues in the PolyTag hampered intramolecular crosslinking owing to structural limitations, and consequently the EGFP-PolyTag was extensively crosslinked by MTG. The researchers further confirmed that heteromeric protein assemblies could also be prepared by simply mixing the PolyTagged proteins. Bioluminescence resonance energy transfer (BRET) analysis of heteromeric assemblies comprising NanoLuc® and EGFP revealed that these proteins are stably located in close mutual proximity in the assembled form. The results suggest that the combination of PolyTag and MTG is a promising molecular tool for the design of scaffold-less protein assemblies. This newly proposed strategy for the preparation of protein polymers is an interesting approach to the production of protein conjugates for detection probes.
Figure 4 Design of the protein of interests (POIs) for MTG-catalyzed polymerization reaction of proteins. (A) Introduction of a mono-reactive peptide tag (K- or Q-tag) to each terminus (N- and C-termini) of the POI that potentially leads to (i) polymerization or (ii) self-cyclization of the POI. (B) Introduction of a peptide tag containing K and Q residues, named PolyTag, for scaffold-less protein assembly of the POI. Reprinted from ref. 48 Copyright 2020 with permission from Elsevier.
2.2.2 Peroxidase-catalyzed formation of polymeric probes

Peroxidases (EC 1.11.1.7) are the most frequently used oxidoreductases in protein polymerization. Their ability to catalyze the oxidation of the phenolic moieties of tyrosine residues to form free tyrosyl radicals makes them useful enzymes for many protein polymerization reactions. By introducing a tyrosine-containing peptide to the target protein, highly site-specific crosslinking or even protein polymerization can be achieved using peroxidases.\textsuperscript{32,49} HRP is the most frequently used peroxidase for the protein polymerization reaction. Polymerizations of bacterial alkaline phosphatase (BAP),\textsuperscript{32,33,50} pG,\textsuperscript{34} the chimera protein of pG and pA,\textsuperscript{49} streptavidin,\textsuperscript{50} and SpyCatcher\textsuperscript{20} have been demonstrated using HRP. The first site-specific protein polymerization was reported by Minamihata et al. in 2011, in which BAP was genetically modified and a flexible peptide tag containing Tyr residues (Y-tag) was introduced to the C-termini of BAP.\textsuperscript{32} The HRP site-specifically recognized the Tyr residues in the Y-tag, and BAP polymers with a high degree of conjugation that retained the enzymatic activity of BAP were obtained. The Y-tagged BAP (CY1-BAP) was further utilized to create polymeric detection probes by co-polymerization with a Y-tagged streptavidin, CY1-SA (Figure 5A).\textsuperscript{33} No polymeric products arising from the heteroconjugation of BAP and SA without Y-tags, or from WT-BAP or WT-SA were detected by SDS-PAGE or native-PAGE (Figure 5B; WT = wild-type; Lane 1 for WT-BAP). Even though the intrinsic Tyr72 of WT-SA—which is located at the tip of the flexible loop region—is recognized by HRP, and contributes to WT-SA crosslinking, its reactivity was too low to be detected. Following the co-crosslinking of WT-SA with CY1-BAP, the reactivity of Tyr72 became
apparent, and resulted in WT-SA fractions co-crosslinked with CY1-BAP (Figure 5B, Lane 1 for CY1-BAP). However, the majority of the WT-SA remained intact. Therefore, the reactivity of Tyr72 was much lower than that of the Y-tags. Moreover, the treatment of CY1-SA with HRP completely consumed the monomeric SA (mSA) (Lane 2 in Figure 5B), indicating the high reactivity of Y-tags. The co-crosslinking of CY1-BAP and CY1-SA yielded copolymers with extremely high molecular weights (Figure 5B, Lane 2 for CY1-BAP). Last, the enzymatic activity of the BAP-SA conjugates was evaluated using a biotin-coated plate (Figure 5C). The BAP-SA conjugate prepared from CY1-BAP and CY1-SA exhibited the highest activity on the biotin-coated plate, revealing that both BAP and SA units were active in the polymeric structures. This demonstrates that highly site-selective crosslinking between Y-tag and HRP is a feasible and versatile strategy for producing polymeric detection probes.

BAP and SA form dimer and tetramer structures, respectively. Therefore, the introduction of a single Y-tag to the C-termini of BAP and SA would result in multiple Y-tags, which would facilitate the formation of protein polymers with high degrees of conjugation. In the case of monomeric proteins with single Y-tags, HRP- and Y-tag-mediated protein crosslinking only yields oligomers and dimers as the main products. The conjugation degree of monomeric proteins can be dramatically increased by simply introducing Y-tags to both their N- and C-termini. Dual-Y-tagged pG was constructed and used as a model monomeric protein. Field-flow fractionation multiangle light scattering (FFF-MALS) revealed that the resulting pG polymers were extremely large and highly branched, with maximum and average molecular weights of approximately 1,600.
kDa and 760 kDa, respectively. Based on this finding, Lili et al. created a SpyCatcher polymer (Figure 6A) that can be utilized as a platform for the assembly of proteins tagged with SpyTag in a ratio-controllable manner (Figure 6B). SpyCatcher-SpyTag is a protein ligation tool derived from Streptococcus pyogenes that was developed by Howarth et al. SpyCatcher specifically and spontaneously forms an isopeptide bond with SpyTag—which is a 13-amino acid peptide—when they are simply mixed together, and no additional elements such as metal ions or enzymes are required. A dual Y-tagged SpyCatcher molecule (Y-SpyCatcher-Y) was constructed, and HRP treatment resulted in the successful formation of highly crosslinked SpyCatcher polymers. The assembly of SpyTag-tagged NanoLuc® luciferase and pG on SpyCatcher polymers created NanoLuc-based bioprobes for ELISA (Figure 6C). The signal from the conjugates increased proportionally as the NanoLuc to pG ratio increased (Figure 6D), demonstrating the ability of the SpyCatcher polymer to assemble various proteins in a ratio-controlled manner. However, the NanoLuc-pG-SpyCatcher conjugates required at least 15 NanoLuc units to 1 pG unit to produce a higher signal than that produced by genetically fused NanoLuc-pG with a conjugation ratio of 1:1. This result suggests that not all the protein units on the SpyCatcher polymer were working properly, probably owing to the steric hindrance caused by the highly branched structure of the SpyCatcher polymer. Therefore, to maximize their functionality, it is essential to control the whole structure of protein polymers.
Figure 5 Heteroconjugation reaction of Y-tagged BAP (CY1-BAP) and Y-tagged Streptavidin (CY1-SA) to create polymeric bifunctional proteins. (A) The schematic illustration of heteroconjugation reaction of CY1-BAP and CY1-SA catalyzed by HRP. (B) SDS-PAGE (left) and native-PAGE (right) analyses of the heteroconjugation reaction of BAPs and SAs. Lane 1: BAP with the addition of WT-SA, HRP, and H₂O₂; lane 2: BAP with the addition of CY1-SA, HRP, and H₂O₂. SA was visualized by biotin-4-fluorescein (in the native-PAGE analysis). (C) Enzymatic activity of the BAP-SA conjugates on a biotin-coated plate. Reproduced from ref. Copyright with permission from the American Chemical Society (ACS).
Figure 6 SpyCatcher polymer and its use as a scaffold for assembling proteins with SpyTag. (A): Molecular image of dual Y-tagged SpyCatcher(Y-SpyCatcher-Y) and formation of the SpyCatcher polymer by HRP reaction.; (B): Assembly of proteins with SpyTag onto the SpyCatcher polymer via isopeptide bond formation between SpyCatcher and SpyTag.; (C): Creation of Nanoluc base bioprobes by assembling SpyTagged Nanoluc and pG onto the SpyCatcher polymer.; (D): Detection of ovalbumin in ELISA using Nanoluc-pG-SpyCatcher polymer conjugates with different ratios of Nanoluc and pG on the SpyCatcher polymer. Genetically fused Nanoluc and pG protein (Nanoluc-pG) was used as positive control. Reproduced from ref.20 Copyright with permission from John Wiley and Sons and Copyright Clearance Center.
2.2.3 Laccase-catalyzed formation of polymeric probes

Laccases (EC 1.10.3.2) are multi-copper-binding enzymes that have primarily been used as catalysts for the degradation of aromatic compounds in the textile and pulp industries. As with peroxidases, laccases catalyze the oxidation of phenolic compounds by O$_2$, and generate free phenoxy radicals and water.$^{52}$ Laccases are good substitutes for HRP in the preparation of protein polymers; they provide the same site-specificity for tyrosine as HRP, but require no H$_2$O$_2$. HRP decomposes H$_2$O$_2$ rapidly. Therefore, in most cases, the addition of H$_2$O$_2$ to initiate the polymerization reaction by HRP does not damage the target proteins. However, when a large amount of H$_2$O$_2$ is required—for example when conducting reactions on a large scale, or in circumstances involving high concentrations of target proteins—excessive amounts of H$_2$O$_2$ can deactivate the target proteins as well as the HRP. Under such circumstances, laccases are preferable to HRP because they catalyze the crosslinking of target proteins without exposing them to the harmful effects of O$_2$, which is always present in air. Laccases have been used to crosslink proteins derived from natural sources,$^{53}$ particularly food proteins such as α-casein, β-casein,$^{54}$ collagen,$^{40}$ and whey protein isolate (WPI).$^{55}$ Recently, our research group demonstrated the feasibility of using laccase for the site-specific crosslinking of recombinant proteins to produce protein polymers.

In the first report of laccase-mediated site-specific protein polymerization, recombinant BAP and IBPs were successfully polymerized using laccase.$^{26}$ BAP with a Y-tag at its C-terminus (BAP-Y), and a chimera protein of pA and pG with a Y-tag at each of its N- and C-termini (Y-pG$_2$pA-Y) were constructed (Figure 7A). The BAP-Y and Y-
pG$_2$pA-Y were co-crosslinked using a *Trametes* spp. laccase (TL) to yield a BAP/pG$_2$pA polymer that can be used as a detection probe in ELISA (Figure 7B). The TL treatment of the Y-tagged proteins yielded polymers with high degrees of conjugation (Figure 7C). The degree of crosslinking of the polymers prepared by TL increased as the reaction progressed from 30 min to 24 h, indicating that TL continued to slowly catalyze the crosslinking of the Y-tagged proteins throughout the course of the reaction. In contrast, the HRP-catalyzed polymerization of Y-tagged proteins was completed within a short time, and there was no clear difference between the band patterns of the products at 30 min and 24 h (Figure 7C). The BAP/pG$_2$pA polymers crosslinked by TL for 30 min and 2 h exhibited higher absorbances than the BAP/pG$_2$pA polymer prepared by HRP (Figure 7D). The polymerization degree of protein polymers influences their effectiveness as detection probes, and must therefore be controlled. Because TL acts more slowly than HRP, it is easier to control protein polymerization using TL, which is another advantage of TL-mediated protein crosslinking.
**Figure 7** Laccase-mediated protein polymerization reaction to create polymeric detection probes. (A) Molecular structure of BAP-Y and Y-pG2pA-Y used in this study. (B) Schematic illustration of co-cross-linking reaction of BAP-Y and Y-pG2pA-Y catalyzed by TL. (C) SDS-PAGE analysis of co-cross-linking reaction of BAP-Y and Y-pG2pA-Y catalyzed by TL and HRP in various incubation time. (E) Results of OVA-detecting ELISA using BAP/pG2pA copolymer prepared by TL and HRP in various incubation time. Reprinted from ref.26 Copyright with permission from Elsevier.
The TL-mediated protein polymerization reaction was then applied to the polymerization of HRP.\textsuperscript{17} The production of a polymeric detection probe composed of HRP is highly significant because HRP is the most commonly used labeling enzyme, and yet there had been no reports of such site-specifically crosslinked HRP polymers. Y-tagged HRPs were expressed using the silkworm-BEVs system, and polymerized by TL. Although Y-tagged HRPs can self-polymerize, the activity of the resulting HRP polymers is lower than that of HRP polymers produced by TL owing to inactivation by H\textsubscript{2}O\textsubscript{2}. Therefore, TL-mediated protein polymerization is an effective strategy for the polymerization of Y-tagged HRPs.

Last, the linear polymerization of BAP has been achieved using TL.\textsuperscript{19} To improve the functionality of bifunctional polymeric probes with regard to ELISA, it is necessary to control the structure of the polymeric probes to increase the efficiency of each protein unit in the probe. By inhibiting the recognition of crosslinked Tyr residues—i.e., dityrosine—by HRP or TL, the formation of branched structures at the conjugation sites can be eliminated, thereby ensuring the formation of a linear protein polymer. A novel peptide loop containing a Tyr residue (the Y-Loop) was designed and introduced to the loop domain of BAP to create BAP-Loop-Y (\textbf{Figure 8A}). The BAP-Loop-Y was co-crosslinked with pG\textsubscript{2}pA containing a single Y-tag, which acts as a capping molecule to terminate the elongation of linear polymers. Consequently, the BAP-Loop-Y/pG\textsubscript{2}pA-Y copolymer comprised a linear BAP polymer tagged with pG\textsubscript{2}pA groups at its termini. The polymerization of BAP-Loop-Y was achieved by TL, which crosslinked the Tyr residues in the Y-Loop and formed linear BAP polymers, as directly confirmed by scanning probe microscopy (\textbf{Figure 8B}). Intriguingly, the signal attributable to the BAP-Loop-Y/pG\textsubscript{2}pA-Y copolymer increased linearly as the ratio of BAP-Loop-Y to pG\textsubscript{2}pA-Y increased,
whereas the signal attributable to the conventional branched BAP/pG₃pA polymer reached a maximum at a ratio of 75:1 (Figure 8C). This indicates the importance of controlling the structure of protein polymers to maximize their functionality as detection probes.
2.3. Protein conjugates and polymers for detection probes made by SpyCatcher/SpyTag Chemistry

Nature always provides excellent and remarkable examples and tools in protein sciences. One example is the spontaneous isopeptide bond formation within the CnaB domain of FbaB protein from *Streptococcus pyogenes*. Howarth *et al.*, split the CnaB domain into two domains, SpyCatcher (138 amino acids) and SpyTag (13 amino acids), to utilize the formation of isopeptide bond formation of CnaB domain as a bioconjugation tool. Upon mixing, the SpyCatcher and SpyTag spontaneously forms iso-peptide bond
with high efficiency and high reaction rate. Unlike other enzyme-mediated conjugation reaction, the SpyCatcher-SpyTag conjugation reaction requires no formation of tripartite complexes, thus it is particularly useful when conjugating large protein complexes, such as virus-like particles (VLPs). Both SpyCatcher and SpyTag can be genetically fused to other proteins thus the SpyCatcher-SpyTag conjugation system has been widely used by many researchers to create diverse kinds of protein conjugates. Here, we summarizes examples of protein conjugates for detection or imaging purposes made by using the SpyCatcher/SpyTag system.

Conjugating binding protein units and signaling protein units, typically enzymes or fluorescent proteins can create detection probes. However, genetically fusing two different proteins often causes misfolding, resulting in low expression levels of the fused proteins and or loss of protein functions. Many proteins have been successfully expressed with SpyCatcher and SpyTag, indicating that fusing SpyCatcher and SpyTag has little effect on the folding of the fused proteins. Therefore, conjugating proteins in a post-translational manner by using SpyCatcher/SpyTag system is an ideal approach to construct functional protein conjugates. Moon et al., demonstrated a plug-and-playable cell imaging systems by conjugating various types of affibodies and fluorescent proteins of different colors by using the SpyCatcher/SpyTag system (Figure 9A). The affibody and fluorescent protein conjugates (AFPCs) can be modularly conjugated depending on the target biomarkers on cells. In their study, anti-epidermal growth factor receptor (EGFR) and anti-HER2 affibodies were used and they were conjugated with enhanced yellow fluorescent protein (eYFP) or mCherry. The AFPCs recognized specific antigens and successfully visualized target cells. Kimura et al., reported creation of affibody-enzyme complex (AEC) using SpyCatcher/SpyTag systems. Glucose dehydrogenase
(GDH) fused with SpyCatcher was conjugated with an anti-EGFR variable domain of heavy chain antibody (VHH) to construct AEC (Figure 9B) and detection of EGFR in sandwich ELISA was demonstrated (Figure 9C).

SpyCatcher is also useful as a connector for chemical conjugation of proteins. A SpyCatcher with an additional Cys residue was chemically modified with maleimide to label the SpyCatcher with a fluorescent molecule, IRDye800CW. The labeled SpyCatcher was then conjugated with antibody with genetically fused SpyTag to make fluorescent-labeled antibody (Figure 9D). Anti-EGFR antibody, nimotuzumab, was labeled with IRDye800CW with this methodology and in vivo imaging was demonstrated by using the IRDye800CW labeled nimotuzumab (Figure 9E). Furthermore, oriented immobilization of single-domain antibodies (sdAb) onto dyed magnetic microspheres was achieved by first immobilizing SpyCatcher to the microspheres and then conjugating SpyTagged sdAb to the immobilized SpyCatcher. The dyed magnetic microspheres functionalized with sdAb was utilized in sandwich assays. The immobilization of sdAb using SpyCatcher/SpyTag system showed significantly higher sensitivity in assays using the dyed microspheres compared with the chemical immobilization of sdAb which results in unoriented immobilization. By either genetically fusing or chemically conjugating SpyCatcher or SpyTag to the target proteins, it is possible to conjugate any combination of proteins in post-translational manners, enabling creation of a wide variety of detection probes.
Figure 9 Detection probes created by using SpyCatcher/SpyTag system. (A) Schematic representation of constructing plug-and-playable fluorescent cell imaging modular toolkits using SpyTag/SpyCatcher protein ligation system. Reproduced from ref.\textsuperscript{61} Copyright with permission from the Royal Society of Chemistry (RSC). (B) Schematic illustration of conjugation method of antibody-enzyme complexes (AECs). Variable domain of heavy chain antibody (VHH) was fused with SpyTag and conjugated with a chimera protein of SpyCatcher and glucose dehydrogenase (GDH). (C) Sandwich ELISA to detect EGFR by using AECs composed of anti-EGFR VHH and GDH. Reproduced from ref.\textsuperscript{62} Copyright with permission from the American Chemical Society (ACS). (D) Schematic diagram of site-specific labeling of antibody with a fluorescent molecule (IRDye800CW) by using SpyCatcher/SpyTag system. (E) In vivo imaging of the EGFR-positive murine xenograft using nimotuzumab labeled with IRDye800CW via SpyTag/SpyCatcher conjugation. Reproduced from ref.\textsuperscript{63} Copyright with permission from the SpringerLink.

Howarth et al., have further engineered the SpyCatcher and split it into two domains, KTag containing the Lys residue which forms isopeptide bond with SpyTag, and SpyLigase containing the catalytic Glu residue required for formation of isopeptide
bond between KTag and SpyTag (Figure 10A). The SpyTag and KTag conjugate with each other by the catalytic activity of SpyLigase and this tripartite system has been applied for the formation of affibody polymers. An affibody against EGFR with KTag and SpyTag at N- and C-termini was constructed (KTag-AffiEGFR-SpyTag) and the affibody polymers were created by using SpyLigase reaction (Figure 10B and 10C). The affibody polymer was immobilized onto magnetic beads and used to capture cancer cell lines expressing different levels of EGFR. The magnetic beads with affibody polymer efficiently captured high-EGFR MDA-MB-468 cells (Figure 10D) as well as low-EGFR BT242 cells (Figure 10E). On the other hand, the magnetic beads with monomeric affibody showed significantly lower capturing efficiency against BT242 cells than the ones with affibody polymer (Figure 10E). These results suggested that the multivalency effect is one promising way to improve the biological function of biomolecules.

**Figure 10** Polymerization reaction of affibody using SpyTag-SpyCatcher chemistry. (A) CnaB2 domain, a domain from the fibronectin adhesion protein FbaB of *Streptococcus pyogenes* can be split into three rational engineered sub domains, SpyTag, KTag, and SpyLigase. (B) Schematic illustration of the creation of affibody polymers by using SpyLigase system. The KTag and SpyTag were introduced to the N- and C-termini of anti-EGFR affibody (KTag-AffiEGFR-SpyTag), respectively. (C) Polymerization reaction of anti-EGFR affibody by SpyLigase
mediated cross-linking reaction of KTag and SpyTag for 24 h. (D) Recovery of high-EGFR MDA-MB-468 using magnetic beads coated with the affibody polymer (polymeric beads, blue) or the affibody monomer (monomeric beads, red). (E) Recovery of low-EGFR BT474 or no-EGFR 721.221 cells. Reproduced from ref. Copyright 2014 with permission from the National Academy of Sciences (NAS).

Conclusions

In the present review, we summarized the strategies for making protein conjugates and described their use as detection probes in EIAs. Genetically fusing proteins can ensure the precisely controlled conjugation and fusion of enzymes with proteins that form multimeric structures, such as streptavidin, which can directly produce multimeric bifunctional proteins. Moreover, the introduction of specific conjugation points for PTMs to the target proteins can facilitate the further functionalization and multimerization of the bifunctional protein conjugates. The use of robust protein expression systems—such as the silkworm-BEVS system featured in the present review—is essential for the genetic fusion of proteins. However, the number of proteins that can be conjugated using this strategy is limited.

Protein conjugates with high degrees of conjugation can be created by enzymatic reactions to produce site-specifically and covalently conjugated protein polymers. Although many enzymes have been proposed for PTMs, few are suitable for the polymerization of proteins. The design of target proteins and conjugation sites is critical for ensuring that enzymatic crosslinking reactions proceed in an intermolecular, but not an intramolecular, manner to obtain protein polymers. Peroxidases and laccases are the most promising enzymes for protein polymerization, and essentially any protein can be polymerized by introducing flexible peptide tags containing Tyr residues. The insights into protein polymerization obtained so far indicate that the ratios of the
component proteins, the degree of polymerization, and the structures involved have a critical impact on the functionality of the protein polymers. Protein polymers are applicable not only as detection probes in diagnostic applications, but also in bioimaging, biosensors, and even bioprocessing. Furthermore, protein polymerization could be an interesting strategy for preparing effective antigens for vaccine development. There are reports regarding the response of immune systems to polymeric antigens\textsuperscript{66,67}, however, the immune response against polymeric antigens that are covalently cross-linked through short peptide tags has not been evaluated enough, thus this strategy is worth pursuing in the near future.

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**Conflicts of interest**

The authors declare no conflict of interest.

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