



## Facile Site-Specific Multiconjugation Strategies in Recombinant Proteins Produced in Bacteria

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### Abstract

For biomedical applications, proteins may require conjugation to small and large molecules. Typical examples are dyes for imaging, cytotoxic effector molecules for cell killing, or half-life extension modules for optimized pharmacokinetics. Although many conjugation strategies are straightforward to apply, most of them do not enable site-specific and orthogonal conjugation, and do not yield a defined stoichiometry. Moreover, techniques offering these desirable features often rely on complex expression procedures and suffer from low production yields. A more promising manufacturing strategy for flexible, site-specific and stoichiometrically defined payloading of proteins is the combination of click chemistry and thiol–maleimide conjugation, which even enables dual labeling when used consecutively. Here, we describe as an example the production of Designed Ankyrin Repeat Proteins (DARPin), a non-IgG binding scaffold, in a specific *E. coli* strain to obtain high yields of protein carrying both a thiol and an azide group. We provide straightforward protocols for strain-promoted azide–alkyne cycloaddition (SPAAC) and thiol–maleimide conjugation, and furthermore compare these conjugation chemistries with existing alternatives like copper-catalyzed azide–alkyne cycloaddition (CuAAC). Finally, detailed instructions for reactivity analysis and yield estimations of the reactions are provided.

**Key words** DARPin, Thiol–maleimide conjugation, Click chemistry, CuAAC, SPAAC, Bacterial expression, PEG stain, Polyethylene glycol, Site-specific conjugation, Bioorthogonal

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## 1 Introduction

To investigate pharmacokinetic properties and biodistribution of proteins in in vivo models, or determine their subcellular localization, proteins can be coupled with various chemical moieties such as dyes for visualization and imaging, or radiotracers. For other applications, such moieties must bring specific functional properties to the protein like an increased hydrodynamic radius for an elevated circulation half-life or cytotoxic activity for cancer therapy. The underlying chemistries for the modification of proteins ideally combine both site specificity and a controlled stoichiometry and

hence need to be fully orthogonal to the functional groups in the protein (e.g., thiols, alcohols, and amines) to prevent unspecific conjugation that might interfere with target binding or protein function. The components used in such conjugations must be stable and small, while the reaction must be fast and conditions mild enough to be tolerated by the protein [1]. A popular concept employs specific enzymes, either for the conjugation reaction itself or for the incorporation of unique functional groups into proteins. These approaches have several benefits but also drawbacks as described elsewhere [2]. Most importantly, they usually require extensive optimizations to reach conjugation efficiencies and yields comparable to chemical modifications. Furthermore, the specific requirements of the coupling enzyme might limit the applicability of these techniques (e.g., by having constraints for the conjugation site, which for some enzymes must be located at the C-terminus, or by requiring particular recognition sequences).

One of the most popular conjugation techniques for protein modification is the reaction between a primary N-terminal amine and an N-hydroxy-succinimide (NHS)-ester (*see* Table 1). However, most proteins contain a number of lysine residues and thus a number of amine nucleophiles. As the reaction relies on the deprotonation of the reacting amine, and since the  $pK_a$  values of the N-terminal amine is only slightly lower than that of the  $\epsilon$ -amino group of a fully exposed lysine (typically 8 vs. 10.5), specificity for the N-terminus is difficult to reach in practice. Thus, the reaction can only be optimized *either* for yield (higher pH) *or* for specificity for N-terminal conjugation (lower pH) [3]. Hence, amine conjugation is rather impractical when *both* the quantitative and the site-specific modifications at the N-terminus are crucial, and when the protein has multiple lysines. The engineering of proteins that are free of lysines except where needed is possible [4], but not many proteins tolerate this strategy.

Alternatively, the maleimide–thiol Michael addition offers a very fast reaction to quantitatively conjugate proteins under mild conditions and independent of particular buffers (*see* Table 1) [5, 6]. Proteins free of cysteine or with no surface-exposed cysteine are suited for this method, but also the intermolecular disulfide bridges of IgG molecules can be exploited to generate drug conjugates upon mild reduction [7–10]. Site-specificity can be assured by the incorporation of a single, surface-exposed cysteine in the protein of interest carrying a unique thiol residue. The maleimide group is then provided by the chemical payload (e.g., a maleimide–dye or maleimide–toxin). In contrast to NHS-chemistry, the maleimide–thiol reaction shows very little to no side reactions when reagent excess and reaction time are kept reasonable, while at long times and/or high reagent excess a slower reaction with primary amines is seen [11]. Because of the rather robust nature of the maleimide–thiol reaction, a comprehensive catalogue of protein-

**Table 1**  
**Conjugation chemistries**

Reaction chemistry	Reaction scheme	Reaction rate	Conjugation site in protein	Incorporation method
Amine-NHS	<p>Primary amine + NHS-Ester <math>\xrightarrow{\text{pH } 7-9}</math> Amide bond + NHS</p>	Fast	Lysine, terminal amine	Natural
Maleimide-thiol Michael addition	<p>Reactive thiol + Maleimide <math>\xrightarrow[\text{Base}]{\text{pH } 6.5-7.5}</math> Thioether bond</p>	Fast	Cysteine	Natural
Cycloaddition CuAAC	<p>Azide + Terminal alkyne <math>\xrightarrow[\text{Cu(I)}]{\text{Physiological pH}}</math> Stable triazole</p>	Slow	Azido-homoalanine	Methionine-auxotrophic <i>E. coli</i>
Cycloaddition SPAAC	<p>Azide + Dibenzocyclooctyne <math>\xrightarrow{\text{physiological pH}}</math> Stable triazole</p>	Slow	Azido-homoalanine	Methionine-auxotrophic <i>E. coli</i>

R<sub>1</sub>: protein (e.g., DARPin), R<sub>2</sub>: payload (e.g., PEG, dye, or cytotoxin). Table adapted from [40]. For reaction details see [16]

modifying agents (e.g., polyethylene glycol (PEG) and a plethora of dyes and chelators) are commercially available. However, a drawback of this reaction is that its efficiency depends on the complete reduction of the exposed cysteine and on avoiding its reoxidation during conjugation. Whereas this is easily achievable for originally cysteine-free proteins where an artificial cysteine is introduced, it is more challenging for proteins carrying internal disulfide bridges because prevention of oxidation of the exposed cysteine must not lead to reduction of the native disulfide bonds.

The principal reversibility of the thiol–maleimide reaction has been much discussed [12], but for most applications the back reaction is slow enough to be irrelevant, and the product can also be further stabilized by ring opening of the maleimide.

A number of bioorthogonal chemistries have been developed [13–16], all with their pros and cons. A very robust bioorthogonal conjugation approach exploits the cycloaddition reaction of an azide and an alkyne forming a stable triazole. Good reaction rates and stability of the reactants have made this type of reaction widely used. The uncatalyzed reaction is very slow, and has thus become useful only when accelerated, either with a terminal alkyne as Cu (I)-catalyzed azide–alkyne cycloaddition (CuAAC) [17, 18] or by using ring-strained cyclooctynes as strain-promoted azide–alkyne cycloaddition (SPAAC) [14], the latter usually being denoted as “click chemistry” (*see* Table 1) [19]. (It might be noted that in some of the literature, all bioorthogonal reactions are collectively termed “click chemistry,” but we will use this term here synonymous with SPAAC). To improve the slow reaction rates of cyclooctyne, various derivatives have been synthesized, of which the cyclopropanated variant bicyclo[6.1.0]nonyne (BCN) and the benzoannulated derivative dibenzocyclooctyne (DBCO), also denoted as aza-dibenzocyclooctyne (DIBAC), are the most important ones [20–22]. Both reactants improve reaction rates ~100-fold over the unmodified cyclooctyne, but in our hands, DBCO could best combine high stability and solubility with useful reaction kinetics. The CuAAC, conversely, has sometimes led to undesired protein precipitation, protein oxidation and comparably low reaction yields and furthermore required optimization for every protein individually (*see* Note 1). Therefore, we discuss here only the use of click chemistry with DBCO to modify proteins. Please note that, despite the use of rate-optimized cyclooctynes like DBCO and BCN, the described reactions are still relatively slow ( $0.1\text{--}0.3\text{ M}^{-1}\text{ s}^{-1}$ ) [16]. Moreover, unspecific coupling to irrelevant reaction partners (e.g., thiol coupling to the triple bond) cannot be excluded, and this limits the orthogonality of the reaction [21–24]. However, since the side reactions are slow and since proteins manufactured for bioconjugation are usually very pure, such side reactions are unlikely to be of concern under the recommended reaction conditions, and furthermore, the stability of the components used for

click chemistry remains as an undisputed advantage. Nonetheless, DBCO has been reported to be unstable to strong acids and strong bases [21, 25], and the azide can in principle be converted to a primary amine under strong reducing conditions [26, 27].

For all protein conjugation reactions, including those using click chemistry, it is beneficial if the conjugation site in the protein can be freely chosen. This can be achieved with nonnatural amino acids containing azides to replace L-methionine. The methionine codon can be incorporated in the coding sequence of the protein at a desired position and the aminoacyl tRNA synthetase for methionine in *E. coli* accepts the methionine-surrogate L-azidohomoalanine (Aha). Thus, in methionine-auxotrophic *E. coli* strains like B834 (DE3), after simple methionine depletion of the culture, Aha can be introduced without any other changes in the *E. coli* strain [28]. However, this technique requires that the protein can be produced in *E. coli* (see detailed protocol below). The amber-suppression technology [29, 30] for the site-specific incorporation of bicyclo[6.1.0]nonyne (BCN)-lysine [31, 32] or other nonnatural amino acids carrying quinones [33, 34] or norbornenes with a nitrile-imine reagent [35, 36] offers an alternative approach. Nevertheless, even if the rate constants of click chemistry can be highly accelerated with these nonnatural amino acids [37], due to its rather low expression yield this technology is better suited to prepare imaging agents, for which only small amounts are needed, than for larger scale preparation of, for example, therapeutic proteins. Another disadvantage is that the efficiency of the amber suppression strongly depends on the surrounding sequences of the amber site and thus requires intensive engineering and optimization for each protein, which limits the freedom of protein design (unpublished data). Although these drawbacks can in principle be overcome using sequence optimization [38] and adapted *E. coli* strains [39], the problem remains that, compared to azide-containing nonnatural amino acids like Aha, nonnatural amino acids like BCN-lysine are expensive, and, to our knowledge, bacterial strains and vectors carrying tRNA–RNA synthase pairs for the incorporation of many relevant nonnatural amino acids have not been made commercially available. Therefore, this technology will not be discussed further herein.

Antibody–drug conjugates (ADCs) are amongst the most popular examples of FDA-approved protein conjugates currently applied in the clinic. However, for reasons described above, site-specific incorporation of bioorthogonal chemical moieties in full-length antibodies produced in eukaryotic systems with high expression yields and without interfering with the multiple functions of the IgG-molecule, is very difficult [40]. Hence, high-affinity binding proteins [41] that are not derived from IgGs and can be produced in bacterial hosts allow the use of these new bioorthogonal chemistries for fast and site-specific drug conjugation with high

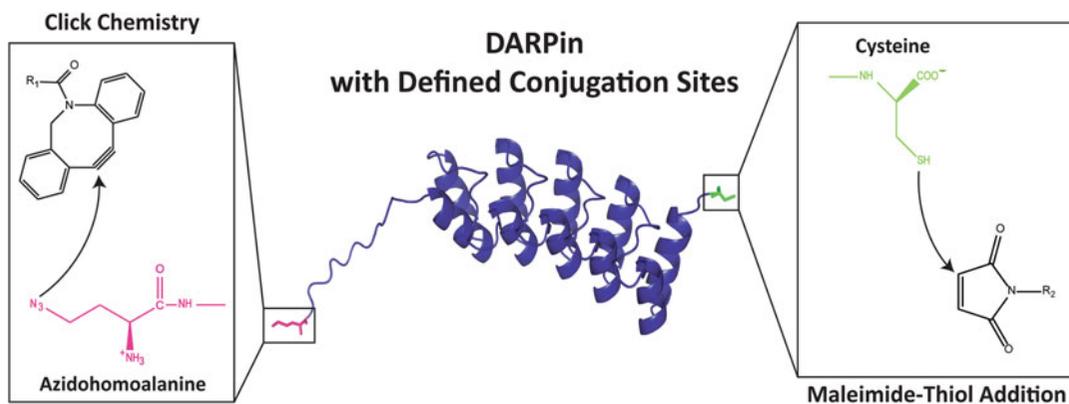
yield. They enable thus the screening of a wide range of pharmacological and molecular properties, and have become of interest and are thus currently under widespread preclinical investigation [42]. One such new scaffold are Designed Ankyrin Repeat Proteins (DARPin) [43]. Their robust nature allows the expression in large quantities in *E. coli*, they can be engineered to various formats from monovalent to bivalent to tetravalent and tolerate the introduction of reactive groups for site-specific and bioorthogonal conjugation [40, 44, 45]. DARPins can be easily selected to bind targets with high specificity and affinity and usually carry neither a free cysteine nor an essential methionine, thus providing a high freedom of engineering using the maleimide–thiol and click chemistry reactions described above [46]. Typically, a cysteine (e.g., at the C-terminus or anywhere else in the sequence) is introduced to allow the maleimide-based conjugation of effector functions, including the cytotoxic payloads known from classical ADCs (see Fig. 1). For production, the DARPin is expressed in minimal medium using a methionine-auxotrophic *E. coli* strain to introduce Aha at the N-terminus (methionine start codon, or if the initiator Met residue is cleaved off in front of a small residue, at a methionine introduced elsewhere). This results in a binding protein carrying two unique functional groups, at freely choosable positions, useful for various combinations of protein-modifying moieties, such as PEG, dyes or cytotoxins (see Fig. 1) [45, 47]. As mentioned, the N-terminus is not the only site allowing Aha incorporation. If the second amino acid is small (e.g., Ala, Gly, or Ser), the N-terminal Aha is cleaved off [48, 49], thereby enabling the introduction of a unique Aha at various desired positions elsewhere in the protein.

Here, we provide a detailed protocol how to express DARPins in minimal medium to incorporate a unique azide (Aha) and thiol (cysteine) residue, and describe how to use both functional groups sequentially for site-specific, bioorthogonal and stoichiometrically defined conjugation reactions. Furthermore, methods for the biochemical and functional analysis of the engineered DARPins and the conjugates will be described.

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## 2 Materials

Prepare all solutions using ultrapure water and analytical grade reagents, if not stated otherwise. Autoclave all media, but make sure to not autoclave the nutrient mix used for the minimal expression medium. Filter all solutions and reagents using a 0.22  $\mu\text{m}$  filter. Handling of bacteria including plating, cultivation, transformation, and cell lysis is conducted according to standard protocols, if not stated otherwise. We only list reagents and media, which are required to employ methods described here in detail without mentioning standard reagents.



**Fig. 1** DARPin protein (blue) equipped with two unique chemical moieties for the bioconjugation of different payloads ( $R_1$  and  $R_2$ ) at defined and freely choosable positions in the DARPin-scaffold. The terminal positions are merely drawn here for the clarity of illustration. A unique azidohomoalanine (pink) (for illustration shown at the  $N$ -terminus) and cysteine (green) (for illustration shown at the  $C$ -terminus) can be used for click chemistry or maleimide thiol coupling, resulting in stoichiometrically defined bioconjugates

## 2.1 Expression of an Azide-DARPin--Cysteine Construct in Minimal Medium

1. Chemically competent cells of the *E. coli* B-strain B834 (DE3) ( $F^- ompT gal hsdS_B (r_B^- m_B^-) met dcm lon \lambda(lacI, lacUV5-T7 gene 1, ind1, sam7, nin5)$ ).
2. Vector: Use a plasmid containing the modified construct with the respective cysteine and/or methionine codon and a His<sub>6</sub>-tag for purification. It should allow IPTG-inducible expression and contain the *lacI* gene under the strong *lacI<sup>q</sup>* promoter (e.g., a modified pQE30 (Qiagen) or pET-28b(+) (Novagen)).
3. M9 pre-expression medium: Mix the medium base (SelenoMet™ Base Medium & Nutrients Glucose Free, Molecular Dimensions) with the nutrient mix according to the manufacturer's instructions and add 100 mg/L ampicillin, 1% (w/v) glucose, and 40 mg/L *L*-methionine. This medium is used for the pre-pre-culture, the o/n culture, and the pre-expression culture.
4. M9 expression medium: Mix the medium base (SelenoMet™ Base Medium & Nutrients Glucose Free, Molecular Dimensions) with the nutrient mix according to the manufacturer's instructions and add 100 mg/L ampicillin, 0.4% (v/v) glycerol, and 40 mg/L of the nonnatural amino acid *L*-azidohomoalanine (H-Dab(N<sub>3</sub>)-HCl, Bapeks, Riga, Latvia). This medium is used for the expression-culture only.
5. 1 M isopropyl- $\beta$ -D-thio-galactopyranoside (IPTG) solution: Dissolve IPTG in water and filter in a sterile vessel using a 0.22  $\mu$ m filter. Aliquot and store at  $-20^\circ\text{C}$ .

## **2.2 Dual Conjugation Using Maleimide–Thiol and Click Chemistry**

1. Appropriate degassing device (e.g., vacuum pump and adapter for flasks or a helium supply).
2. Inert gas supply (e.g., argon or N<sub>2</sub>) to flush reaction vessels and buffer flasks.
3. Dimethyl sulfoxide (DMSO) to dissolve chemical reagents like short PEG linkers. Use DMSO of very high quality (e.g., for molecular biology, LC/MS grade) to not interfere with the desired reaction.
4. Desalting column or device allowing the fast and efficient depletion of reducing agent (e.g., a PD-10 or PD-Minitrap™ G25 column, GE Healthcare).
5. DBCO and/or maleimide component solutions either in PBS or in DMSO. Solutions in DMSO should be prepared at high concentrations (e.g., 5–10 mM) to keep DMSO concentrations in the reaction mix low.

## **2.3 Analyzing Reactivity and Conjugation Yields**

1. 1–5 mM PEG solutions for analytical reactions: Make sure the respective PEG is soluble in PBS; therefore, use long PEG molecules (e.g., PEG<sub>10k</sub> or PEG<sub>20k</sub>) with high homogeneity and quality. We obtained good results with the manufacturers JenKem Technology, Laysan Bio Inc. and Click Chemistry Tools. For a 1 mM solution of a PEG<sub>20k</sub> molecule, dissolve 20 mg in 1 mL PBS and mix thoroughly. Always prepare thiol or maleimide-containing PEG solutions freshly.
2. 0.1 M perchloric acid solution: Add 407 μL of a 70% perchloric acid solution (commercially available) to 50 mL water. Do not filter afterward.
3. 5% (w/v) BaCl<sub>2</sub> solution: Dissolve 5 g BaCl<sub>2</sub> in 100 mL 1 M HCl solution and mix thoroughly. Do not filter afterward.
4. 0.05 M iodine (I<sub>2</sub>) solution (commercially available).
5. Appropriate SDS-PAGE gels. We tested a variety of suppliers and could not detect differences in PEG-staining efficiency.
6. 5× SDS-PAGE sample buffer to prepare protein samples: 175 mM Tris–HCl pH 6.8, 50% (v/v) glycerol, 10% (w/v) sodium dodecyl sulfate, 5% (v/v) 2-mercaptoethanol, 0.15% (w/v) bromophenol blue.

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## **3 Methods**

### **3.1 Expression of an Azide–DARPin–Cysteine Construct in Minimal Medium**

The expression of a clickable DARPin requires the incorporation of the nonnatural amino acid L-azidohomoalanine (Aha) by cultivating the bacteria in minimal medium. Both the pre-expression and the expression medium are based on the glucose-free synthetic M9 minimal medium SelenoMethionine Medium Base supplemented

with a nutrient mix. After reaching a particular cell density, the cells are transferred from the pre-expression medium to the expression medium.

1. Transform the methionine-auxotrophic *E. coli* B-strain B834 (DE3) with the expression plasmid coding for the azide-DARPin-cysteine construct and containing the *lacI* gene under the strong *lacI<sup>q</sup>* promoter control (e.g., pQE30 (Qiagen) modified as in [45, 47, 50]).
2. Inoculate a 5 mL pre-pre-culture in M9 pre-expression medium from a single colony or glycerol stock. Incubate it for 8 h at 37 °C during the day and use this culture to inoculate a 100 mL o/n-culture for each 1 L of expression culture. Incubate this culture with agitation at 37 °C (*see Note 2*). Use a flask-to-medium volume ratio of at least 5:1 for all pre-, o/n, and expression cultures for sufficient aeration.
3. The next morning, inoculate 1 L M9 pre-expression medium to an OD<sub>600</sub> of 0.1 (approximate dilution 1:25). The cells are cultivated at 37 °C with agitation.
4. Once an OD<sub>600</sub> of 1.0–1.2 is reached (usually after 3–4 h), centrifuge the expression culture at 4000 × *g*, 10 min, 4 °C. Wash the pellet thoroughly three times by resuspending in ice-cold PBS (30 mL for a 1 L culture pellet) to deplete all extracellular L-methionine. Cells should be constantly cooled during the wash procedure.
5. Resuspend the pellet in 30 mL M9 expression medium and inoculate 1 L M9 expression medium with the solution.
6. The expression culture is agitated for 15–20 min at 37 °C in a shaker to additionally deplete all intracellular L-methionine pools of *E. coli*.
7. Induce the expression of the clickable DARPin using 1 mM isopropyl-β-D-thio-galactopyranoside (IPTG) from a sterile 1 M stock solution and incubate for 4–5 h at 37 °C with agitation.
8. Harvest the expression culture by centrifugation for 10 min at 4000 × *g*, 4 °C to pellet the cells. Wash the pellet three times in ice-cold PBS as described above and subsequently snap-freeze it in liquid nitrogen. Store the pellet at –20 °C for short-term storage or at –80 °C for longer storage times.

As the expressed DARPins contain a hexa-His-tag, their purification can be performed by immobilized metal ion affinity chromatography (IMAC) as a single-step purification (*see Note 3*). The reader is referred to [3, 51] for a detailed description.

Most DARPin constructs used for bioconjugation contain a unique cysteine residue at the C-terminus, hence, it is recommended to add 5 mM 2-mercaptoethanol to all washing buffers

to reduce disulfide bonds that might have formed between individual DARPins and with other endogenous cellular components. In addition, it is advantageous to store the purified protein in a reducing buffer by dialyzing it after the elution step to, for example, PBS supplemented with 5 mM 2-mercaptoethanol (*see Note 4*).

After this IMAC purification, a yield of 30–50 mg/L can be expected, which depends strongly on the individual construct and the employed expression conditions. If low expression yields are encountered, expression optimizations should be performed by varying first the expression temperature (e.g., to 30 °C [50]) and then the time before harvest.

### **3.2 Dual Conjugation Using Maleimide–Thiol and Click Chemistry**

The expressed and purified proteins (here an azide–DARPin–cysteine construct) carry either a thiol group (introduced by cysteine) or an azide group (introduced by Aha) or both, which can subsequently be used for the conjugation of various payloads. The payload can, for example, be a dye for visualization and imaging, a cytotoxin for tumor cell killing or a half-life extension module like PEG or serum albumin [45, 47]. In addition, the use of bifunctional chemical linkers carrying a DBCO and a maleimide group to, for example, link an azide–DARPin to a thiol–DARPin to generate bispecific binders or other protein–protein conjugates, have also been used. Here we only describe in detail the conjugation of a DARPin carrying an *N*-terminal azide and a *C*-terminal thiol to two reactants with the respective counter group (DBCO or maleimide) as examples illustrating the procedure. All provided protocols can be adapted to either protein–protein conjugations using a small DBCO–maleimide linker or single conjugations when proteins carrying only either an azide or a thiol group are expressed, since all described workflows and considerations are applicable.

Theoretically, click chemistry offers an orthogonal coupling reaction that does not interfere with other functionalities present in proteins such as thiols. It is, hence, possible to perform the maleimide–thiol and click chemistry conjugation simultaneously in a one-step approach. However, as recently shown [23, 24] side reactions cannot be completely avoided and the simultaneous approach complicates the troubleshooting if a reaction step does not work or is incomplete. We thus recommend performing the two reactions separately to maximize the yield by minimizing side-reactions. As the maleimide–thiol reaction exhibits the much faster kinetics, this reaction should be performed first (*see Note 5*). The conjugated protein is then purified before performing the azide–alkyne reaction. For the latter, we will only explain the click chemistry (SPAAC) as we found CuAAC to result in much lower reaction product yields and large amounts of side products (*see Note 1*).

1. Reduce the protein to provide a reactive thiol group not blocked by disulfide bridges (e.g., between proteins or between

the protein and media components). Omit this step if the protein was stored in reducing buffer after purification (*see* Subheading 3.1). Incubate the protein in an appropriate buffer (e.g., PBS) supplemented with 20 mM 2-mercaptoethanol for 1 h at 25–30 °C. Keep in mind that for the dual-conjugation approach neither DTT nor TCEP can be used as both reduce the azide group fast, making click chemistry impossible (*see* **Note 4**). In addition, always degas all buffers used and flush reaction tubes with argon or another inert gas to avoid reoxidation of the protein.

2. Remove the reducing agent by loading the reduced protein to an appropriate desalting column flushed with degassed buffer, collect fractions and identify the protein-containing fraction by absorbance measurements at 280 nm. It is crucial to establish a desalting method that depletes the reducing agent completely as it interferes with the subsequent maleimide–thiol conjugation. Perform an analytical PEGylation (*see* Subheading 3.3) or a mass spectrometry analysis to confirm complete reactivity of the desalted protein and the absence of remaining reducing agent. If the reduction is not complete and not all thiols are reactive, consider **Note 6**.
3. Immediately mix the desired protein fraction with a 2–4 × molar excess of the maleimide component (e.g., a dye) and incubate it for 1–2 h at 25–30 °C or 4 °C for 24 h with agitation (*see* **Note 7**). Optionally, quench the reaction by adding 2-mercaptoethanol at a 10–30 × molar excess over the maleimide component and incubate for 10 min at 25–30 °C with agitation.

Note that if the maleimide component is expensive or tedious to synthesize, the protein can also be applied in 2–4 × molar excess, and the derivatized protein must then be separated from unreacted proteins (e.g., by ion exchange chromatography). Make sure the protein concentration is at least in the micromolar range or increase the concentration of the maleimide component to maintain the fast reaction kinetics. If the maleimide component is dissolved in organic solvents (e.g., DMSO) make sure to have a highly concentrated (millimolar range) stock solution to keep the DMSO concentrations in the final reaction low (5–10% maximum).

4. Investigate the yield of your reaction by either performing mass spectrometry, or an analytical PEGylation on unreacted starting material or other analytical methods (*see* Subheading 3.3). If the yield is not satisfactory, increase reaction time, concentrations and temperature. Note that some thiols might be unreactive since they are hidden in a cavity thus decreasing the  $pK_A$  (making it less reactive at lower pH) or resulting in sterically blocking the thiol group. This can be tested using

smaller maleimide components and performing subsequent analytical reactions (*see* Subheading 3.3).

5. Purify the conjugated protein from remaining educts. For many dyes or other small maleimide components, this can be done using appropriate desalting columns or dialysis membranes. Further purification options are ion exchange chromatography if a charge was introduced (e.g., with a charged dye) or size exclusion chromatography if the hydrodynamic radius was significantly increased (e.g., by PEG conjugation). These separation steps are described in detail elsewhere [45, 47, 50].
6. Perform the click chemistry reaction by mixing the purified protein with a 2–4 × molar excess of the DBCO component (dye, PEG, etc.) and incubate for 24–48 h at 4 °C or at least 4 h at 25 °C with agitation. Note that click chemistry has slow reaction rates; hence, increasing reactants concentrations (high micromolar range) as well as the temperature and reaction time has a large influence on the reaction yield. Thereafter, use suitable purification methods if needed to deplete the remaining DBCO component [45, 47, 50]. Again, the protein can also be applied in molar excess over the DBCO component, if the latter is expensive or a complex synthesis is needed, and then the conjugated protein must be separated from the unconjugated one (*see* Note 7).

### 3.3 Analyzing Reactivity and Conjugation Yields

Proteins that were expressed and purified as described above carry a unique azide group, a unique thiol group, or a combination of both. Prior to the following conjugation of the desired molecule, which might be costly or laborious to produce, the reactivity of the respective reactive group incorporated into the protein should be tested. A convenient option is the conjugation of a PEG molecule carrying the respective counter group, followed by a gel-shift assay. If the SDS-PAGE gel band of the protein is shifted to higher molecular weights, then the respective azide or thiol group incorporated in the protein is reactive. By subsequent PEG staining of the same gel, the PEGylation of the protein can be confirmed, thereby excluding that the band shift was caused by, for example, the formation of covalent protein multimers. Hence, this facile assay shows whether the incorporation of the reactive group into the protein was successful and whether its reactivity was maintained after the various protein expression and purification steps.

Several functionalized PEG molecules are commercially available which greatly facilitates the testing of all possible types of reactions with the respective counter group. The following PEG variants are particularly useful to test the modified proteins for their reactivity: maleimide-PEG (to test the reactivity of the thiol group in the protein), DBCO-PEG (to test the reactivity of the azide group in the protein), thiol-PEG (to test the reactivity of a

maleimide group incorporated in the protein by a second functionalization reaction), or azide-PEG (to test the reactivity of a DBCO incorporated in the protein by a secondary functionalization reaction). Proceed as follows:

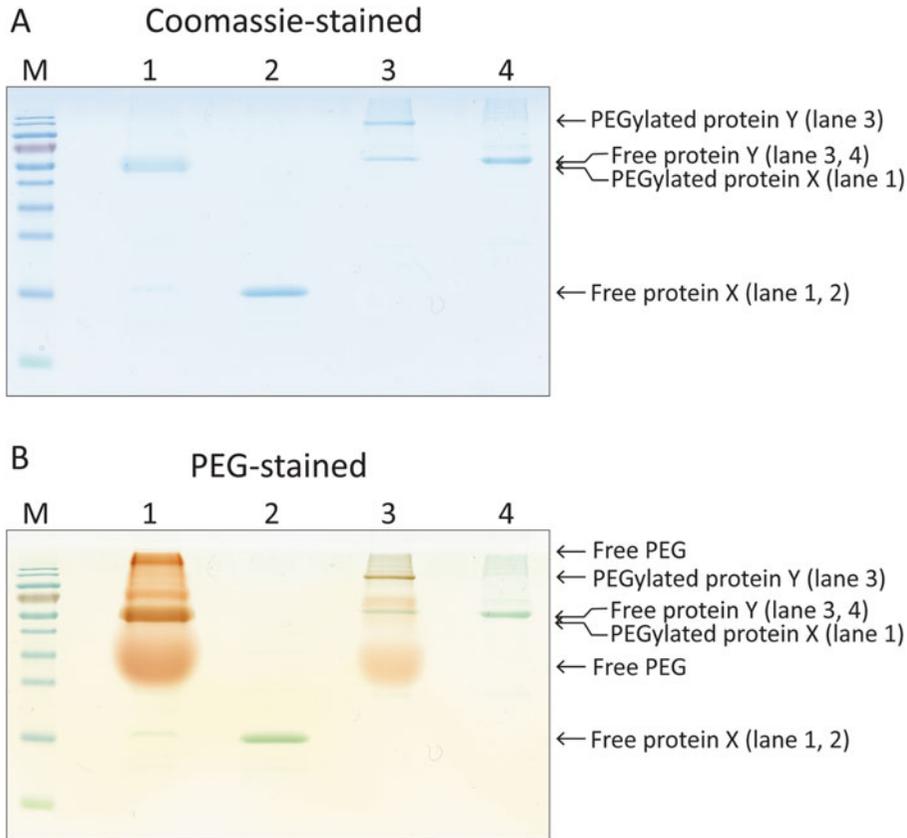
1. Prepare a 1 mM solution of the respective polyethylene glycol (PEG) in PBS and, if the maleimide-thiol should be investigated, degas the buffer thoroughly (*see Note 8*).
2. To test the reactivity of the protein thiol group, prepare a freshly reduced protein aliquot using 2-mercaptoethanol (*see Notes 4 and 6*) as described previously. Use degassed buffers during the reduction and desalting process and flush reaction vessels with argon or a similar inert gas. Ensure that the reducing agent is completely removed as thiol components interfere with the analytical maleimide-PEG conjugation, for example, by using a desalting column with very good separation capability (*see Subheading 2.2*) in a very conservative manner collecting only the main protein peak fractions. This reduces recovery but allows more efficient protein conjugations.
3. Add maleimide-PEG in 4–8 × molar excess (*see Note 8*) and mildly shake the reaction at 25 °C for 2 h or at 4 °C for 24 h. Degas all buffers and flush the reaction vessels with argon or another inert gas to avoid reoxidation of thiols during the test reaction. Note that this reaction can also be inverted, that is, a thiol-PEG solution can be added and the reactivity of a maleimide incorporated into the protein can be examined.

To test the reactivity of an azide group incorporated in the protein, add a 4–8 × molar excess of DBCO-PEG and mildly agitate at 4 °C for 24 h. Again, this reaction can be inverted, that is, the incorporation of a DBCO group in a protein can be tested by adding azide-PEG. As the click chemistry reaction is not oxidation sensitive, no degassing or use of inert gas is required. If two reactive groups in one protein should be tested (e.g., the bifunctional DARPin carrying an azide and a thiol group), we recommend to perform two separate test reactions for each reactive group.

4. Analyze the complete reaction mix by SDS-PAGE. Load higher amounts of protein (~5 µg) than usual, as otherwise remaining unreactive educts might not be visible. Inspect and scan the gel after Coomassie staining. A gel shift will show a successful reaction (*see Fig. 2*) and the gel can be PEG-stained as described below.

The staining of PEG in a previously Coomassie stained gel is performed at 25 °C and was adapted from [52, 53]:

1. Rinse the gel 3× with ~50 mL water.
2. Cover the gel with ~20 mL 0.1 M perchloric acid and shake mildly for 15 min.



**Fig. 2** 15% SDS-PAGE gel for testing the quality of model proteins, (“X,” a cys-containing model protein, and “Y,” a maleimide-containing model protein) for their efficiency in conjugation, by coupling maleimide-PEG<sub>20k</sub> as an easily detectable test compound. For protein X (lanes 1 and 2), maleimide-PEG<sub>20k</sub> was used, while for Y, thiol-PEG<sub>20k</sub> was used. 6 μg of each model protein was loaded and stained first with Coomassie (**a**) and subsequently for PEG (**b**) using the protocol described in the text. 1: Protein X reacted almost quantitatively with maleimide-PEG<sub>20k</sub>, 2: Protein X w/o maleimide-PEG<sub>20k</sub>, 3: Protein Y reacted partially with thiol-PEG<sub>20k</sub>, 4: Protein Y w/o thiol-PEG<sub>20k</sub>. A substantial portion of protein Y did not react with the thiol-PEG, presumably due to undesired side reactions in previous modification steps, compromising the integrity of the maleimide group

3. Add 5 mL 5% BaCl<sub>2</sub> in 1 M HCl to the solution and mix well.
4. Add 2 mL 0.05 M iodine solution and mix well.
5. Incubate on a shaker for ~10–15 min until the gel shows a dark brown color.
6. Destain the gel by discarding the solution and cover it with ~25 mL water, frequently check the destaining process and exchange the water. It takes approx. 10–15 min to destain the gel. As the destaining cannot be reverted, it is important to permanently check for a satisfying stain. Replace the water again before scanning the gel. *See Fig. 2* for a PEG-stained example. Note that PEG molecules can form multimers and

be heterogeneous in length depending on the available PEG quality. Therefore, the free PEG can be visible as multiple bands at different heights in SDS-PAGE. Consequently, it is important to add the respective PEG in excess to the reaction and observe the free protein band.

Analytical PEGylation is a fast and convenient method to investigate reaction yields and reactive groups. However, the assessment of a gel band intensity and its comparison are not quantitative and SDS-PAGE exhibits a limited sensitivity. Hence, more precise, alternative methods are also listed in the following sections. These also enable the analysis of protein expression and integrity and the investigation of its conjugation to different chemical moieties and provide a broader dataset for final assessments.

The quantitative and exclusive incorporation of Aha in the protein can be investigated by *N*-terminal Edman analysis [45, 50]. Consider that blotting the protein to a PVDF membrane prior to Edman analysis might add interfering components to the analysis reaction. Try to use the protein in solution directly and apply only buffer components of very high-grade purity. The non-natural amino acid Aha usually elutes 1–2 min earlier than *L*-methionine [3]. Furthermore, the amino acid composition of the expressed protein can be analyzed with amino acid hydrolysis using commercial kits. Subsequently, the resulting amino acid mix is analyzed on an UHPLC system, where no *L*-methionine should be detectable [3, 45]. Finally, mass spectrometry analysis offers a facile method to determine the incorporation of Aha, as its mass is –5 Da different from *L*-methionine. For example, ESI-MS yields good results with DARPin as these proteins are comparably small. However, keep in mind that the analysis of large proteins in mass spectrometry might be complicated and such small mass shifts might not be detectable. Furthermore, it should not be used to compare conjugation rates quantitatively as peak heights might vary with the biochemical properties of the conjugate and the nonconjugated protein, and thus the peak heights cannot be used to compare molar amounts. Nevertheless, mass spectrometry is well suited for the analysis of any remaining educts in the reaction mix.

The conjugation of a chemical moiety (e.g., a dye or a small linker like maleimide–biotin or maleimide–DBCO for the cross-linking of proteins) can be quantified with various methods. Keep in mind that before using these methods, it is important to determine the quality of the respective conjugation partner itself, for example, by mass spectrometry, NMR, or RP-HPLC. An inhomogeneous reaction partner (e.g., dye, maleimide–DBCO linker) will otherwise result in ambiguous results. For the investigation of dyes and DBCO-containing molecules, it is possible to use the absorption of the respective moiety at a given wavelength for conjugation quantification. For example, for a maleimide–DBCO linker, we

calculated the molar absorption coefficient in DMSO to be  $12,388 \text{ L}\cdot\text{mol}^{-1} \text{ cm}^{-1}$  at 280 nm and  $13,375 \text{ L}\cdot\text{mol}^{-1} \text{ cm}^{-1}$  at 309 nm. Next to its use in conjugation quantification, these high absorption coefficients can also impede protein quantification and must thus be taken into account here. Again, for homogeneous small molecules, mass spectrometry offers a viable method to determine mass shifts and quantify conjugation (see above). The conjugation of a small moiety often also changes the hydrophilicity of the modified protein, which can thus be analyzed by reverse phase HPLC, for example, using a C18-column as described in [47] (see **Note 9**). If the conjugated molecule contains a biotin residue, for example, when a maleimide–biotin linker is conjugated, a streptavidin gel shift assay as described in [54] can be performed. Finally, many chemical moieties like dyes and also, for example, a maleimide–PEG4–DBCO linker add detectable mass to the protein, large enough to be detectable in a gel-shift assay using high-quality commercial SDS-PAGE gels run at low voltage (e.g., 4–12% Bis-/Tris with MOPS running buffer or 10% Bis/Tris with MES buffer). Mass differences down to 0.5–1 kDa can be detected on such gels. This method offers a cheap screening method and furthermore, fluorescent moieties can additionally be quantified by a gel documentation device equipped with a fluorescence detector [45].

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## 4 Notes

1. The azide-alkyne Huisgen cycloaddition reaction has an inherently very low reaction rate [16] which can be increased to a reasonable level by using either strained alkynes (SPAAC) like DBCO or copper catalysis (CuAAC). Using DARPin, we also performed the latter according to Presolski et al. [55], but found that the proteins contained multiple oxidation species in mass spectrometry analysis, high amounts of covalently linked protein by side-reactions and precipitates. Furthermore, we observed reaction yields of a maximum of 50%. Please note that the amount of copper and other components must be optimized for every protein individually when using CuAAC, whereas SPAAC offers a broadly applicable technology with yields of almost 100% in our hands and no detectable side reactions or undesired protein modifications, when following the recommended protocols. Currently, more strained alkynes are available such as BCN-based reagents. These reagents show decreased lipophilicity while maintaining high reaction rates. Furthermore, these compounds provide very high reaction rates also with, for example, aromatic azides. However, the incorporation of such azides into proteins is unfortunately much more laborious and the reaction with Aha does not show faster kinetics, whereas side-reaction rates might be

facilitated. Finally, a number of DBCO components such as dyes are now commercially available in good qualities and at affordable prices. We therefore recommend using the SPAAC reaction (click chemistry) with DBCO and an azide group provided by Aha.

2. In theory, 2YT or other rich growth media can also be used for the o/n and pre-expression cultures when thorough washing steps are applied. However, the pre-expression minimal medium offers the advantage that the metabolism of the bacteria has already been adapted to the growth conditions in minimal medium, resulting in significantly higher expression yields in our experience.
3. It is important to prevent the copurification of endogenous *E. coli* proteins which might bind to the Ni-NTA resin due to a native metal-binding function or the presence of surface clusters of histidine residues [56]. Therefore, next to avoiding the use of an oversized IMAC column, we highly recommended to include both a low salt washing step (20 mM NaCl, counteracting hydrophobic interactions) and a high salt washing step (at least 1 M NaCl, counteracting ionic interactions).
4. In principle, the cysteine residue can be reduced by using either dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP). However, for the dual-conjugation approach described here, DTT and TCEP must not be used as they react with the azide-group and inactivate it for click chemistry. Both molecules efficiently reduce azides to amino groups [26, 55] and TCEP furthermore undergoes a Staudinger ligation by providing a phosphine group. However, it seems that 2-mercaptoethanol can be used as a reducing agent for disulfide bridges keeping the azide reactive as the rate constants of the undesired reduction are much slower [27]. Furthermore, 2-mercaptoethanol is more stable in solution and, hence, also in the storage buffers used after IMAC purification [57].
5. The maleimide-thiol reaction exhibits much faster reaction kinetics compared to the strain-promoted click chemistry [16]. We found that when performing protein-protein conjugation using a small maleimide-DBCO linker with one protein species ("X") carrying an exposed thiol and another protein species ("Y") carrying an exposed azide group, performing the maleimide-thiol reaction first and then conjugating protein X-DBCO to protein Y-azide, greatly increased the reaction yields (unpublished data). Presumably, this is due to maleimide hydrolysis and/or side-reactions taking place during the lengthy DBCO-azide reaction, which is performed at 4 °C for at least 24 h.

6. If the reduction is incomplete, we recommend optimizing the reduction conditions by either increasing the reaction temperature, time, concentration of reducing agent or the pH of the used buffer. At higher pH the amount of thiolate ions is increased, which is the reactive species in the reduction of disulfides, and thus higher pH strongly increases the reaction rate (e.g., instead of PBS pH 7.4 use an appropriate buffer at pH 8.0–8.5 if your protein is stable under these conditions). It is also crucial to establish a fast and efficient desalting method. This must be fast (within 10–20 min) to avoid reoxidation of the previously reduced thiols and efficient so that the reducing agent 2-mercaptoethanol is *completely* depleted as it competes with the desired maleimide–thiol conjugation.
7. If the maleimide component is expensive or laborious to synthesize, the protein can also be applied in a 2–4 × molar excess, provided that an effective method to separate the conjugated from the nonmodified protein is available (e.g., by preparative anion exchange chromatography). Also make sure that the protein concentration is at least in the micromolar range or increase the concentration of the maleimide component to maintain the fast reaction kinetics. If the maleimide component is dissolved in organic solvents (e.g., DMSO), we recommend to prepare a highly concentrated (millimolar range) stock solution to keep the DMSO concentrations in the final reaction low (5–10% maximum, depending on the stability of the target protein).
8. Highly concentrated PEG solutions are viscous and difficult to pipet and mix. They reduce reaction rates and, consequently, yield. We therefore do not recommend to prepare PEG solutions above 5 mM PEG concentration. Consider using a high molecular weight PEG of at least 10–20 kDa to ensure solubility in PBS and to obtain a clearly visible gel shift also for larger proteins. Especially when maleimide–PEG or thiol–PEG is used, solutions should be prepared freshly before each experiment as the hydrolysis of the maleimide or disulfide formation of thiols can occur. Degas the PBS prior to dissolving thiol–PEG to avoid undesired oxidation reactions. If the protein is highly concentrated (>100 μM) the molar excess of PEG can be reduced or the protein diluted. However, we do not recommend using less than 4 × molar excess of PEG as, depending on the quality of the PEG and its storage conditions, not all molecules carry the desired reactive group. This can possibly lead to a false interpretation of the conjugation results, if the effective concentrations of the reactive group provided by the PEG molecule are too low.

9. Many modifying molecules like dyes or small linkers are dissolved in DMSO and then mixed with the respective protein for conjugation. If this reaction mix is loaded to RP-HPLC for analysis, high DMSO concentrations will influence the running behavior on the column. Therefore, the sample must be diluted (<1% final DMSO concentration) before loading it on the column to minimize this effect.

## References

- van Berkel SS, van Delft FL (2013) Metal-free bioconjugation reactions. *Drug Discov Today Technol* 10:45–51
- Rashidian M, Dozier JK, Distefano MD (2013) Enzymatic labeling of proteins: techniques and approaches. *Bioconjug Chem* 24:1277–1294
- Tamaskovic R, Simon M, Stefan N et al (2012) Designed ankyrin repeat proteins (DARPin)s from research to therapy. *Methods Enzymol* 503:101–134
- Hansen S, Stüber JC, Ernst P et al (2017) Design and applications of a clamp for Green fluorescent protein with picomolar affinity. *Sci Rep* 7:16292
- Gorin G, Martic PA, Doughty G (1966) Kinetics of the reaction of N-ethylmaleimide with cysteine and some congeners. *Arch Biochem Biophys* 115:593–597
- Makmura L, Hamann M, Areopagita A et al (2001) Development of a sensitive assay to detect reversibly oxidized protein cysteine sulfhydryl groups. *Antioxid Redox Signal* 3:1105–1118
- Panowski S, Bhakta S, Raab H et al (2014) Site-specific antibody drug conjugates for cancer therapy. *MAbs* 6:34–45
- Hamblett KJ, Senter PD, Chace DF et al (2004) Effects of drug loading on the antitumor activity of a monoclonal antibody drug conjugate. *Clin Cancer Res* 10:7063–7070
- Pillow TH, Tien J, Parsons-Reponte KL et al (2014) Site-specific trastuzumab maytansinoid antibody-drug conjugates with improved therapeutic activity through linker and antibody engineering. *J Med Chem* 57:7890–7899
- Junutula JR, Raab H, Clark S et al (2008) Site-specific conjugation of a cytotoxic drug to an antibody improves the therapeutic index. *Nat Biotechnol* 26:925–932
- Sharpless NE, Flavin M (1966) The reactions of amines and amino acids with maleimides. Structure of the reaction products deduced from infrared and nuclear magnetic resonance spectroscopy. *Biochemistry* 5:2963–2971
- Fontaine SD, Reid R, Robinson L et al (2015) Long-term stabilization of maleimide-thiol conjugates. *Bioconjug Chem* 26:145–152
- McKay CS, Finn MG (2014) Click chemistry in complex mixtures: bioorthogonal bioconjugation. *Chem Biol* 21:1075–1101
- Dommerholt J, Rutjes FP, van Delft FL (2016) Strain-promoted 1,3-dipolar cycloaddition of cycloalkynes and organic azides. *Top Curr Chem* 374:16
- Hu QY, Berti F, Adamo R (2016) Towards the next generation of biomedicines by site-selective conjugation. *Chem Soc Rev* 45:1691–1719
- Lang K, Chin JW (2014) Bioorthogonal reactions for labeling proteins. *ACS Chem Biol* 9:16–20
- Rostovtsev VV, Green LG, Fokin VV et al (2002) A stepwise Huisgen cycloaddition process: copper(I)-catalyzed regioselective “ligation” of azides and terminal alkynes. *Angew Chem Int Ed Engl* 41:2596–2599
- Tornøe CW, Christensen C, Meldal M (2002) Peptidotriazoles on solid phase: [1,2,3]-triazoles by regioselective copper(I)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. *J Org Chem* 67:3057–3064
- Agard NJ, Prescher JA, Bertozzi CR (2004) A strain-promoted [3 + 2] azide-alkyne cycloaddition for covalent modification of biomolecules in living systems. *J Am Chem Soc* 126:15046–15047
- Ning X, Guo J, Wolfert MA et al (2008) Visualizing metabolically labeled glycoconjugates of living cells by copper-free and fast Huisgen cycloadditions. *Angew Chem Int Ed Engl* 47:2253–2255
- Debets MF, van Berkel SS, Schoffelen S et al (2010) Aza-dibenzocyclooctynes for fast and efficient enzyme PEGylation via copper-free (3+2) cycloaddition. *Chem Commun* 46:97–99
- Dommerholt J, Schmidt S, Temming R et al (2010) Readily accessible bicyclononynes for bioorthogonal labeling and three-dimensional

- imaging of living cells. *Angew Chem Int Ed Engl* 49:9422–9425
23. van der Linden WA, Li N, Hoogendoorn S et al (2012) Two-step bioorthogonal activity-based proteasome profiling using copper-free click reagents: a comparative study. *Bioorg Med Chem* 20:662–666
  24. van Geel R, Pruijn GJ, van Delft FL et al (2012) Preventing thiol-yne addition improves the specificity of strain-promoted azide-alkyne cycloaddition. *Bioconjug Chem* 23:392–398
  25. Witte MD, Theile CS, Wu T et al (2013) Production of unnaturally linked chimeric proteins using a combination of sortase-catalyzed transpeptidation and click chemistry. *Nat Protoc* 8:1808–1819
  26. Faucher A, Grand-Maitre C (2003) Tris(2-carboxyethyl)phosphine (TCEP) for the reduction of sulfoxides, sulfonylchlorides, N-oxides, and azides. *Synth Commun* 33:3503–3511
  27. Handlon AL, Oppenheimer NJ (1988) Thiol reduction of 3'-azidothymidine to 3'-aminothymidine: kinetics and biomedical implications. *Pharm Res* 5:297–299
  28. Kiick KL, Saxon E, Tirrell DA et al (2002) Incorporation of azides into recombinant proteins for chemoselective modification by the Staudinger ligation. *Proc Natl Acad Sci U S A* 99:19–24
  29. Plass T, Milles S, Koehler C et al (2011) Genetically encoded copper-free click chemistry. *Angew Chem Int Ed Engl* 50:3878–3881
  30. Wang L, Brock A, Herberich B et al (2001) Expanding the genetic code of *Escherichia coli*. *Science* 292:498–500
  31. Borrmann A, Milles S, Plass T et al (2012) Genetic encoding of a bicyclo[6.1.0]nonyne-charged amino acid enables fast cellular protein imaging by metal-free ligation. *ChemBioChem* 13:2094–2099
  32. Lang K, Davis L, Wallace S et al (2012) Genetic encoding of bicyclononynes and trans-cyclooctenes for site-specific protein labeling in vitro and in live mammalian cells via rapid fluorogenic Diels-Alder reactions. *J Am Chem Soc* 134:10317–10320
  33. Alfonta L, Zhang Z, Uryu S et al (2003) Site-specific incorporation of a redox-active amino acid into proteins. *J Am Chem Soc* 125:14662–14663
  34. Borrmann A, Fatunsin O, Dommerholt J et al (2014) Strain-promoted oxidation-controlled cyclooctyne-1,2-quinone cycloaddition (SPOCQ) for fast and activatable protein conjugation. *Bioconjug Chem* 26:257–261
  35. Kaya E, Vrabel M, Deiml C et al (2012) A genetically encoded norbornene amino acid for the mild and selective modification of proteins in a copper-free click reaction. *Angew Chem Int Ed Engl* 51:4466–4469
  36. Lang K, Davis L, Torres-Kolbus J et al (2012) Genetically encoded norbornene directs site-specific cellular protein labelling via a rapid bioorthogonal reaction. *Nat Chem* 4:298–304
  37. Dommerholt J, van Rooijen O, Borrmann A et al (2014) Highly accelerated inverse electron-demand cycloaddition of electron-deficient azides with aliphatic cyclooctynes. *Nat Commun* 5:5378
  38. Pott M, Schmidt MJ, Summerer D (2014) Evolved sequence contexts for highly efficient amber suppression with noncanonical amino acids. *ACS Chem Biol* 9:2815–2822
  39. Johnson DB, Xu J, Shen Z et al (2011) RF1 knockout allows ribosomal incorporation of unnatural amino acids at multiple sites. *Nat Chem Biol* 7:779–786
  40. Merten H, Brandl F, Plückthun A et al (2015) Antibody-drug conjugates for tumor targeting—novel conjugation chemistries and the promise of non-IgG binding proteins. *Bioconjug Chem* 26:2176–2185
  41. Owens B (2017) Faster, deeper, smaller—the rise of antibody-like scaffolds. *Nat Biotechnol* 35:602–603
  42. Deonarain MP, Yahioğlu G, Stamati I et al (2015) Emerging formats for next-generation antibody drug conjugates. *Expert Opin Drug Discovery* 10:1–19
  43. Plückthun A (2015) Designed ankyrin repeat proteins (DARPs): binding proteins for research, diagnostics, and therapy. *Annu Rev Pharmacol Toxicol* 55:489–511
  44. Boersma YL, Plückthun A (2011) DARPs and other repeat protein scaffolds: advances in engineering and applications. *Curr Opin Biotechnol* 22:849–857
  45. Simon M, Zangemeister-Wittke U, Plückthun A (2012) Facile double-functionalization of designed ankyrin repeat proteins using click and thiol chemistries. *Bioconjug Chem* 23:279–286
  46. Binz HK, Stumpp MT, Forrer P et al (2003) Designing repeat proteins: well-expressed, soluble and stable proteins from combinatorial libraries of consensus ankyrin repeat proteins. *J Mol Biol* 332:489–503
  47. Simon M, Frey R, Zangemeister-Wittke U et al (2013) Orthogonal assembly of a designed ankyrin repeat protein-cytotoxin conjugate with a clickable serum albumin module for half-life extension. *Bioconjug Chem* 24:1955–1966

48. Hirel PH, Schmitter MJ, Dessen P et al (1989) Extent of N-terminal methionine excision from *Escherichia coli* proteins is governed by the side-chain length of the penultimate amino acid. *Proc Natl Acad Sci U S A* 86:8247–8251
49. Wang A, Winblade Nairn N, Johnson RS et al (2008) Processing of N-terminal unnatural amino acids in recombinant human interferon-beta in *Escherichia coli*. *Chembiochem* 9:324–330
50. Stefan N, Zimmermann M, Simon M et al (2014) Novel prodrug-like fusion toxin with protease-sensitive bioorthogonal PEGylation for tumor targeting. *Bioconjug Chem* 25:2144–2156
51. Block H, Maertens B, Spriestersbach A et al (2009) Immobilized-metal affinity chromatography (IMAC): a review. *Methods Enzymol* 463:439–473
52. Kurfürst MM (1992) Detection and molecular weight determination of polyethylene glycol-modified hirudin by staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Anal Biochem* 200:244–248
53. Skoog B (1979) Determination of polyethylene glycols 4000 and 6000 in plasma protein preparations. *Vox Sang* 37:345–349
54. Petris G, Vecchi L, Bestagno M et al (2011) Efficient detection of proteins retrotranslocated from the ER to the cytosol by in vivo biotinylation. *PLoS One* 6:23712
55. Presolski SI, Hong VP, Finn MG (2011) Copper-catalyzed azide-alkyne click chemistry for bioconjugation. *Curr Protoc Chem Biol* 3:153–162
56. Bolanos-Garcia VM, Davies OR (2006) Structural analysis and classification of native proteins from *E. coli* commonly co-purified by immobilised metal affinity chromatography. *Biochim Biophys Acta* 1760:1304–1313
57. Hansen RE, Winther JR (2009) An introduction to methods for analyzing thiols and disulfides: reactions, reagents, and practical considerations. *Anal Biochem* 394:147–158