

Site-selective protein-modification chemistry for basic biology and drug development

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Nature has produced intricate machinery to covalently diversify the structure of proteins after their synthesis in the ribosome. In an attempt to mimic nature, chemists have developed a large set of reactions that enable post-expression modification of proteins at pre-determined sites. These reactions are now used to selectively install particular modifications on proteins for many biological and therapeutic applications. For example, they provide an opportunity to install post-translational modifications on proteins to determine their exact biological roles. Labelling of proteins in live cells with fluorescent dyes allows protein uptake and intracellular trafficking to be tracked and also enables physiological parameters to be measured optically. Through the conjugation of potent cytotoxicants to antibodies, novel anti-cancer drugs with improved efficacy and reduced side effects may be obtained. In this Perspective, we highlight the most exciting current and future applications of chemical site-selective protein modification and consider which hurdles still need to be overcome for more widespread use.

Over billions of years of evolution, nature has developed an intricate machinery to covalently attach diverse functional groups to proteins after their synthesis by the ribosome — a process called post-translational modification (PTM). Naturally occurring protein PTMs are now understood to play important roles in tuning the physicochemical properties of proteins, modulating enzymatic activity, controlling protein–protein recognition and imparting enzymes with chemical functionalities that are not covered by the standard proteinogenic amino acids¹. For example, the attachment of carbohydrates to proteins — a process known as glycosylation — can improve the solubility of proteins and modulate ligand–receptor interactions¹. Through the reversible addition of phosphate groups at serine (Ser), threonine (Thr) or tyrosine (Tyr) residues, enzymes can be switched between their active and inactive states. In a similar manner, the formation of complexes between two or more proteins can be controlled¹. Covalently bound co-factors (so-called prosthetic groups), such as flavins or inorganic metal clusters, can impart metabolic enzymes with the ability to perform redox chemistry¹. Collectively, these examples highlight how, through the introduction of small modifications, nature often re-designs the structure and function of existing proteins to impart immense biological diversity.

In an attempt to follow nature's example, chemists have developed tools to covalently modify proteins with diverse functionalities in the laboratory. Initial methodologies typically offered poor control over the precise position of modification (that is, they exhibited poor site-selectivity). Water-stable electrophilic carboxylic acid derivatives such as *N*-hydroxysuccinimidyl (NHS) esters were among the first reagents used for covalent protein-modification applications². NHS esters react with diverse nucleophiles on protein surfaces, most importantly the ϵ -amine of lysine (Lys) residues. Given the abundance of surface Lys on most proteins, the reaction typically results in a mixture of products modified at different protein positions. This in turn makes functional characterization of modified proteins difficult, because one is dealing with a heterogeneous protein sample rather than a single, homogenous

molecule. Therefore, reactions that enable chemists to selectively install modifications on protein surfaces at pre-defined positions would facilitate both the chemical characterization of these modifications and investigation of their exact biological function. In the context of this Perspective, we will refer to methods that permit the formation of a covalent bond between a protein and a synthetic organic molecule at a pre-defined residue as site-selective protein-modification reactions.

The challenges associated with the development of site-selective protein-modification reactions are manifold. Reactions have to be chemoselective for one amino acid side chain over all others on the protein surface. Amino acids that are lowly abundant on protein surfaces and have unique reactivity lend themselves particularly well to site-selective modification chemistries. The most important natural amino acid fulfilling these criteria has proven to be surface exposed cysteine (Cys) residues³. Certain natural amino acids may also have unique properties when introduced at the N-terminal position of a protein. For instance, an N-terminal Ser can be oxidized to generate an aldehyde that can subsequently be used for site-selective modification⁴. Also, at the N-terminus, Cys can react selectively with thioester derivatives to give a native peptide bond in a process called native chemical ligation (NCL)⁵. Site-selective protein-modification reactions must furthermore be compatible with aqueous buffered systems, not interfere with proper protein folding, and proceed at near neutral pH and moderate temperatures (that is, 20–37 °C). Because the chances of encountering multiple proteins that carry even lowly abundant natural amino acids on their surface increases considerably in complex protein mixtures, it is typically only possible to site-selectively label purified recombinant proteins on naturally occurring amino acids (Fig. 1).

One method of achieving site-selective modification of a particular protein target within a complex mixture of proteins relies on recent advances in genetic code expansion techniques^{6,7}. These techniques allow for the ribosomal incorporation of non-canonical amino acids into proteins that display functionalities that are normally not present in living cells⁷. These include side chains featuring

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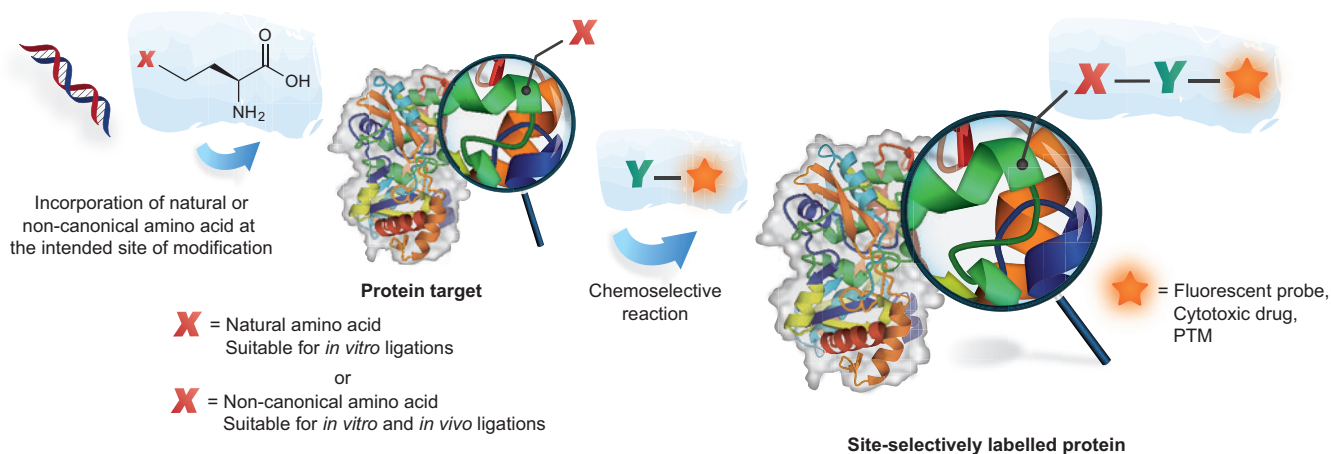


Figure 1 | Conceptual strategy to achieve site-selective protein-modification using biocompatible chemical reactions *in vitro* and *in vivo*. Typically, site-selective chemical modification of proteins can be achieved either (1) on a natural amino acid side chain with unique reactivity (for example, Cys) of which a single copy is already present on the protein of interest or may be introduced by site-directed mutagenesis or (2) on a non-canonical amino acid featuring a side chain with a functional group that is normally not present in living systems but can be introduced using genetic encoding procedures (for example, azide, alkyne, ketone, alkene and tetrazine). In the case of natural amino acid side chains, one is limited to the modification of purified proteins in a test tube, as many other biomolecules may display the same functionality in a complex protein mixture. Using non-canonical amino acids, on the other hand, one can devise reactions that are selective over all naturally occurring amino acid side chains thus permitting site-selective modification of individual proteins in complex mixtures and living organisms. Upon choice of the functionality to be chemically targeted, reaction with a suitable modified molecule of interest (for example, post-translational modification (PTM), fluorescent label or cytotoxic drug) must proceed in a chemoselective manner under physiological conditions.

azide, alkyne, ketone, alkene or tetrazine functional groups⁸. For each of these functionalities, reactions can be envisioned that are highly chemoselective or even mechanistically specific for the non-canonical amino acid over all other functionalities present in living cells. This concept is referred to as bioorthogonal chemistry and is now permitting chemists to site-selectively install modifications on individual proteins in complex biological mixtures, including in living organisms (Fig. 1)^{9–11}. Bioorthogonal ligation reactions may furthermore be used on purified protein preparations when bond stability or labelling accuracy beyond that achievable with native amino acids is required (for example, construction of antibody–drug conjugates (ADCs)).

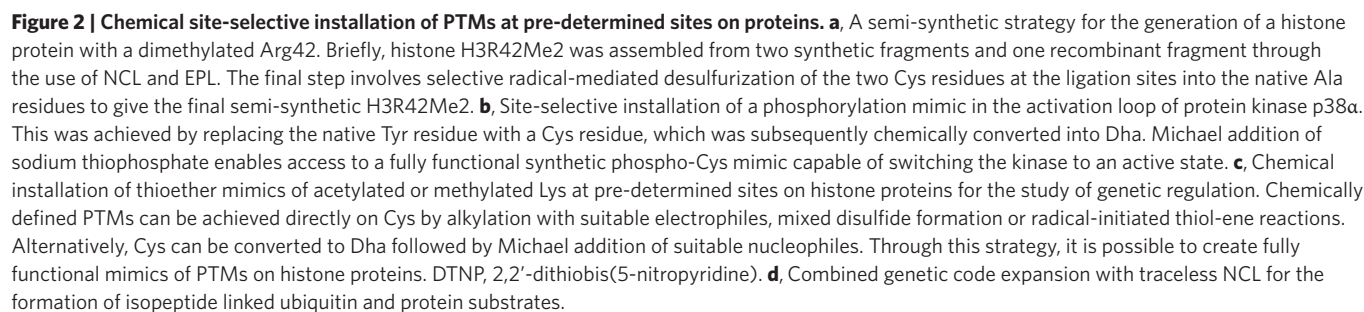
Chemists and biologists are now increasingly using site-selective protein-modification tools to study the function of naturally occurring PTMs. In addition, by attaching synthetically prepared modifications that are typically not found in nature, they are attempting to confer novel functionality to proteins of bacterial or eukaryotic origin. The aim of this Perspective is to discuss key developments in the field of chemical site-selective protein-modification in the context of their biological and therapeutic applications, rather than exhaustively present a large number of reactions concerning this topic^{8,12}. The methods covered in this Perspective involve ligation reactions between the side chain of a natural or non-canonical amino acid and a suitable small molecule derivative (Fig. 1).

Study of protein PTMs

Elucidating the effect of PTMs on protein function can be greatly assisted by studying pure protein preparations with precise modifications *in vitro*. Access to modified proteins could permit raising modification-specific antibodies¹³, studying enzymatic activities *in vitro* or potentially affinity-purifying modification-specific interaction partners from cell lysates, much like established affinity purification protocols¹⁴. However, isolation of single isoforms of modified proteins from natural sources in sufficient quantities is often challenging. Efficient chemical means to prepare proteins with defined PTMs *in vitro* are thus required. Achieving high precision with respect to the site of the modification is typically the prime concern.

One approach towards the chemical preparation of post-translationally modified proteins that meets the requirement for precision involves the total or semi-synthesis of proteins with defined PTMs *in vitro*⁵. Typically, peptide fragments bearing PTMs are prepared by solid-phase peptide synthesis, joined through NCL reactions and folded *in vitro* to give the full-length protein⁵. In one example, through the use of NCL, Danishefsky and co-workers assembled a single glycoform of human erythropoietin with the consensus glycosylation motifs placed at their native sites¹⁵. While this approach is feasible for relatively short polypeptide chains, it becomes excessively cumbersome for large proteins. Alternatively, recombinantly expressed protein fragments with N-terminal Cys or C-terminal protein thioesters obtained from split-intein fusions can be directly conjugated to small peptide fragments using NCL. This technique, termed expressed protein ligation (EPL)¹⁶, is particularly useful when the modification is relatively close to the N- or C-terminus of a protein. EPL has been widely employed by Muir and co-workers for the study of the effect of histone tail methylation and acetylation on chromatin structure and function¹⁷. In one example, a semi-synthetic strategy was used to generate a dimethylated arginine (Arg) at position 42 of histone H3. Specifically, the sequence from alanine 1 (Ala1) to valine 46 (Val46) of histone H3 was prepared by total synthesis and then joined to a recombinantly expressed fragment representing Cys47 to Ala135 (Fig. 2a). Dimethylation of Arg42 was shown to stimulate transcription *in vitro*¹⁸. In many other cases, however, PTMs are located at sites in the inner regions of the protein, and a totally synthetic or semi-synthetic approach for the preparation of the modified protein becomes particularly difficult. In such cases, tools that permit the site-selective installation of PTMs on fully folded proteins may allow easier access to the desired modified protein product.

Site-selective protein-modification chemistry promises to allow the facile preparation of proteins with defined PTMs from full-length recombinantly produced precursors while still meeting the requirement for introducing the PTM at a precise position. So far, however, most chemical site-selective protein-modification approaches only allow the introduction of PTM mimics. For many applications (for example, raising antibodies) mimics may, however, be fully sufficient¹⁹.



modified via Michael addition with sodium thiophosphate (Fig. 2b) to mimic a native phosphate group²¹. In this case, the phospho-Cys variant of p38 α was sufficiently similar to the natural phospho-Tyr to switch the kinase into its active state. The introduction of

phosphorylated Tyr analogues has also been achieved through the Staudinger-phosphite reaction of azides²².

Furthermore, other PTM mimics, such as acetylated or methylated Lys, can be installed at Dha by reacting the Michael acceptor with a suitable thiol precursor (Fig. 2c)²³. Other approaches to prepare histones with thioether mimics of Lys methylation and acetylation involve direct Cys alkylation with suitable electrophiles^{24,25}, mixed disulfide formation²⁶ and radical-initiated thiolene reactions (Fig. 2c)²⁷.

The chemical introduction of native PTMs into full-length proteins at defined positions is more difficult, but has found particular success in site-selective installation of ubiquitin into recombinant substrate proteins. One strategy consisted of site-directed incorporation of a δ -thiol-Lys derivative into a recombinant protein that reacts with a C-terminal thioester of ubiquitin through NCL; subsequent desulfurization yields a naturally occurring isopeptide bond between substrate proteins (for example, small ubiquitin-like modifier) and ubiquitin (Fig. 2d)²⁸. Alternatively, a genetically encoded orthogonal protection and activated ligation strategy consisting of genetic encoding of a protected Lys residue has been developed. All the other Lys residues present on the protein are then chemically protected followed by orthogonal deprotection of the Lys that was genetically introduced. The free single Lys then reacts with a C-terminal thioester of ubiquitin through NCL to yield a natural isopeptide bond. Final global deprotection affords the ubiquitinated protein²⁹. This approach has been used to prepare, for example, mono-ubiquitinated human Dvl2 DIX domain at Lys54 and Lys58 providing insight into the effect of ubiquitination into oligomerization of these domains³⁰. Protein substrates may contain multiple ubiquitin chains. Polyubiquitination of target proteins at specific sites through defined chemical linkages has been achieved, for example, by Cu(I)-catalysed azide-alkyne cycloaddition-based polymerization of a ubiquitin containing two orthogonal non-canonical amino acids, which led to the formation of a protease-resistant polyubiquitinated protein³¹. Alternatively, Brik and co-workers have introduced suitable electrophiles at the proximal end on ubiquitin for chemoselective reaction with a Cys-tagged α -globin protein substrate via disulfide or thioether bonds³².

To add further complexity, nature often introduces more than one distinct PTM at pre-determined sites on a protein. Yet, with all of the abovementioned approaches it is currently difficult to site-selectively install two distinct modifications *in vitro* when modifying only natural amino acids. Therefore, further work is needed to develop reactions that allow for the facile installation of native PTMs on recombinantly produced full-length proteins and the installation of two different PTMs on the same protein substrate.

The ability to genetically encode unnatural amino acids into pre-defined sites on proteins can help in achieving site-selective protein-modification at multiple positions. As an example of such a strategy, Davis and co-workers have built a reconstituted and fully functional mimic of P-selectin glycoprotein ligand 1 (PSGL-1) containing both a sulfo-Tyr mimic and a trisaccharide moiety. This was possible through the placement of a Cys residue and an unnatural azidohomoalanine (Aha) amino acid, which allowed for sequential mixed disulfide and Cu(I)-catalysed azide-alkyne cycloaddition reactions, on a bacterially expressed protein scaffold³³.

As a possible alternative, techniques are now emerging that allow for the ribosomal incorporation of amino acids that already carry PTMs into proteins^{7,34}. These approaches could allow for the expression of precisely post-translationally modified proteins in living cells and have great potential to facilitate the study of PTMs in their native environment.

Site-selective protein modification for imaging

Modern biology would be impossible without the ability to label biological macromolecules with visible-light dyes or radionuclides

for diverse imaging applications. Very often, purified proteins are conjugated to fluorophores or radionuclides *in vitro* and then used to track the localization of the labelled protein or its binding partners in an experimental system *in vitro* (for example, immunofluorescence) or *in vivo* (for example, nuclear imaging techniques). For *in vitro* applications, site-selective labelling is not a strict requirement. Labelled protein reagents are typically used in excess and non-binding species are removed in washing steps. For *in vivo* applications, on the other hand, it has been shown that the position in which a modification is made to a protein can have an influence on pharmacokinetics and biodistribution^{35,36}. So far, however, it is poorly understood how much, or whether at all, site-selectivity would improve the specificity or sensitivity of an imaging agent *in vivo* and further work is required to address this question.

Site-selectivity becomes strictly important when optical imaging techniques are used to obtain a more complex functional readout beyond the mere localization of a single protein. For example, measuring the association of two proteins by Förster resonance energy transfer (FRET) will require specific placement of the FRET donor and acceptor in sites that are not involved in protein-protein interactions, yet are close enough for FRET to take place. Site-selective labelling of purified protein preparations with FRET dyes can nowadays routinely be achieved using commercially available maleimide, sulfone, Michael-acceptor and haloacetamide reagents *in vitro*.

Few approaches, however, exist so far to site-selectively label the same protein with two different dyes on two different native amino acids³⁷. Double labelling is required if one, for example, wants to follow conformational changes of a protein by FRET. To achieve this aim, it is very often necessary to first introduce an unnatural amino acid into the protein of choice that can bioorthogonally react with a suitably modified dye. The second label is then site-selectively introduced using, for example, the reaction of maleimides with free Cys (ref. 38). Alternatively, the introduction of two non-canonical amino acids that are suitable precursors for protein double-labelling with a FRET pair is now possible using genetic code expansion techniques (Fig. 3d)³⁹.

Bioorthogonal chemistry also allows for the site-selective labelling of proteins in complex mixtures, including live cells and whole organisms. Using an inverse electron demand Diels-Alder cycloaddition reaction, Chin and co-workers were able to site-selectively fluorescently label the non-native amino acid norbornene, which was genetically encoded into a protein expressed on the membrane of a mammalian cell (Fig. 3a)⁴⁰. As an additional benefit, fluorophores can also be engineered such that their fluorescence markedly increases on bioorthogonal reaction with the protein bearing the unnatural amino acid handle. It is thought that this 'turn-on' mechanism may give better signal to background ratios than those found when labelling proteins with constitutively fluorescent dyes. Bioorthogonal labelling of specific proteins may also be used to detail protein uptake and internalization in the context of disease. Using a Cu(I)-catalysed azide-alkyne cycloaddition strategy, it was possible to label anthrax lethal factor in a quantitative manner with a small fluorophore⁴¹. This enabled the time-lapse monitoring of anthrax lethal factor internalization and membrane translocation processes in living cells. The abovementioned and other recent studies⁸ provide exciting examples of the localization and tracking of bioorthogonally labelled proteins in living cells or *in vivo* with minimal disruption of the native protein and with an unprecedented molecular precision.

Weissleder and co-workers recently extended the use of bioorthogonal chemistry on proteins to nuclear imaging applications. They demonstrated that a tumour-homing antibody bearing a *trans*-cyclooctene (TCO) ring on its surface could selectively and rapidly react with a radiolabelled variant of a tetrazine ring in an inverse electron demand Diels-Alder reaction *in vivo* in a mouse xenograft tumour, thus making the antibody visible in a positron

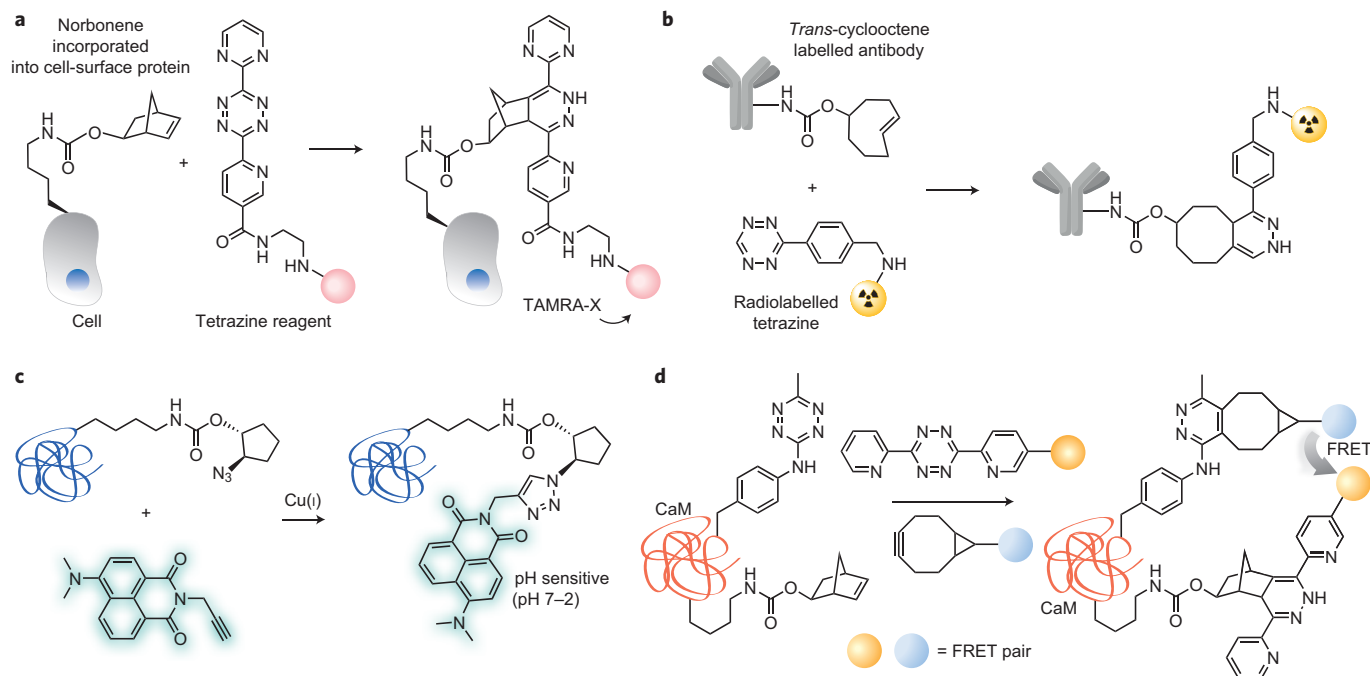


Figure 3 | Bioorthogonal protein-modification chemistry for live cell imaging applications. **a**, Genetic encoding of a norbornene amino acid into a mammalian cell-surface protein followed by selective labelling with a tetrazine-modified version of the fluorescent dye TAMRA-X. **b**, *Trans*-cyclooctyne (TCO) was conjugated to a tumour-homing antibody and injected into a tumour-bearing mouse. Once the antibody had accumulated inside the tumour and cleared from healthy organs, a radiolabelled tetrazine was injected. The tetrazine and TCO rapidly reacted leading to retention of radioactivity at the tumour site that could be visualized by radio-imaging approaches. **c**, Encoding of an azido-amino acid into the acid-sensitive chaperone HdeA inside the *E. coli* periplasm. Bioorthogonal attachment of an alkyne derivative of the environmentally sensitive dye 4-*N,N*-dimethylamino-1,8-naphthalimide (4-DMN) to the protein was achieved using Cu(I)-catalysed azide-alkyne cycloaddition. On pH dependent conformational changes in HdeA, 4-DMN changes its fluorescence properties enabling the measurement of changes in local pH. **d**, Simultaneous genetic encoding of two distinct non-natural amino acids into the same protein. The tetrazine and norbornene amino acids did not react with each other but the tetrazine selectively reacted with a strained cyclooctyne ring and the norbornene with a different tetrazine. This mutually orthogonal and bioorthogonal pair of reactions allowed for the site-selective placement of two distinct fluorescent dyes onto the surface of CaM in *E. coli* and its conformational changes in response to Ca^{2+} stimuli were measured by FRET.

emission tomography (PET) scanner (Fig. 3b)⁴². More recently, ^{18}F -labelled antibody fragments for PET imaging were generated by installing TCO into an anti-class II MHC single domain antibody followed by reaction with a tetrazine-labelled ^{18}F -2-deoxyfluoroglucose (FDG)⁴³. The method was found to be particularly rapid and efficient with a radiochemical yield of >25% enabling imaging of pancreatic tumours *in vivo* with an increased level of specificity compared with conventional ^{18}F -FDG imaging. Furthermore, Robillard and colleagues used a similar approach where, before injection of the radiolabelled tetrazine, residual amounts of antibody-TCO in circulation were first removed by injection of a tetrazine-functionalized clearing agent⁴⁴. This led to a significant increase in tumour to healthy tissue ratios, and a predicted eightfold higher tumour dose compared with the directly labelled antibody. While speed is not the primary objective for most protein-modification reactions that occur *in vitro*, the extremely fast kinetics of the inverse electron demand Diels-Alder reaction makes it particularly suitable for *in vivo* applications where reagents may rapidly be excreted through the liver or kidney; however, an improved understanding of the stability of the *trans* double bond of TCO that may undergo isomerization *in vivo* will be required. Alternatively, it may also be possible to encode tetrazine motifs into the antibody followed by ligation with strained alkene probes, avoiding long exposure of the *trans* double bond to *in vivo* conditions⁴⁵.

In the coming years, one of the most exciting applications of bioorthogonal protein-labelling chemistry may well be the ability to construct functional optical sensors to measure diverse physiological parameters inside the dynamic environment of living cells and even in animals. For example, Chen and co-workers have recently

described the modification of the acid-sensitive chaperone HdeA with an environment-sensitive fluorophore inside the periplasm of *Escherichia coli* to create a biosensor for extremely low pH values (Fig. 3c)⁴⁶. On lowering the pH from 7 to 2, HdeA adopts a highly plastic structure making hydrophobic residues accessible to the fluorophore, which is reflected in an increase in fluorescence. Even bioorthogonal dual modification of a single protein with two distinct dyes has recently been achieved. Impressively, Chin's group has developed an optimized translational system for proteins containing non-natural amino acids in *E. coli* that enable the placement of two distinct non-native amino acids at two pre-determined sites that could be modified with mutually and bioorthogonal reactions (Fig. 3d). Site-selective, bioorthogonal dual labelling of calmodulin (CaM) with a FRET pair made it possible to follow alterations in CaM conformation inside the living cell in response to changes in Ca^{2+} concentrations³⁹.

These examples demonstrate that bioorthogonal protein-modification chemistry is a very promising tool for the construction of protein biosensors *in vivo*. Protein biosensors have the unique potential to give an optical readout in response to physiological changes inside living cells and organisms. These sensors will allow biologists to use imaging techniques to study complex processes inside living cells and even whole organisms in a spatiotemporally controlled manner.

Modulating the properties of protein therapeutics

Therapeutic proteins are a rapidly growing class of drugs⁴⁷. Unfavourable pharmacokinetics and immunogenicity, however, often hamper the efficacy of recombinant protein therapeutics

in vivo. Therapeutic proteins may be quickly degraded by proteases or excreted by the kidneys leading to a rapid drop in plasma concentration after administration of the drug. A strong immune reaction may also be a life-threatening complication akin to an allergic shock. Alternatively, anti-drug antibodies may inactivate the therapeutic protein and lead to treatment resistance. One of the first applications of protein-modification chemistry in the context of drug development was the conjugation of protein therapeutics with long polyethylene glycol (PEG) chains to extend their plasma circulation half-life and attenuate their immunogenicity *in vivo*⁴⁸. By attaching a long, unstructured polymer chain, the hydrodynamic radius of the macromolecule is increased, thus reducing renal filtration. At the same time, the PEG polymer physically shields the protein from degradation by proteases and recognition by the immune system.

Many Food and Drug Administration-approved PEGylated protein drugs are still produced using non-site-selective modification chemistry⁴⁹, which can potentially produce inactive protein species. Indeed, many marketed PEGylated drugs are less active than their unmodified counterparts⁵⁰. For this reason, site-selective PEGylation should be preferred and site-selective protein-modification chemistry is increasingly used for the PEGylation of proteins⁵¹.

In one of the first examples of site-selective PEGylation, a N-terminal Ser residue was oxidized to the corresponding aldehyde using sodium periodate followed by subsequent oxime ligation with an aminoxy PEG derivative (Fig. 4a)⁴. The modified proteins, interleukin (IL)-8, granulocyte-colony stimulating factor (G-CSF) and IL-1 α , fully retained their activity after PEGylation. Alternatively, Francis and co-workers reported a pyridoxal phosphate (PLP) mediated transamination reaction that introduces

reactive aldehydes at the N-terminus (Fig. 4a)⁵². In a similar manner, subsequent reaction of the aminoxy PEG derivative at acidic pH 6.5 provides the corresponding oxime product. These methods require, however, chemical manipulation of the N-terminal groups to introduce a reactive aldehyde functionality. More recently, the same research group reported a direct site-selective method that enabled PEGylation of native proteins⁵³. The method uses 2-pyridinecarboxylaldehydes that selectively react at the N-terminus at neutral pH. The reaction proceeds via the formation of the imine followed by attack of the neighbouring amide nitrogen in the protein backbone leading to the formation of a stable imidazolidinone conjugate (Fig. 4b). This methodology may be applied to a wide range of native proteins, with the exception of those where the N-terminus is acylated, those with proline in the second position or those where the N-terminus is hindered. However, in some instances, the modification of internal sites on the protein may be preferred. Site-selective PEGylation of proteins may also be achieved using genetic encoding non-canonical amino acids bearing unique reactive handles. Incorporation of *para*-acetylphenylalanine (*pAF*) into human growth hormone (hGH) followed by reaction with aminoxy-PEG derivatives at pH 6 enabled the construction of hGH variants with PEG at 20 different pre-determined sites (Fig. 4c)⁵⁴. The PEGylated construct of hGH modified at site 35 was shown to have improved pharmacokinetics in rats and comparable efficacy in clinical studies performed in GH-deficient adults but with reduced injection frequency. This example is illustrative of the advantage of using site-selective PEGylation methods that allow the structure–activity relationships of proteins to be modified for optimal pharmacokinetics while retaining their biological activity and improving clinical application.

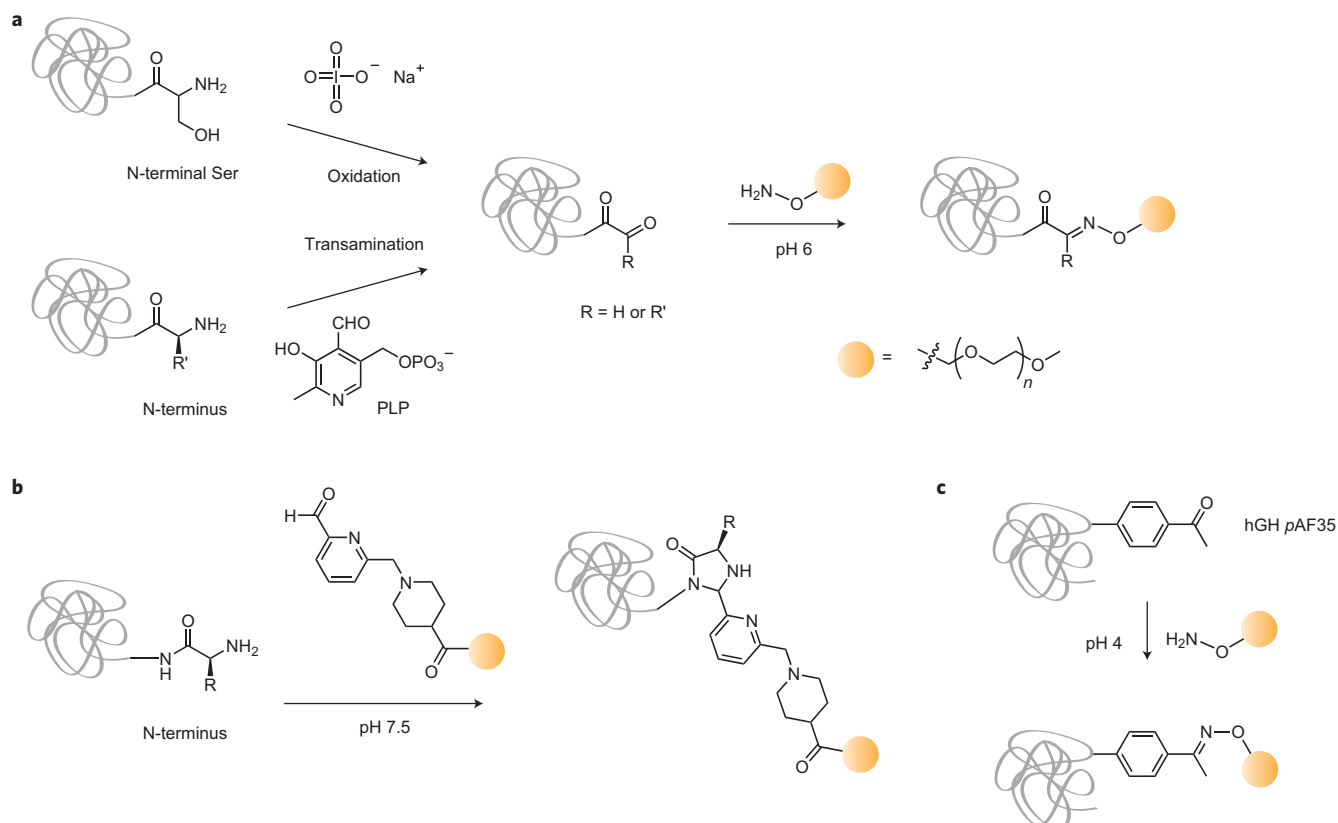


Figure 4 | Example of strategies to achieve site-selective PEGylation of proteins. a, Examples of PEGylation of the N-terminus using oxime ligation. N-terminal aldehydes can be either generated through oxidation using sodium periodate or using pyridoxal phosphate (PLP) promoted transamination. Subsequent oxime ligation with aminoxy PEG derivatives afford the N-terminally PEGylated protein. **b,** Site-selective attachment of 2-pyridinecarboxylaldehyde PEG derivatives to native proteins at neutral pH. **c,** Genetic encoding of *pAF* into hGH at pre-determined site 35 followed by oxime ligation with an aminoxy PEG derivative at acidic pH.

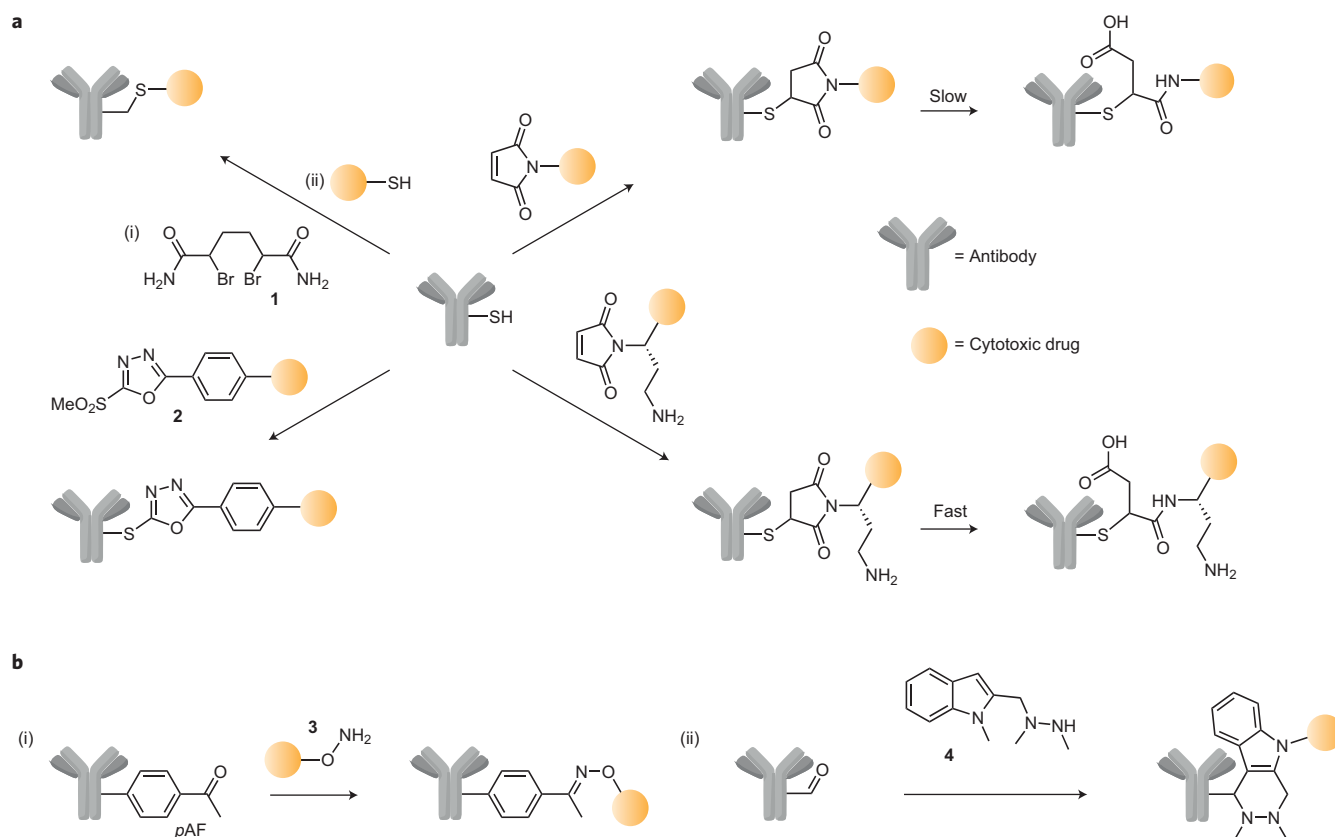


Figure 5 | Site-selective protein-modification reactions suitable for the synthesis of antibody-drug conjugates (ADCs). **a**, Reactions selective for Cys.

The classical chemoselective reaction of maleimides with thiols is widely used for the site-selective synthesis of ADCs. The initial product of the reaction is now known to be unstable and can either regenerate the starting materials through a retro-Michael addition or hydrolyse to form the more stable ring-opened product. Ring opening can be facilitated by placing a positively charged amino acid in close proximity to the modified Cys on the protein surface. Alternatively, a primary amine can be placed next to the maleimide on the linker between drug and antibody. Conversion of Cys into Dha on protein surfaces using **1** followed by Michael addition with thiol-containing drugs results in a very stable thioether bond. Similarly, the reaction of a protein surface Cys with a Julia-Kocienski-like methylsulfonylbenzothiazole reagent **2** yields a site-selective protein conjugation product that is intrinsically very stable in plasma and may be very useful for the synthesis of ADCs. **b**, Bioorthogonal reactions selective for non-natural amino acids. (i) Genetically introduced pAF on the antibody surface can selectively react with hydroxylamine-containing drugs **3** to form a very stable linkage. (ii) Genetically or enzymatically introduced aldehydes on the antibody surface can selectively react with **4** in a Pictet-Spengler type reaction to form a very stable ADC.

Antibody conjugation for delivery of cytotoxic drugs

More recently, the conjugation of monoclonal antibodies directed against tumour marker proteins with highly potent cytotoxic drugs is emerging as a strategy to create antibodies with improved killing potential towards malignant cells or even turn antibodies lacking any cytotoxic activity into potent antineoplastic agents⁴⁷. Initial work on ADCs relied on poorly site-selective conjugation strategies to attach drugs to antibodies yielding a heterogeneous mixture of antibodies with different drug loading at different sites^{55–57}.

It was soon found that both the number of drugs per antibody and the attachment site could have a profound effect on the pharmacokinetics, efficacy and toxicity of an ADC (refs 36,58). A high drug-to-antibody ratio (DAR; typically > 2) may lead to reduced thermostability of the ADC (ref. 56) and result in a faster clearance from the bloodstream⁵⁷. ADCs with a high DAR were also found to have a smaller therapeutic window than ADCs with low DARs⁵⁷. In addition, the site of drug attachment can have a profound effect on clearance kinetics and off-target ADC toxicity^{36,58} as well as in plasma stability and efficacy⁵⁹. These observations demonstrate the importance of using site-selective protein-modification chemistries for the synthesis of homogenous protein conjugate therapeutics.

At present, the most commonly used approach for the site-selective generation of antibody-conjugate therapeutics is the Michael addition reaction of the thiol side-chain of genetically engineered

Cys residues on the antibody's surface with maleimides (Fig. 5a)³. Protocols have been established that allow for the selective reduction of engineered Cys while leaving native disulfide bonds on the antibody intact³⁶. Using carefully optimized conditions, ADC preparations containing >90% ADCs with exactly two drugs per antibody could be prepared³⁶.

A potential drawback of maleimide chemistry is related to the fact that the thioether bond formed between the protein's Cys and the maleimide may not be fully stable in plasma (Fig. 5a)⁵⁸. Conjugates can undergo a retro-Michael addition releasing the maleimide from the carrier protein under physiological conditions⁶⁰. In the case of ADCs, the product of this reaction would be a highly potent cytotoxic drug that can kill cells in healthy tissues. Indeed, off-target toxicity is a pressing problem in the clinical development of ADCs. Different strategies have thus been pursued to improve the stability of maleimide conjugates. Conjugation sites have been placed in close proximity to positively charged amino acid residues, which promote the hydrolysis and ring-opening of maleimides to give more stable species⁵⁸. Linkers containing primary amines in close proximity to a maleimide that undergo rapid hydrolysis to the more stable ring-opened form have also been proposed (Fig. 5a)^{61,62}. The resulting ADCs were demonstrated to be more stable in plasma and more efficacious *in vivo* while reducing the side effects observed when using traditional maleimides.

A number of reactions for the modification of Cys that inherently give more stable thioether bonds, such as those using Julia–Kocienski-like reagents developed by Barbas and co-workers (Fig. 5a)⁶³ or the Michael addition of thiol nucleophiles to Dha developed by Davis and co-workers (Fig. 5a)⁶⁴, may also be exploited for the synthesis of ADCs. These technologies are fairly novel, however, and we are not aware of any industrial ADC discovery programmes using them thus far.

As an alternative to the chemical modification of genetically incorporated Cys residues on monoclonal antibody (mAb) surfaces, one can incorporate unnatural amino acids into mAbs and modify them using bioorthogonal reactions. This approach has the potential advantage of yielding highly homogeneous protein conjugate preparations and antibody–drug linkages with high stability.

Schultz and co-workers have genetically incorporated *p*AF into mAbs and reacted the side chain ketone with an aminoxy-modified version of the potent cytotoxic drug monomethyl auristatin E (MMAE) **3** for the preparation of ADCs (Fig. 5b(i))⁶⁵. The same antibody was expressed with two Cys in the same positions as the *p*AF and conjugated with a maleimide-containing version of MMAE. The *p*AF-modified ADC had a DAR of exactly 2.0, whereas attempts to selectively modify the two genetically introduced Cys on the mAb using a maleimide-containing derivative of MMAE gave a DAR of 2.3. The homogenous *p*AF-modified ADCs were found to be more stable than conventional maleimide linkages *in vivo*⁶⁶. These characteristics resulted in an improved efficacy of the bioorthogonally modified ADC in xenograft tumour-bearing mice.

Bertozzi and co-workers have reported a site-selective protein-ligation where aldehyde-containing proteins rapidly react with 3-methylhydroxylamino indoles in a Pictet–Spengler type mechanism⁶⁷. A variation of the methodology, the hydrazino-*iso*-Pictet–Spengler (HIPS) ligation, yields highly stable C–C linkages between aldehyde-containing antibodies and drugs containing

3-methylhydrazino indole-groups **4** (Fig. 5b(ii)). As such, this methodology has been used for the construction of ADCs⁶⁸. By performing HIPS conjugations at different locations on an immunoglobulin G1 backbone, it was shown that the site of conjugation influences dramatically the *in vivo* efficacy and pharmacokinetic parameters⁶⁸. In addition, the stable conjugates built using the HIPS conjugation strategy exhibited an improved safety profile in rats when compared with those conjugates prepared using a non-site-selective lysine conjugation strategy.

Collectively, these observations highlight the fact that site-selective protein-conjugation reactions can vastly improve the therapeutic efficacy of protein-conjugate therapeutics when compared with protein conjugates that were synthesized using non-selective bioconjugation chemistry, at least within animal models of disease. Although clinical proof is not likely within the next few years, we expect that protein-conjugate therapeutics synthesized using site-selective modification reactions that yield homogenous and more stable products will also be more efficacious and tolerable to patients than currently used heterogeneous and potentially unstable conjugates.

The future of bioorthogonal chemistry on protein surfaces

Evidence is now accumulating that chemistry on protein surfaces will in the future go far beyond bioconjugation. In particular, the combination of genetic code expansion for the incorporation of non-canonical amino acids into proteins with bioorthogonal chemistry could enable tantalizing new applications. For example, the genetic incorporation of caged amino acids that can be deprotected using bioorthogonal reactions holds great potential for the precise spatiotemporal control of protein function *in vivo*. Indeed, a palladium-mediated depropargylation has recently been achieved within a living cell and could be used to restore the function of a propargyl-protected protein (Fig. 6a)⁶⁹. In another example, a tetrazine-triggered protein decaging method based on the inverse

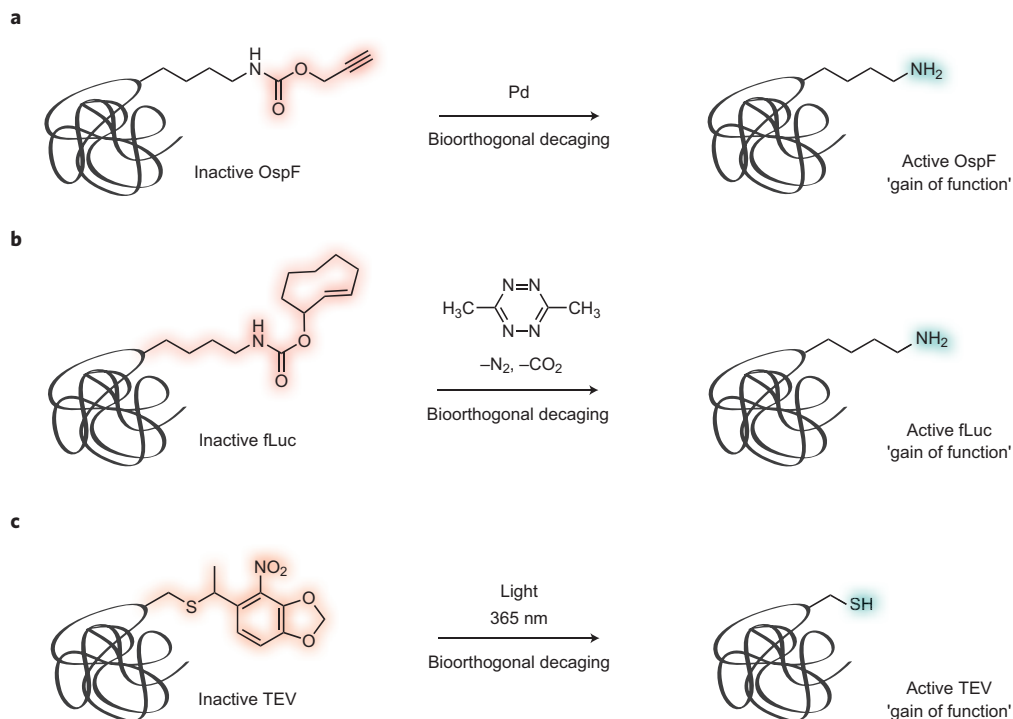


Figure 6 | Bioorthogonal approaches to *in situ* protein activation. **a**, A biocompatible palladium catalyst that cleaves the propargyl carbamate group of a protected Lys analogue to generate a free Lys was used to modulate the function of an intracellular protein in a gain-of-function fashion. **b**, A TCO-caged Lys on the active site of a protein renders the protein inactive. On reaction with 3,6-dimethyl-1,2,4,5-tetrazine, rapid inverse electron demand Diels–Alder elimination took place enabling bioorthogonal decaging and intracellular activation of the protein target in its native cellular context. **c**, Photodeprotection of a caged Cys that was genetically encoded into tobacco etch virus (TEV) to reveal the native protein in live cells.

electron demand Diels–Alder reaction was successfully employed for the bioorthogonal deprotection of a caged Lys within living cells (Fig. 6b)⁷⁰. The inverse electron demand Diels–Alder reaction elimination occurs through the conversion of the dihydropyridazine adduct, formed from the [4+2] cycloaddition between a 1,2,4,5-tetrazine and TCO, to a pyridazine with elimination of CO₂ and an amine derivative.

This inverse electron demand Diels–Alder elimination reaction was also used by Robillard and co-workers in a small molecule pro-drug strategy. A carbamate between 1-hydroxy-*trans*-cyclooctene (HO-TCO) and the amino sugar of doxorubicin (DOXO), a chemotherapeutic agent commonly used for the treatment of cancer, was prepared⁷¹. When reacting the DOXO derivative with a tetrazine reagent in an inverse electron demand Diels–Alder fashion, the dihydropyridazine product undergoes conversion into a conjugated pyridazine with consequent elimination of the NH₂-substituted DOXO and CO₂ (ref. 71). It remains to be demonstrated that this strategy can be expanded to antibodies for *in vivo* bioorthogonal elimination and delivery of drugs. For example, by incorporating a DOXO HO-TCO carbamate into a tumour-homing antibody, one could selectively transport the inactivated drug into the tumour and release it selectively by injecting tetrazine as a trigger substance. This in turn would decrease exposure of healthy tissues to DOXO and reduce treatment-associated side effects. Bioorthogonal chemistry on protein surfaces may thus permit the development of novel medicines with a spatially and temporally defined action for the treatment of cancer and other debilitating diseases.

Alternatively, genetic encoding of photocaged variants of natural amino acids (for example, Cys and Tyr) could be used to the same aim. In one case, photodeprotection of photocaged Cys results in rapid activation of tobacco etch virus (TEV) protease within living cells in their native environment (Fig. 6c)⁷². Similarly, deprotection of a key photocaged Tyr residue was used to control phosphorylation and signal transduction⁷³. It will emerge over the coming years how far and in which settings these techniques are superior to currently used methods for the control of protein function, for example, at the transcription level with doxocycline-inducible promoters and for *in situ* pro-drug antibody-based delivery strategies.

Conclusions and outlook

Site-selective protein-modification chemistry can enable the *in vitro* and *in vivo* study of PTMs, construction of protein-based sensors for biological applications and the preparation of powerful new protein therapeutics. Other applications that were not addressed in detail here may be found in the fields of materials science or regenerative medicine, where the synthesis of chemically defined scaffolds containing protein building blocks (for example, factors for cell attachment and growth) may play an important role.

Site-selective protein-modification chemistry comes in two flavours: reactions with naturally occurring, but lowly abundant, amino acids are typically limited to the modification of isolated proteins *in vitro*. Methods that allow the modification of proteins with diverse synthetic molecules ranging from PTMs or their mimics to fluorophores and drugs with high accuracy with respect to the site of modification are now available. The use of low reagent concentration and the stability of the bond between the protein and the modification are primary concerns for the modification of proteins *in vitro* (for example, for ADCs) and methods that satisfy these requirements are now becoming available. The advantage of modifying naturally occurring amino acids lies in the fact that standard methods of protein engineering can be easily employed to generate the protein starting material.

Bioorthogonal ligation reactions, on the other hand, are a type of site-selective protein-modification chemistry directed at unnatural functionalities introduced into pre-determined sites on proteins often using genetic code expansion techniques. Bioorthogonal

ligation reactions are generally more versatile than classical site-selective protein-modification reactions directed at natural proteinogenic amino acids. In particular, bioorthogonal ligation chemistry may allow for the site-selective modification of proteins even in complex protein mixtures, where rare amino acids traditionally used for site-selective protein-modification (that is, most commonly Cys) are present in multiple copies. It now even allows for the site-selective modification of individual proteins in cells and living organisms where high reaction speeds on top of high selectivity may be required.

Bioorthogonal protein-modification chemistry may also be particularly suited to site-selectively introduce two or even more modifications on the same protein, opening new doors to interrogate protein structure, dynamics and function in their native environment. The main drawback of bioorthogonal approaches remains the need for introducing non-natural amino acids into the target protein before modification, but efficient methods, even for the large-scale production of proteins incorporating non-native amino acids, are now becoming available^{66,74}. Together with efforts to integrate optimized genetic encoding expression systems for researchers, including the Unnatural Protein Facility, located at Oregon State University⁷⁵, such techniques will hopefully find increased implementation in laboratories.

Modern site-selective, bioorthogonal protein-modification techniques have shown potential to provide molecular insights in numerous proof-of-concept studies and enabled the construction of more efficient and safer protein therapeutics. As the number of reactions at our disposal for the site-selective and bioorthogonal modification of proteins increases, we anticipate that the widespread use of bioorthogonal protein-modification will generate new fundamental biology knowledge, imaging and therapeutic applications with unprecedented precision. It is, however, key to make these methodologies available in an easily usable format to as wide a research community as possible.

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Author contributions

N.K. and G.J.L.B. developed the concept, researched and wrote the manuscript. F.P.C. designed and produced the figures, and F.P.C. and O.B. assisted with writing the manuscript.

Additional information

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Competing financial interests

The authors declare no competing financial interests.