

Chapter 2

Site-Specific Protein Labeling in the Pharmaceutical Industry: Experiences from Novartis Drug Discovery

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Abstract

Chemically modified proteins play an important role in several fields of pharmaceutical R&D, starting from various activities in drug discovery all the way down to biopharmaceuticals with improved properties such as antibody–drug conjugates. In the first part of the present chapter the significance and use of labeled proteins in biophysical methods, biochemical and cellular assays, in vivo imaging, and biopharmaceuticals is reviewed in general. In this context, the most relevant methods for site-specific modification of proteins and their application are also described. In the second part of the chapter, in-house (Novartis) results and experience with different techniques for selective protein labeling are discussed, with a focus on chemical or enzymatic (Avi-tag) biotinylation of proteins and their application in biophysical and biochemical assays. It can be concluded that while modern methods of site-specific protein labeling offer new possibilities for pharmaceutical R&D, classical methods are still the mainstay mainly due to being well established. However, site-specific protein labeling is expected to increase in importance, in particular for antibody–drug conjugates and other chemically modified biopharmaceuticals.

Key words Biophysical methods, Biochemical assay, Cellular assays, In vivo imaging, Biopharmaceutical, Antibody–drug conjugates, Biotin ligase, Avi-tag, SNAP-tag, Transglutaminase, Lipoic acid ligase, Click chemistry, Sortase, Phosphopantetheinyl transferase

1 Introduction

Modern drug discovery in pharmaceutical research is a highly diverse, protracted, and intricate process encompassing many activities such as target identification/validation, development of in vitro assays, screening for active compounds, structural studies, biophysical methods, medicinal chemistry, and in vivo pharmacology. Several of these disciplines are absolutely dependent on the supply of purified proteins in order to deliver meaningful results. For many applications like enzymatic assays or structure determination, nonmodified, native proteins are perfectly suitable. On the contrary, chemically modified proteins are needed or at least preferred for various experimental techniques such as certain

types of fluorescence-based assays, biophysical methods which rely on immobilization of proteins, in vivo imaging approaches, or chemically modified biopharmaceuticals with improved properties like antibody–drug conjugates. Therefore pharmaceutical research labs have made use of labeled proteins for quite a long time. Originally, the classical nonspecific approach by attachment of small molecules to reactive groups (mainly amino and thiol groups) on proteins was used exclusively for the production of labeled proteins. Because of the intrinsic disadvantages of the chemical labeling approach such as inhomogeneous incorporation of the chemical label and a potential impairment of the function and/or stability of the protein, the need for more specific and controllable methods to modify proteins was recognized. The last decade has witnessed the invention of several new techniques for site-specific protein labeling as reviewed generally in [1, 2] and more specifically for enzyme-catalyzed approaches in [3]. These latter methods include for instance the already well-established Avi-tag approach enabling site-specific attachment of biotin to a 15 aa long peptide tag catalyzed by the enzyme biotin ligase BirA as described in [4]. In analogy to the Avi-tag approach, other methods relying on short peptide tags for enzyme-catalyzed modification were developed as well. For example, recognition sequences have been designed and optimized for enzymes such as lipoic acid ligase [5, 6] or phosphopantetheinyl-transferases [7, 8] enabling the selective conjugation of lipoic acid analogues or coenzyme A derivatives to a specified Lys or Ser residue, respectively. Other enzyme-based labeling methods with short recognition sequences rely on enzymes such as transglutaminase [9, 10] or sortase [11, 12]. Finally, if no additional amino acids are tolerated or desired at all on a certain protein, incorporation of nonnatural amino acids with specific linking chemistries [13, 14] can be considered as well.

These novel technologies are increasingly being explored by the industry, and may become a part of standard procedures within drug discovery and development. However, the well-established classical reactive chemistry remains the dominant labeling method in many areas. In the following sections, I discuss several applications in which chemically modified proteins play an important role, and to what extent site-specific methods may provide an advantage over classical labeling techniques in the respective fields. In the final section, experiences made with several selected labeling techniques performed in several labs mainly in Novartis drug discovery will be described, with the aim of providing the reader with an idea whether we considered a given technique easy to implement and successful for our purposes. I am aware, of course, that this is my personal view and experience, which may therefore be biased and not congruent with the experiences of others.

2 Biophysical Methods

Biophysical methods are used to determine a direct binding of a pure target protein to a ligand which can either be another protein, a peptide, or a low molecular weight (LMW) compound. The most frequently used methods are for instance differential scanning fluorimetry, isothermal calorimetry, affinity-based mass spectroscopy, ligand or protein observed NMR spectroscopy, and techniques based on surface plasmon resonance (SPR). While the first methods are label-free as they work with proteins that are not chemically modified at all, the SPR-related approaches need a covalent or non-covalent immobilization of the target protein onto a surface as indicated in Fig. 1 and reviewed in [15, 16]. Covalent immobilization is mostly performed by nonspecific coupling of amino groups on the protein to carboxyl groups present on the dextran surface of the chip which is very similar the nonspecific protein labeling with *N*-hydroxy-succinimide (NHS) derived reagents. The nonspecific immobilization approach leads to a random orientation of the immobilized proteins which might result in a population of molecules where the binding site is not accessible or functional anymore. In addition, an efficient immobilization of a protein requires enrichment at the negatively charged SPR chip surface, which can be challenging for proteins with a low iso-electrical point. In order to circumvent these problems a strong non-covalent and/or directed covalent immobilization approaches can be a useful alternative. Ideally a strong non-covalent interaction should have a very low dissociation constant K_d in order to be reasonably stable over time. This requirement is perfectly fulfilled by the interaction between biotin and avidin/streptavidin with a K_d of 10^{-15} M, making biotinylated proteins highly important for SPR applications as described in [17, 18]. Besides classical modification of amino or

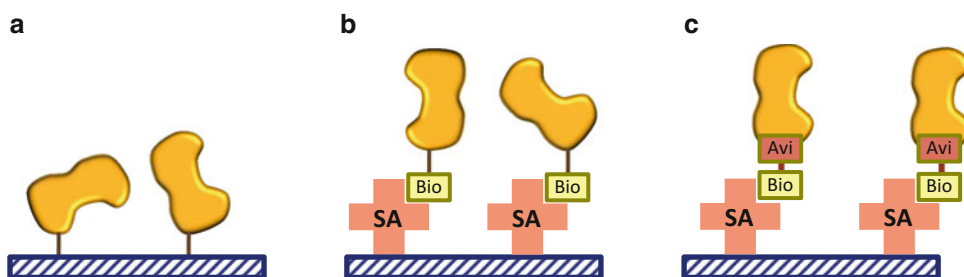


Fig. 1 Different approaches for immobilization of protein onto solid surfaces: Scheme (a) represents the direct immobilization of a protein through coupling of amino groups, resulting in random orientation. Scheme (b) shows the so-called minimal biotinylation technique, in which chemically biotinylated proteins are bound to a streptavidin surface. The biotinylated proteins are thus also oriented in a random manner but in contrast to the first method the immobilization procedure is less harsh. In scheme (c) specific biotinylated proteins (Avi-tagged) are immobilized onto streptavidin in a directed manner

thiol groups with biotin, site-specific biotinylation has become more and more important and mostly relies on the Avi-tag technology resulting usually in a complete incorporation of biotin [4]. Incomplete labeling is not problematic, as nonmodified protein molecules do not bind to the streptavidin surface and will be washed away. More recent approaches for attachment of proteins to surfaces such as click chemistry or binding of poly-histidine-tagged proteins on specific metal chelates can be considered as interesting alternatives as described in [19–22].

3 Biochemical Assays

A prominent example requiring modified proteins is the study of protein–protein interactions (PPI), where low molecular weight compounds or larger biomolecules are employed that disrupt or enhance the binding between two proteins. For measuring PPIs, proximity-based assays relying on fluorescence energy transfer (FRET, [23]) or time-resolved (TR-FRET or HTRF [24]) are widespread assay formats [25]. As depicted in Fig. 2, there are several possibilities to build up a proximity assay for a PPI, with the simplest setup being that two proteins are labeled directly with either donor or acceptor fluorophores. On the other hand, more complex setups exist in which one or more of the proteins contain “recognition handles” for helper molecules such as antibodies (epitope tags) or streptavidin (biotin). In this context, biotinylation is widely used for protein modification and can be performed either in

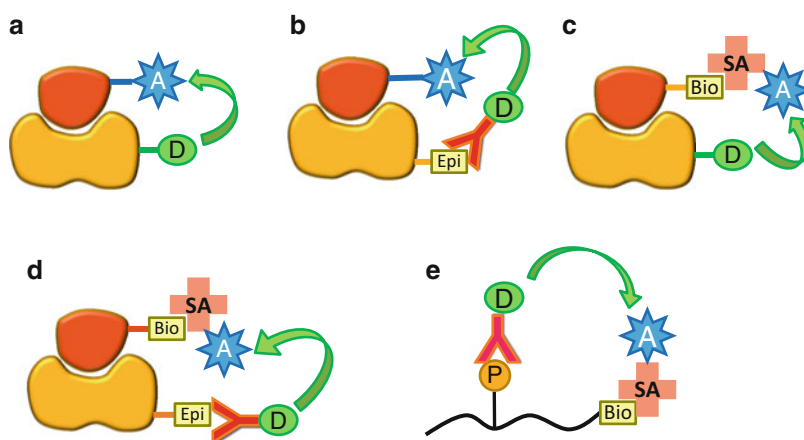


Fig. 2 Various layouts for TR-FRET assay formats in biochemical screening. Pictures (a–d) represent the mainly used formats for protein–protein or protein–peptide interaction assays, starting from directly labeled components (A) to most complex layout with two accessory tags/molecules (D). Picture (e) represents a format mainly used in protein kinase assays in which a biotinylated substrate peptide becomes phosphorylated and is recognized by a phospho-specific antibody

a specific manner (Avi-tag) or through reactive chemistry [26, 27]. The same considerations apply to helper antibodies and streptavidin with attached acceptor or donor molecules. However, this modification is usually done with chemical modification of amino groups. Since antibodies and streptavidin are large and stable proteins with many reactive amino groups (about 80–100 in the case of an mAb), classical chemical labeling remains the main method, since interference with the binding site and/or destabilization are of less relevance. In a typical layout for a PPI assay, the target protein (against which the inhibitors are searched for) is labeled with the donor (lanthanide chelate in case of the widely used TR-FRET) in order to obtain better signal/noise ratios in the read-out. Until recently these reagents were available only with limited reactive groups (as isothiocyanate derivatives for amino group labeling and iodoacetamide derivatives for thiol group labeling). However, lanthanide chelate reagents have been also developed for site-specific attachment with proteins containing a SNAP, CLIP, or Halo-tag from CisBio (<http://www.httf.com/tag-lite-toolbox>). These so-called self-labeling protein tags allow the covalent and irreversible attachment of a large set of labels containing either benzyl-guanine/cytosine moieties reacting with alkyl-guanine/cytosine transferase (SNAP/CLIP tag [28, 29]) or chloro-alkane moieties reacting with haloalkane-dehalogenase (Halo-tag [30]). On the other side, there are many acceptor fluorophores such as Cy5 or various Alexa dyes available with a large variety of reactive chemistries making them amenable for newer site-specific labeling techniques such as for the aforementioned self-labeling tags. In cases where these large fusion tag encompassing around 200 aa might pose a problem for biochemical assays alternative approaches with shorter tags such as the aldehyde tag [31], a trans-glutaminase acceptor tag [10], or non-natural amino acids [32, 33] have been published.

4 Structural Biology

Another cornerstone in industrial drug discovery is structural biology for solving the 3D-structure of isolated drug targets and in-depth elucidation of the binding/active site of targets in complex with chemical compounds or biopharmaceuticals. Especially the latter has become a very important tool for systematic exploration of structure–activity relationship (SAR) in order to optimize hit and lead compounds in medicinal chemistry based on rational and structure-guided design. X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy are the two state-of-the-art technologies for structural investigation of protein–drug interactions. The incorporation of NMR-active isotopes into proteins for 2D-NMR is an interesting field of application for site-specific labeling, in particular using methods of unnatural amino acid

incorporation. The standard method for incorporation is isotope labeling with ^{13}C and/or ^{15}N labeled precursor compounds such as the 20 proteinogenic amino acids in the case of eukaryotic cell culture, or more simple molecules like NH_4Cl and glucose for the metabolically more competent *E. coli* bacteria [34]. Besides labeling all amino acids uniformly, isotope labeling is also performed with selected amino acid residues having distinct spectroscopic properties such as methyl groups of the aliphatic amino acids like Met, Ile, Leu, Val, the aromatic rings of Phe, Tyr or nitrogen atoms of side chains in Trp, His, Lys, and Arg residues [35, 36]. This more selective approach allows monitoring specifically the binding of ligands if one or several of selectively labeled amino acids are located in sufficient proximity of the interaction site on the protein. As an alternative to metabolic labeling, chemical probes with NMR active moieties such as spin labels (unpaired electron in a stabilized radical), ^{19}F bearing molecules, or other chemically distinct amino acids can be incorporated into the protein [37]. Since it is advantageous to attach such NMR active labels in the proximity of the active/binding site, modification at selected residues can for instance be achieved by using incorporation of nonnatural amino acids as described in [38], mutation of suitable residues to cysteine for specific thiol modification [39], or by using the transglutaminase reaction [40].

5 Cellular Assays

Besides looking at isolated and purified protein targets in structural biology and biochemical assays, it is equally important to study them within a more natural environment which is by definition either the whole cell, an isolated organ, or even the whole organism. Additionally, certain important classes of targets such as GPCRs, ion channels, or large protein complexes are very challenging to be produced as isolated proteins and therefore need to be investigated within a cellular context. Visualization and tracking of proteins in response to a stimulus is performed by (immuno-)fluorescence microscopy. In the classical approach, the proteins of interest are detected with specific antibodies and visualized by fluorescent dyes that are attached directly to these antibodies or to secondary antibodies used for detection. With new techniques in microscopy and the introduction of automated acquisition and analysis of images, the so-called high-content screening (HCS) technology was developed. HCS allows the investigation of cellular imaging at medium to high-throughput and is now well established within pharmaceutical R&D as an important tool in profiling and optimization of compounds, secondary screening, and in some cases even for primary screening. In a typical HCS experiment not only the entire appearance of cells in terms of size, morphology, and organelle

distribution, but also the individual fate (expression, degradation, and translocation) of distinct proteins can be investigated at once. For the observation of individual proteins the detection with fluorescently labeled antibodies is still a widely used approach; however, the direct labeling of the target protein is gaining importance as reviewed in [41]. Especially the development of fluorescent proteins based on green fluorescent protein (GFP) with various improved and different properties such as wavelengths for excitation/emission, stability, and reactivity has enabled a lot of new possibilities in cellular imaging [42]. In addition to the genetically encoded auto-fluorescent proteins, other methods for protein labeling in a cellular environment were introduced like the self-labeling enzymes tags (SNAP-tag, Halo-tag) or small cysteine-rich peptide tags such as the FAsH-tag [41, 43]. More recently it was also shown that a mutated version of lipoic acid ligase is able to attach a coumarin-based fluorescent dye on intracellular proteins that contain the corresponding lplA acceptor peptide sequence [44]. Especially the SNAP-tag technology has now become a quite popular alternative to the fluorescent proteins, since it has some advantages such as a greater variety of fluorescent labels with improved properties (sensitivity, stability, wavelengths) or the possibility of labeling of cell surface proteins with non-cell permeable dyes [45]. As a newer development the so-called cooper-free or strain-promoted click chemistry approach becomes interesting for cell-based applications as it utilizes reagent that do not need cooper ions for efficient coupling between azide and alkyne moieties [46] or even more recently trans-cyclooctene and tetra-azine derived labels [47, 48].

6 In Vivo Imaging

Besides imaging of single cells, whole-body imaging (animal or even human) has a very high importance not only in medical diagnostics but also within pharmaceutical research and development. One of the main goals of whole organism imaging is to determine pharmacodynamics and pharmacokinetics aspects of a given drug. This involves answers to the questions where drugs act in the body, how they reach their target, what organs are affected, at which doses side or toxicological effect becomes relevant, how long the drug stays on the target organ, and how fast it is eliminated from the body. Generally molecular imaging techniques are based on radioactive nuclides, like positron emission tomography (PET) with ^{11}C , ^{18}F , or ^{124}I as the most common isotopes and single photon emission computerized tomography (SPECT) using gamma radiation emitting isotopes such as ^{99}Tc or ^{111}In . Nonradioactive methods like optical imaging with fluorescent dyes in the long wavelength range and magnetic resonance imaging (MRI) with contrast enhancing metals (e.g., Gd) are also established [49, 50].

If the drug is a biopharmaceutical, the specific label must be linked stably to the protein during the whole residence time within the organism to avoid unspecific and high background signals caused by dissociated label. The radioactive or nonradioactive metals are normally present as cations and therefore need to be integrated into a stable complex with an organic chelator such as EDTA or similar molecules. For covalent attachment of the metal-chelator complex or the fluorescent dye to the protein of interest usually classical nonspecific modification techniques are used, and as mentioned previously concerns regarding negative effects caused by unspecific labeling are of lower importance. Several radionuclide labeled mAbs currently are commercialized and used especially in diagnostic oncology [51, 52]. However, if the protein of interest is a smaller biomolecule such as an antibody fragment, protein hormone, cytokine, or growth factor, a more selective and controllable conjugation could be desirable for protein preparation with improved stability and activity. Therefore site-specific labeling approaches have also emerged for applications around in vivo imaging as reviewed [53]. For instance selective labeling has been achieved by reaction with engineered thiols [54, 55] or selenocysteine [56]. Further, in vivo imaging has been performed with proteins fused both to SNAP-tag [57] and Halo-tag [58].

7 Biopharmaceuticals

A lot of recombinant endogenous proteins and specific antibodies acting on extracellular targets became available to patients in the last decades. In particular, monoclonal antibodies in the field of oncology and autoimmune diseases such as Herceptin, Avastin, and Humira generate multibillion revenues. While in the beginning most of the proteins were produced in their native form with only cell-derived posttranslational modifications like glycosylation, there was a growing need to obtain chemically modified biopharmaceuticals with improved properties. For instance, rather small proteins with a mass below 30–50 kDa suffer fast clearance from the circulation by excretion through the kidneys. Proteolytic degradation and decreased activity by denaturation or aggregation can also be problematic for proteins used as therapeutics. As a remedy to overcome these shortcomings, it was found that the covalent attachment of hydrophilic polymers such as poly-ethylene-glycol (PEG) to proteins not only resulted in larger molecules with a lower clearance rate but also improved properties in terms of stability, solubility, and overall bioavailability [59, 60]. This modification strategy was also applied to several biopharmaceuticals such as the cytokines interferon alpha 2a or 2b (Pegasys or PEG-Intron) or erythropoietin (Mircera), leading to commercial success. However, these modifications were and are still performed with

classical nonspecific conjugation techniques using reactive amino, thiol, or carbohydrate groups on the surface of the protein, which usually yield an inhomogeneous product in terms of location and number of the attached PEG chains, unless there are only single reactive groups such as thiols present on the protein. Such randomly modified proteins might be problematic in terms of characterization, batch to batch variations, and a decreased potency caused by masked binding sites. Therefore, strategies to incorporate PEG chains in a site-specific manner to biopharmaceutical protein are of a great interest. For instance, a PEG molecule can be conjugated only to the N-terminal amino group of a protein based on its lower pK_a compared to ϵ -amino groups of lysine residues; selectivity is achieved by a careful adaptation of the reaction conditions. This method was successfully established with the commercial product Neulasta through reductive alkylation of the N-terminal amino group with PEG-aldehyde under acidic conditions [61]. Other approaches include the site-directed exchange of lysine residues to arginine residues which preserve the charge and functionality but do not react with PEG reagents as demonstrated for TNF- α [62], or introduction of unique cysteine residues at selected sites of the protein such as interferon alpha 2 [63] or thyroid-stimulating hormone [64]. While these procedures still use classical conjugation chemistry, newer technologies relying on truly targeted modification were also evaluated: For instance PEG was attached to glutamine residues catalyzed by the enzyme transglutaminase [65] or by using the sortase technology [66]. In another example nonnatural amino acids such as azido-homoalanine were incorporated into the polypeptide chain and coupled with alkyne labels by using click chemistry [67].

Another emerging and highly interesting topic of protein modification related to biopharmaceuticals is the so-called antibody-drug conjugates (ADCs) in which cytotoxic drugs are covalently attached to specific antibodies. The purpose of this approach is the selective delivery of cytotoxic compounds to tumor cells without affecting noncancerous cells. Since the antibody part of an ADC typically binds to extracellular proteins nearly exclusively expressed on tumor cells, the ADCs are selectively internalized, followed by release of the attached cytotoxic agent into the cytoplasm and cell killing. The marketed products Adcetris (conjugate between the anti-CD30 mAb brentuximab and monomethyl-auristatin) [68] and Kadcyla (anti-HER2 mAb trastuzumab coupled to a derivative of maytansine) [69] have demonstrated that this strategy is highly promising and many other ADCs are now in several phases of clinical studies [70, 71]. Adcetris and Kadcyla are generated based on traditional reactive chemistry: in the case of Kadcyla, the antibody and cytotoxic drug are linked through a bifunctional reagent with an NHS moiety forming amide bonds with NH₂ groups of the antibody and a maleimide group reacting with a thiol group on the

maytansine derivative. These linkers are non-cleavable and the drug gets released in the cell only by lysosomal digestion of the whole antibody. Other linkers contain cleavable parts: the linker of the ADC Adcetris contains a specific protease cleavage site that is cleaved by a lysosomal protease thus enhancing the liberation of the drug [72]. Other approaches rely on disulfide or hydrazone moieties which are labile against reducing or acidic conditions which occur within the cell [72]. At the present the generation of ADC with classical chemical modification is the most widespread approach since the large size of the antibody might mitigate the potential negative impact of random labeling. Nevertheless, the first ADC (Gemtuzumab ozogamicin; Mylotarg) was removed from the market due to safety and efficacy issues which may have also been caused by the conjugation technique [73]. In order to improve the properties of ADCs approaches for a more directed conjugation such as introduction of additional specifically reactive cysteine residues [74], nonnatural amino acids [75, 76], or the conjugation with the help of transglutaminase [77, 78] have been described, highlighting the potential of site-specific modification in this highly competitive and commercially attractive field.

Another important application for site-specific labeling in the field of biopharmaceuticals is not the direct modification of biopharmaceutical proteins but rather the generation of monoclonal antibodies against pharmacologically relevant targets. Besides the classical hybridoma approach with immunization of mice, the mainly used technology is the selection of specific antibodies with the phage display technology [79]. In this method usually the antigen against which antibodies need to be selected are immobilized onto a solid surface, enabling the binding of well-binding phages and washing away of weakly binding phages in several rounds. Since this immobilization step should preserve the conformation and accessibility of the antigen, there are similar requirements and challenges as in the SPR technologies described in the section above. Hence, either chemical or site-specific biotinylation of the antigen and immobilization on streptavidin-coated surfaces or beads are widely used in phage display selection technique [80, 81].

8 Efforts and Experiences from In-house (Novartis)

8.1 Example 1: *Biotinylation and SPR*

Site-specific biotinylation with the Avi-tag technology was evaluated quite early in Novartis Research labs [4], and since then its use has been continuously expanded and it is now established as a standard technology for various applications like SPR, immobilization for phage display, and biochemical assays. In our department close to 20 proteins from various families and different lengths (full-length vs. single or multiple domains) were used for site-specific labeling using the Avi-tag approach. In our experience we found

this method easy to implement and straightforward in its routine usage. In the beginning we mostly relied on the in vitro labeling approach in which the isolated target protein is incubated with biotin, ATP, and purified biotin ligase (BirA). We have seen, however, that this method was detrimental to some of our target proteins as even these rather mild conditions (incubation at room temperature for several hours and buffers with rather low salt concentration) were too harsh for some proteins. Therefore we invested considerable effort to optimize biotinylation during expression in *E. coli* as described before [4], especially by varying location of Avi-tag (N-terminal vs. C-terminal), concentrations of biotin and arabinose (induction for co-expression of BirA) in the cultivation medium, and optimizing expression times and temperatures during expression. With this optimization a vast majority of different proteins as listed in Table 1 could be biotinylated with an incorporation >95 % as determined by LC-MS. Interestingly the

Table 1

Examples of proteins expressed in *E. coli* and modified by in vivo biotinylation with the Avi-tag method

Protein	Location of Avi-tag	Expression conditions	Biotin incorporation (%)
Bromodomain 1 (15 kDa)	C-terminal N-terminal	200 μ M biotin, 8 mg/ml arabinose, TB, 20 °C ON	>95 40
Bromodomain 2 (15 kDa)	C-terminal N-terminal	400 μ M biotin, 8 mg/ml arabinose, TB, 20 °C ON	65 >95
Bromodomain 2 (16 kDa)	C-terminal N-terminal	400 μ M biotin, 8 mg/ml arabinose, TB, 20 °C ON	>95 >95
Catalytic domain of histone-methyl-transferase 1 (28 kDa)	C-terminal N-terminal	100 μ M biotin, 4 mg/ml arabinose, TB,	>95 >95
Internal fragment of histone-methyl-transferase 2 (7 kDa)	C-terminal N-terminal	200 μ M biotin, 4 mg/ml arabinose, TB, 20 °C ON	80 50
Catalytic domain of protein deacetylase 1 (50 kDa)	C-terminal N-terminal	50 μ M biotin, 2 mg/ml arabinose, TB, 20 °C ON	>95 >95
Hydrolase (65 kDa)	C-terminal N-terminal	100 μ M biotin, 4 mg/ml arabinose, TB, 20 °C ON	>95 >95
Ligand binding domain of nuclear receptor 1 (28 kDa)	N-terminal	100 μ M biotin, 4 mg/ml arabinose, TB, 20 °C ON	90
N-terminal part of E3 ligase (21 kDa)	C-terminal	200 μ M biotin, 2 mg/ml arabinose, LB, 25 °C, 5 h	>95
Ser/Thr kinase (35 kDa)	C-terminal	100 μ M biotin, 4 mg/ml arabinose, LB, 20 °C ON	>95

The expression conditions are described in terms of amount of biotin added to the cultivation medium, arabinose for co-expression of biotin ligase (BirA). TB and LB refer to the cultivation media (terrific broth or Luria broth)

only three cases in which the maximal biotin incorporation reached less than 90 % were observed with small domains or stretches within a protein, whereas it worked very well with larger domains or full-length proteins.

A successful biotinylation via Avi-tag technology is no guarantee for a successful application in an SPR experiment, however. We therefore routinely apply at least two of the three different protein immobilization approaches (amine coupling, minimal chemical biotinylation, and site-specific biotinylation with Avi-tag) with the proteins under study. Examples are provided in Table 2. In our experience, the outcome with the three methods can be quite different, and there is no clear favorite single approach. Sufficient and stable immobilization was achieved in all examples with the selected methods and proteins. However, in terms of binding efficiency

Table 2

Outcome of SPR-based assays with different proteins and immobilization methods, immobilization is referring whether sufficient amounts of the protein could be bound on the surface; outcome of the assay describes whether a useful assay with detection of low molecular weight compounds could be established

Protein	Immobilization approach	Outcome assay
Bromodomain 1 (15 kDa)	Amino-coupling, Avi-tag	Worked well with amino coupling, low binding efficiency with all Avi-tagged variants
Bromodomain 2 (15 kDa)	Amino-coupling, Avi-tag	
Bromodomain 2 (16 kDa)	Amino-coupling, Avi-tag	Results better with Avi-tag than amino-coupling, assay implemented
Ligand binding domain of nuclear receptor 2 (27 kDa)	Avi-tag (in vitro)	Assay implemented and used in SPR pilot studies
Catalytic domain of protein deacetylase 2 (50 kDa)	Avi-tag (in vitro)	Low binding efficiency, no assay implemented
Protease (25 kDa)	Amino coupling, minimal biot	Low binding efficiency, no assay implemented
Ser/Thr kinase 2 (75 kDa)	Amino coupling, minimal biot	Assay implemented and used
Hydrolase (65 kDa)	Avi-tag	Low binding efficiency, no assay implemented
Catalytic domain of Ser/Thr kinase 3 (37 kDa)	Avi-tag (in vitro)	Assay implemented and used
N-terminal part of E3 ligase (21 kDa)	Avi-tag	Assay implemented and used
Catalytic domain of histone-methyltransferase 1 (28 kDa)	Avi-tag, minimal biotinylation	No assay implemented yet, rather low signals
Hydroxylase (45 kDa)	Minimal biotinylation	Assay implemented and used

(percentage of the protein molecules which are still able to bind the ligand after immobilization) and sensitivity, the picture is much more mixed. For instance, with two different bromodomains SPR measurements could only be performed when they were immobilized through random amino coupling, whereas with a third bromodomain or the catalytic domain of Ser/Thr kinase the Avi-tag approach worked perfectly well. In other cases, different immobilization techniques all enabled a good assay, while with still other proteins no useful assay could be developed regardless of the biotin attachment method. The quintessence of these various experiments is that no single “gold standard” immobilization technique exists and the result seems to depend on various properties of the proteins, such as size, charge distribution, accessibility of the binding state, and thermal stability.

8.2 Example 2: Protein Labeling and Protein–Protein Interactions

In our company many protein–protein interactions were subjected to assay development and HTS in order to find modulating compounds and some of them are listed in Table 3. We usually employ pairs of donors and acceptors where one contains a directly attached label, while the other contains one or even two accessory detection partners such as streptavidin or a specific antibody (cf. Fig. 2). In a majority of the examples one of the proteins could be replaced by a short peptide without compromising the binding properties, thus facilitating the assay by the straightforward synthesis and labeling of the peptide during synthesis.

Table 3
Examples of assays that assessed protein–protein interactions in a proximity assay format such as TR-FRET

Protein 1	Label/tag (donor)	Protein/peptide 2	Label/tag (acceptor)
E3-ligase complex	Eu-chelate (NH ₂ groups)	Peptide	Cy5 synthetic
Ubiquitin	Biotin (single Cys)	C-terminal part of E3 ligase 2	Cy5 (NH ₂ groups)
N-terminal part of E3 ligase 1	Biotin (Avi-tag)	Peptide	Cy5 synthetic
Immunoglobulin	Biotin (NH ₂ groups)	Extracellular part of Ig receptor	Alexa647 (NH ₂ groups or carbohydrate)
Ligand binding domains of nuclear receptors	His ₆ -tag	Co-activator derived peptides	Cy5 synthetic
Cytokine	His ₆ -tag	Cognate receptor	Cy5 (NH ₂ groups)
Various bromodomains	His ₆ -tag	Histone-derived peptides	Biotin synthetic

Labeling/detecting whether the donor/acceptor moiety was either conjugated directly to the protein or through an accessory molecule such as an antibody. In case of protein labeling on amino groups either *N*-hydroxy-succinimide-derived fluorescent dyes or isothiocyanate-derived Eu-chelate was used. For peptides the label was attached during synthesis at specific site (usually N-terminal NH₂ group)

Labeling proteins using classical chemical modifications (via lysine, cysteines, or sugar side chains) usually works sufficiently well. Although an optimization of reaction conditions is required in many cases, we have as of now not had any examples in which random chemical labeling did not result in proteins that were functionally active in the TR-FRET assay. Due to a lack of necessity, the use of site-specifically modified proteins in biochemical assays is therefore all in all still quite limited in our in-house drug discovery.

Nonetheless, we have investigated the use of modern labeling methods: In one instance, we biotinylated the N-terminal domain of an E3 ligase with the Avi-tag technology and used it in a setup as shown in Fig. 2b with Eu-chelate streptavidin as the helper molecule. Additionally, a specifically biotinylated protein served as the substrate for a protein kinase in a functional assay. The format of this assay was based on a TR-FRET readout between a Eu-chelate labeled antibody directed against a phosphorylated residue within the protein and streptavidin binding to the biotin on the Avi-tag, and worked.

We have also tested selective labeling of several chemokines with fluorescent dyes with the help of transglutaminase. These specifically labeled chemokines were intended for binding experiments to their receptors (GPCR) present either on intact cells or on membrane preparations. In the case of the chemokine MCP1 the approach worked quite well since just one distinct glutamine residue near the C-terminus of the protein was modified with the label tetra-methyl-rhodamine-cadaverine when transglutaminase from liver extracts was used. In consequence, this modified chemokine showed similar affinities in the binding assay when compared to a scintillation proximity assay format. However, in other chemokines such as IL-8 and SDF1, no glutamine residue was reactive or accessible enough to enable a specific attachment of label to a sufficient extent. We tried to overcome this problem with microbial transglutaminase. However, this enzyme led to highly cross-linked proteins in which several glutamine and lysine residues reacted with each other. As an alternative, we tried to fuse small peptide sequences containing reactive glutamine residues such as the first seven amino acids of substance P to the C-terminus of the chemokine. After extensive optimization of labeling conditions using liver transglutaminase, specific modification was achieved, but cross-linked side products were still obtained. Due to the rather poor predictability and variability of optimal reaction conditions, we abandoned the transglutaminase-catalyzed approach for site-specific modification and pursued other approaches.

One of the alternative methods was the SNAP-tag technology as described earlier [4] with an E2 ubiquitin conjugating enzyme as the target protein. Expression of the fusion protein and site-specific modification on the SNAP-tag moiety worked well, and in

consequence we performed some preliminary TR-FRET experiments with labeled ubiquitin, showing rather low FRET signals. Even though the cause of these modest results could not be explained with the properties of the SNAP-tag we did not see an obvious advantage of this approach in the context of biochemical assays and did not perform further studies.

In the course for exploration of the ubiquitin pathway we attached fluorescent peptides through an isopeptide linkage specifically to the C-terminus of ubiquitin with the help of ubiquitin activating and conjugating enzymes [82]. These specifically modified ubiquitins served then as substrates for deubiquitinating enzymes in fluorescence-based assays. Even though this approach enabled successful assay development and screening it is clearly restricted to ubiquitin and related molecules and cannot be expanded to other classes of proteins.

Most recently we also started to explore the short ACP and LAP tags for selective labeling. These two tags are comparable in size to the well-characterized Avi-tag; thus based on the experiences with Avi-tagged proteins we assume they are not problematic in terms of interference with the properties of the protein under study. Internal efforts to use these tags for specific modification of proteins with fluorescent dyes have been initiated and some first preliminary results show a potential for these novel techniques, especially for the LAP2 technology in conjunction with copper-free click chemistry.

In conclusion, Avi- and SNAP-tag technology worked well in our hands and LAP2 labeling shows promise, while transglutaminase-mediated labeling required excessive optimization due to either nonreactiveness or the formation of covalent protein–protein aggregates. Nonetheless, classical reactive labeling remains the main method applied in biochemical assays at Novartis so far. The main reason for the reluctant use of site-specific labeling methods is that so far the established approaches using labeled accessory molecules have worked well and reliably, reducing the pressure for switching to different methods.

8.3 Example 3: Cellular Imaging

In the field of cellular imaging, the established methods rely on detection through labeled protein-specific antibodies and fusion to fluorescent proteins. These approaches are very reproducible, robust, and also reliable in higher throughput applications, and are therefore also the main approach used by Novartis. In one exploratory study a key member of a signaling pathway was fused to GFP or three self-labeling tags (SNAP, Halo, and FIAsh-tags) in either N- or C-terminal position and compared in terms of labeling efficiency and biological functionality of the fused protein. In our experience the labeling worked equally well for both SNAP and Halo-tags with low background staining, and fluorescence signals were comparable to eGFP. In contrast, fusion proteins with the

short FlaSH tag resulted in significant background signals, most likely caused by nonspecific interaction between Cys-rich sequences of cellular proteins and the biarsenic fluorescent dye. In terms of biological activity as determined by a luciferase-based reporter gene assay there were some differences between the different fusion proteins: Generally if the fluorescent partners such as GFP and SNAP-tag were fused to the N-terminus of the target protein, the signal of the reporter gene assay was significantly higher when compared to an attachment at the C-terminus. Interestingly we observed the opposite behavior with the Halo-tag showing a higher signal when fused to the C-terminus.

8.4 Example 4: Antibodies for Diagnostic Imaging and ADCs

For whole-body imaging the specific labeling of a therapeutic antibody or of antibody fragments like Fab or single-chain Fv was explored with incorporation of a nonnatural amino acid using the PCL approach [14]. For that purpose, different labels such as long wavelength fluorescent dyes and metal chelates were selected. The goal of this study was to evaluate whether specifically labeled antibody and especially the smaller fragments behave more stably and robustly within a whole organism compared to random labeled proteins; however no results are yet available.

In another very recent publication from the Novartis Institute for Functional Genomics, the successful production and preclinical studies of an ADC using a site-specific modification approach have been published [83]. In these experiments, selected peptide sequences serving as substrate for the enzyme phosphopantetheinyl transferase were inserted at defined locations within the Fc part of the well-known mAb Trastuzumab (Herceptin). The most promising Trastuzumab variants were then conjugated with various derivatives of coenzyme A, including some coupled to the cytotoxic drug mono-methyl-auristatin. Well-behaving ADCs with a drug to antibody ratio of about 2 were then tested in cellular assays and in animal xenograft tumor models where they proved to be efficacious. These site-specifically modified ADCs might be a truly valuable alternative to ADCs generated with a nonspecific modification approach.

9 Concluding Remarks

The different previous sections demonstrated that modified proteins play an important role in pharmaceutical research and development in many different areas. Starting from early discovery activities with biochemical assays and biophysical methods, through imaging in cells or whole organisms, to marketed products such as labeled antibodies for diagnostics and antibody–drug conjugates, chemically labeled proteins have become indispensable. At this point in time, it appears that the vast majority of applications using

modified proteins still relies on classical chemical chemistry. Various methods for site-specific labeling have been explored at Novartis, but are not yet broadly applied. The lack of publications from pharmaceutical companies in this area in general indicates that the situation is similar in the industry as a whole. This is most likely due to the time-pressure and result-driven environment of the pharmaceutical industry, where there is a certain reluctance to alter processes unless established methods fail to deliver results. Nonetheless, site-specific labeling is expected to become more important in different applications as methods and applications mature, and new areas are explored where there is a proven advantage of using specifically labeled proteins over randomly labeled protein. In the case of Novartis drug discovery, Avi-tagged based site-specific biotinylation has now become a standard method, as it works very well and has proven superior over chemical biotinylation in various examples. Other, more recent approaches for site-specific labeling of proteins with other moieties than biotin are also being applied, but only occasionally and on an opportunistic basis, as the traditional techniques work reasonably fine. Probably the greatest potential for site-specific modification lies in chemically modified biopharmaceuticals such as PEGylated proteins, antibodies for diagnostic imaging, and especially ADCs, as several examples described above already point in this direction. But also in this commercially highly attractive field, the adaptation of the labeling procedure to a more site-directed approach needs to overcome some hurdles, and a clear benefit over the existing methods in terms of pharmacological properties such as efficacy safety and/or cost still needs to be demonstrated.

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