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Abstract

Immobilization of proteins onto surfaces is of great importance in numerous applications, including protein analysis, drug screening, and medical diagnostics, among others. The success of all these technologies relies on the immobilization technique employed to attach a protein to the corresponding surface. Non-specific physical adsorption or chemical cross-linking with appropriate surfaces results in the immobilization of the protein in random orientations. Site-specific covalent attachment, on the other hand, leads to molecules being arranged in a definite, orderly fashion and allows the use of spacers and linkers to help minimize steric hindrances between the protein and the surface. The present work reviews the latest chemical and biochemical developments for the site-specific covalent attachment of proteins onto solid supports.

Key words:

Protein splicing, protein α-thioester, expressed protein ligation, native chemical ligation, protein micro-arrays, chemoselective ligation, "click" chemistry, Staudinger ligation, site-specific protein derivatization

Introduction

Many experimental approaches in biology and biophysics as well as applications in diagnosis and drug discovery require proteins to be immobilized on solid substrates.[1-6] Immobilized proteins are instrumental in identifying protein–protein,[3] protein–DNA,[7] and protein–small molecule interactions;[8] they can also be used for a variety of diagnostic and profiling purposes.[9,10]

Enormous progress has been made in immobilizing DNA biomolecules onto different types of solid supports.[11] But the immobilization of proteins has been a particularly challenging task, mainly due to the heterogeneous chemical nature of protein surfaces and the marginal stability of the native, active tertiary structure over the denatured, inactive random coil structure.

Most of the available methods for immobilizing proteins onto solid supports have traditionally relied on non-specific adsorption[12,13] or on the reaction of naturally occurring chemical groups within proteins (mainly amines and carboxylic acids) with complementary reactive groups chemically introduced onto the solid support.[13-15] In both cases, the corresponding proteins are attached to the surface in random orientation which may cause the loss of the protein's biological activity.[3] The use of recombinant affinity tags addresses the orientation issue.[2,16-23] However, in most cases the interactions of the tags are reversible and not stable over time.[22,24-27] Site-specific covalent immobilization, on the other hand, allows the proteins to be arranged in a definite, controlled fashion. This process requires the presence of two unique and mutually reactive groups on the protein and the support surface. The reaction between these two groups should be highly chemoselective, thus behaving like a molecular "velcro."[28,29] Also, the reaction should

work efficiently under physiological conditions (i.e., in aqueous buffers around neutral pH) to avoid the denaturation of the protein during the coupling step. Finally, it is desirable that the reactive group on the protein be obtained using recombinant protein expression techniques. The present mini-review examines the latest developments for the site-specific immobilization of functional proteins onto solid supports.

Chemoselective immobilization of functional proteins onto solid supports

Most of the methods suitable for the chemoselective attachment of proteins to surfaces are based on ligation methods originally developed for the synthesis, semi-synthesis, and selective derivatization of proteins by chemical or biochemical means.[30-36] All of these methods involve derivatizing a protein with a unique chemical group at a defined position, which will later react chemoselectively with a complementary group previously introduced into the surface (see Fig. 1).

Surface modification

Silicon, metals (mainly Au and Ag), and semiconductor (i.e., Ag_2S, CdS, and CdSe)-based substrates are among the most common materials used to immobilize proteins in micro- and nano-biotechnology. Si-based substrates are usually modified using derivatized trialkoxysilanes such as (3-aminopropyl)-trialkoxysilane (APS) or (3-mercaptopropyl)-trialkoxysilane, which are able to introduce an amino or thiol group, respectively. These functionalities can be further chemically modified to introduce appropriate linkers where the proteins can be covalently attached in a chemoselective fashion.

Sulfur- and selenium-containing compounds can also be used for the modification of substrates based on several transition metals (Au, Ag and Pt)[37,38] or semiconductor

materials (e.g., Ag_2S, CdS and CdSe).[14] The most studied system, however, uses alkanethiols on gold surfaces. Chemisorption of alkanethiols as well as alkyl disulfides on clean gold gives rise to similar levels of surface coverage, although thiols react faster than disulfides.[37,39] Our group has developed a new efficient solid-phase synthetic scheme (see Fig. 2) for the rapid generation of modified alkanethiols.[40,41] Our group and others have used this approach for the chemical synthesis of different modified alkane thiols that have been successfully used to immobilize different biological functional proteins onto Si-based and Au surfaces.[40-43]

Protein immobilization using Expressed Protein Ligation

Our group has pioneered the use of Expressed Protein Ligation (EPL) for the chemoselective attachment of biologically active proteins to produce protein microarrays[41] (Fig. 3). Key to this approach is the use of recombinantly produced protein α-thioesters, which can be efficiently attached to surfaces containing N-terminal Cys residues through Native Chemical Ligation (NCL, Fig. 3). In this reaction, independently developed by Kent [44] and Tam,[45] two fully unprotected polypeptides, one containing a C-terminal α-thioester group and the other a N-terminal Cys residue, react chemoselectively under neutral aqueous conditions to form a native peptide bond at the ligation site. The initial step in this ligation involves the formation of a thioester-linked intermediate, which is generated by a transthioesterification reaction involving the C-terminal thioester moiety of one fragment and the N-terminal Cys thiol group of the other. This intermediate then spontaneously rearranges to produce a native peptide bond at the ligation site.

We have recently used this process to immobilize several biologically active proteins onto modified glass surfaces through their C-termini.[41] In this work, two fluorescent proteins (EGFP and DsRed) and a SH3 domain protein C-terminal α-thioesters were readily expressed in *E. coli*, using an intein expression system.[46] The α-thioester proteins were then immobilized onto a N-terminal Cys-coated glass slide. The chemical modification of the glass slide was accomplished first by silanization with (3-acryloxypropyl)-trimethoxysilane and then reacting with a mixture of PEGylated thiol linkers **1** and **2**, shown in Fig. 3B, in a molar ratio of 1:5, respectively. Linker **1** contained a protected N-terminal Cys residue for the selective attachment of the α-thioester proteins. Linker **2** was used as a diluent to control the number of reactive sites on the surface. Linker **1** contains a longer PEG moiety than linker **2** to ensure that the reactive Cys groups were readily available to react with the corresponding protein α-thioester in solution. When the derivatization was complete, the corresponding protecting groups of the Cys residue from linker **1** were removed by a brief treatment with trifluoroacetic acid. Typically, the ligation reactions were kept in the dark at room temperature for 36 h, and the minimal protein concentration required for acceptable level of immobilization was found to be ≈50 μM.

Yao and co-workers have also used NCL and EPL, for the selective immobilization of N-terminal Cys-containing polypeptide[47] and proteins[48] onto α-thioester-coated glass slides. In this case, the polypeptide–proteins are site-specifically immobilized through their N-termini, which may be convenient in cases where the C-terminal immobilization, described earlier, affects the activity of the protein.

Protein immobilization using the Staudinger ligation reaction

Azido-containing proteins can be chemoselectively immobilized onto solid supports modified with a suitable phosphine via a modified version of the Staudinger ligation reaction.[30,34,49-52] This reaction allows the formation of an amide bond between an arylphosphine moiety and azide group (Fig. 4). This ligation reaction is highly chemoselective and works with better yields when Z is $–CH_2-$ and X is sulfur (i.e., a thioester function). The reactive arylphosphine derivative can be easily introduced onto carboxylic- or amine-containing containing surfaces.[24,51]

The azido function is not present in any naturally occurring protein. Tirrell, Bertozzi, and co-workers, however, have reported a novel method for incorporation of azido groups into recombinant proteins.[53,54] Unnatural azido-containing aminoacids were incorporated in recombinantly expressed proteins using engineered methionyl-tRNA synthetases in combination with auxotroph *E. coli* strains. This approach, however, is not site specific for proteins containing more than one methionine residue.

An elegant way to overcome this limitation was recently developed by Waldmann and co-workers[51] using *in vitro* EPL for the site-specific introduction of an azido group at the C-terminus of a protein. This approach also allows the incorporation of other functional tags, labels, or reporter groups. More recently, Raines and co-workers[55] have shown that the thioester linkage between a target protein and intein can also be efficiently cleaved by bifunctional hydrazides bearing an azido group. This procedure appends an azido group to the target protein in a single step without the need to use strong reducing conditions.

Protein immobilization using "click" chemistry

The Cu(I)-catalized Huisgen 1,3-dipolar azide-alkyne cycloaddition, also known as "click" chemistry,[35] has also been successfully used to immobilize azido- or alkyne-

containing proteins onto alkyne- or azido-coated surfaces, respectively.[56-58] (see Fig. 5) This cycloaddition reaction appears to be very forgiving and does not require any special precautions. In the presence of Cu(I) as catalyst, the reaction proceeds to completion in 6 to 36 h at ambient temperature in aqueous buffers at pH 7-8. Under these conditions, the cycloaddition is highly regioslective producing the corresponding 1,4-disubstituted tetrazole as the only product (Fig. 5A). It has been found that meanwhile a number of Cu(I) sources can be used directly, the catalyst is better prepared *in situ* by reduction of Cu(II) salts, such as $CuSO_4 \bullet 5H_2O$. Among the different reducing agents that can be used in this cycloaddition, TCEP is one of the most competent reagents for the *in situ* reduction of Cu(II) and has been shown to react only very slowly with aliphatic azides.[36] The site-specific incorporation of an alkyne group at the C-terminus of proteins has also been accomplished using *in vitro* EPL[56] or nucleophilic cleavage of intein fusion proteins with derivatized hydrazines.[55] More recently, Poulter and Distefano have independently reported the use of protein farnesyltransferases (PFTase) for the selective alkylation of C-terminal Cys residues of proteins with farnesyl analogues containing the alkyne function.[57,58] PFTases catalize the alkylation of the thiol function in the Cys located in C-terminal CaaX motifs, where X = Ala, Ser by farnesyl diphosphate. This reaction is general for any soluble protein bearing a C-terminal CaaX motif and works very well for the chemoenzymatic incorporation of alkyne and azido groups in the C-terminus of proteins.

Although this cycloaddition reaction can in principle be used for the chemoselective immobilization of alkyne- or azide-modified proteins onto azide- or alkyne-coated surfaces, respectively, Lin and co-workers[56] have found that alkyne-modified proteins

react more efficiently with azide-coated surfaces. This effect has been attributed to the fact that Cu(I) coordinates with alkynes in solution more rapidly and with higher affinity than with the azide group, thereby enhancing the rate of the cycloaddition reaction with the surface azido group. Using these conditions, the minimal concentration of protein required for acceptable level of immobilization was found to be in the low μM range.[56,57]

Chemoenzymatic methods for the site-specific immobilization of proteins

All the methods described so far rely on pure chemoselective reactions with little or no activation at all. That means that the efficiency of these reactions depends on the concentration of the reagents (i.e., on the concentration of the protein to be attached to the corresponding surface) to bring both reactants close enough to allow them to react in an efficient way.

One way to overcome this intrinsic barrier and make ligation reactions more efficient is to introduce complementary moieties in the protein and the surface, which can form a stable and specific intermolecular complex. Once formed, this complex brings both reactive groups in close proximity, thus increasing the local effective concentration of both reactants.

Protein immobilization using active site-directed capture ligands

The idea of using reactive ligands to capture proteins has been used by Meares and co-workers[59] for creating antibodies with infinite affinity. In this interesting work, the authors created an antibody against a metal-complex ligand, which contained a reactive electrophile close to the binding site. When the antibody and the ligand are apart, their complementary groups do not react, mainly due to the dilution effect. However, when the

antibody specifically binds the ligand, the effective concentration of their complementary groups is greatly increased, leading to the irreversible formation of a covalent bond. Mrksich and co-workers[60] have used this same principle for the selective attachment of proteins onto surfaces with total control over the orientation. They used the protein calmodulin fused with the enzyme cutinase as a capture protein. Cutinase is a 22 kDa serine esterase that is able to form a site-specific covalent adduct with chlorophosphonate ligands.[61] The chlorophosphonate group mimics the tetrahedral transition state of an ester hydrolysis. When it binds specifically to the active site of the enzyme, the hydroxyl group of the catalytic serine residue reacts covalently with the chlorophosphonate to yield a stable covalent adduct that is resistant to hydrolysis.

In this case, the authors use a gold surface to immobilize the cutinase inhibitor. The attachment is extremely selective and can be carried with the whole crude *E. coli* periplasmic lysate containing the cutinase fusion protein.[60] This approach has also been used to prepare antibody arrays on self-assembled monolayers presenting a phosphonate capture ligand. [62]

Walsh and co-workers[26] have also recently reported a very elegant scheme for the chemoenzymatic site-specific modification of proteins. In their approach, the target proteins are expressed as fusions to a peptide-carrier protein (PCP) excised from a nonribosomal peptide synthetase (NRPS). NRPS PCPs are relatively small (8-10 kDa), autonomously folded, compact, and stable domains. These domains contain one specific Ser residue that can be catalytically phosphorylated by the phosphopantetheinyl (Ppant) transferase SFP using CoA (Coenzyme A) as a substrate. Using the Ppant transferase SFP from *B. subtilis*, the authors were able to specifically label proteins with Ppant-biotin

using biotin-CoA as substrate. These biotin-labeled proteins were used to produce protein microarrays onto an avidin-coated glass slide. Johnsson and co-workers[63] have used a similar approach involving the transfer of phosphopantetheine derivatives to a peptide-acyl carrier protein fused to the protein of interest. In a similar way, this approach could be used for the site-specific immobilization of PCP-fusion proteins onto surfaces derivatized by CoA.

Johnsson and co-workers[64] have also developed a novel approach for the site-specific labeling of recombinant proteins using a mutant of human O^6-alkylguanine-DNA alkyltransferase (AGT). This modified enzyme can efficiently transfer a benzyl group to itself when presented with O^6-benzylguanine (BG) derivatives. The mutant enzyme is promiscuous with respect to the substituents appended to the benzyl group, enabling a range of probes to be used for site-specific labeling. The same group has recently reported the use of this active site-directed capture approach for the selective immobilization of different AGT-fusion proteins onto O^6-benzylguanine-coated slides.[65] The fact that the AGTs from *E. coli* and yeast do not react with BG derivatives allows direct immobilization without purification of AGT-fusion proteins expressed in the above microorganisms. It should be noted, however, that for AGT-fusion proteins expressed in mammalian cells, the use of specific inhibitors is required to prevent the unwanted attachment of cognate AGTs. These inhibitors have been designed to inhibit endogenous AGTs without affecting the engineered AGT used for the site-specific attachment.[66]

Protein immobilization by protein trans-splicing

One of the main limitations of site-specific capture methods for site-specific immobilization of proteins is that the linker between the protein of interest and the

surface is always another protein or protein domain. In some cases, the presence of such a large linker could give rise to problems, especially in those applications where the immobilized proteins will be involved in studying protein–protein interactions with complex protein mixtures.[2,67]

To address this problem, our group has developed a new traceless capture ligand approach for the selective immobilization of proteins to surfaces based on the protein trans-splicing process.[42] (Fig. 6) This process is similar to protein splicing[68,69] with the only difference being the intein self-processing domain is split in two fragments (called N-intein and C-intein, respectively).[70,71]

In our approach, the C-intein fragment is covalently immobilized onto a glass surface through a PEGylated-peptide linker while the N-intein fragment is fused to the C-terminus of the protein to be attached to the surface. When both intein fragments interact, they form an active intein domain, which ligates the protein of interest to the surface at the same time the split intein is spliced out into solution (see Fig. 7).

Key to our approach is the use of the naturally split DnaE intein from *Synechocystis sp*. PCC6803.[72] The C- and N-intein fragments of the DnaE intein are able to self-assemble spontaneously (K_d = 0.1-0.2 μM), not requiring any refolding step.[42,73] The DnaE intein-mediated trans-splicing reaction is also very efficient under physiological-like conditions ($\tau_{1/2} \approx 4$ h and trans-splicing yields ranging from 85% to almost quantitative).[42]

Using this strategy, we have successfully immobilized several proteins to chemically modified SiO_2-based substrates. Immobilizing the proteins using protein trans-splicing is highly specific and efficient. It allows the use of protein mixtures and eliminates the need for the purification and/or reconcentration of the proteins prior to the immobilization

step. The required minimum protein concentration for efficient immobilization was estimated to be sub-micromolar.[42] More importantly, once the protein is immobilized to the surface, both intein fragments are spliced out into solution, providing a completely traceless method of attachment. All these features allow this methodology to be easily interfaced with cell-free protein expression systems with rapid access to the high-throughput production of protein chips and other types of biosensors.

Summary

We have reviewed some of the latest developments for the covalent site-specific immobilization of active proteins onto solid supports. The ability to interface active biomolecules such as proteins with solid supports is of great importance for the development of new technologies in biotechnology and biophysics. For example, functional protein microarrays are starting to become a key research tool in proteomics research.[3,4,41,42,74,75] Like DNA microarrays, protein microarrays allow for high-throughput analysis of thousand of proteins simultaneously for rapid characterization of new protein–protein, protein–nucleic acid, and protein–small molecule interactions as well as enzymatic activity and post-translational modifications.[74,76,77]

Other potential applications for site-specific immobilization of protein onto surfaces involve the creation of optimized biosensors.[78,79] An ordered protein film will have a higher activity density that a random protein film, where a significant percentage of the protein molecules may be bound to the surface in potentially inactive conformations. With ordered proteins, biosensors can be miniaturized without losing sensitivity. Also, the combination of recent advances in nano-lithography technique [80] combined with the ability to bind proteins in an extremely ordered fashion allows for the creation of

molecular nanopatterns that can be used to better understand the processes involved in macromolecular assembly.[40,81]

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Figures and Figure Legends

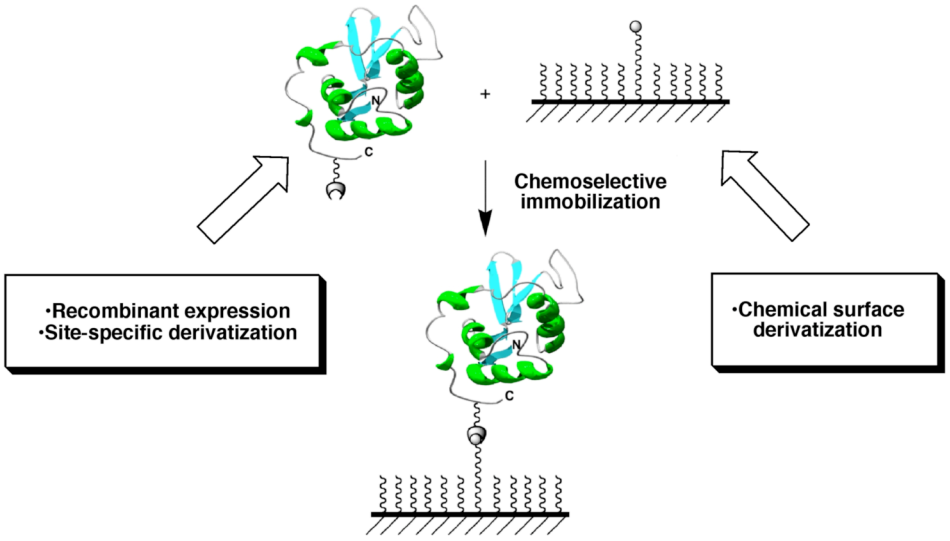

Figure 1. General concept of a chemoselective reaction between a protein and an appropriately modified surface.

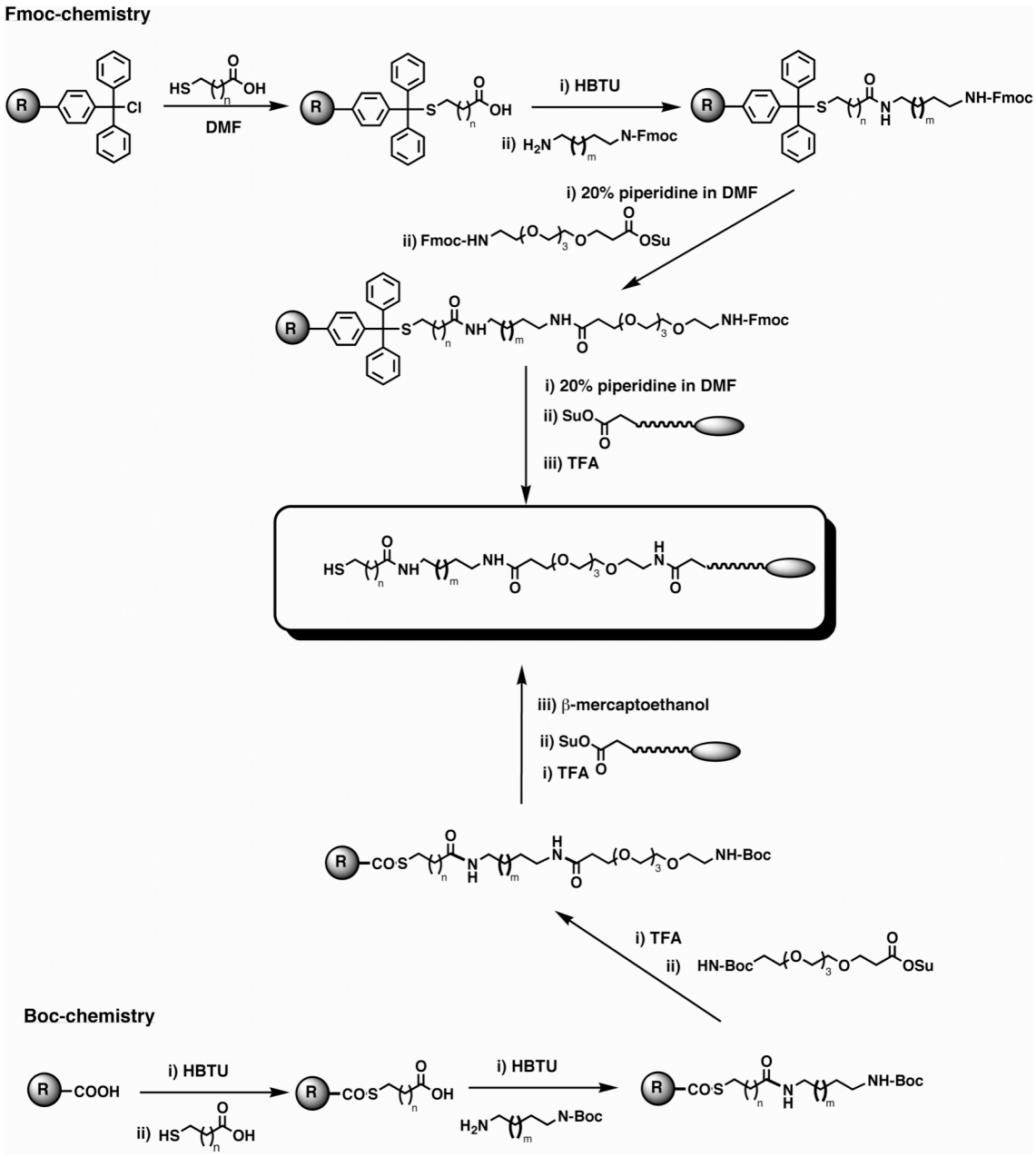

Figure 2. Synthetic scheme for the rapid and efficient preparation of chemically modified thioalkanes.[40-42,81] The solid ellipsoid represents the different molecular functionalities that can be incorporated in the thioalkane for surface modification.

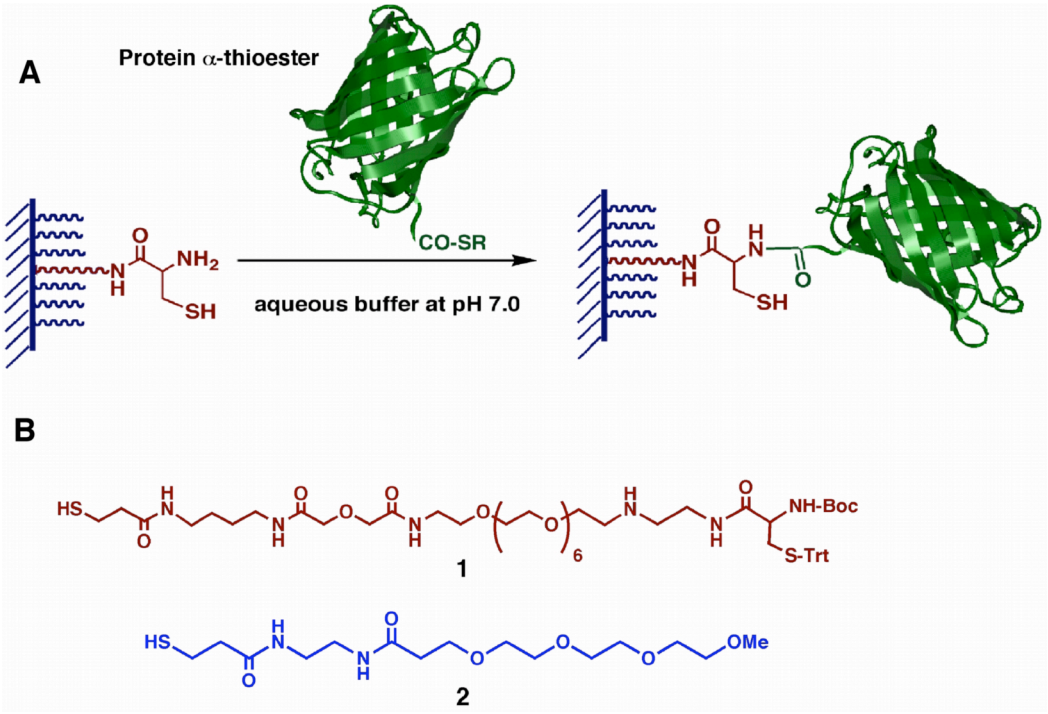

Figure 3. (A) Site-specific attachment of a protein α-thioester through its C-terminus. (B) Pegylated thiol linkers used to attach C-terminal α-thioester proteins onto acryloxy-modified glass surfaces.[41]

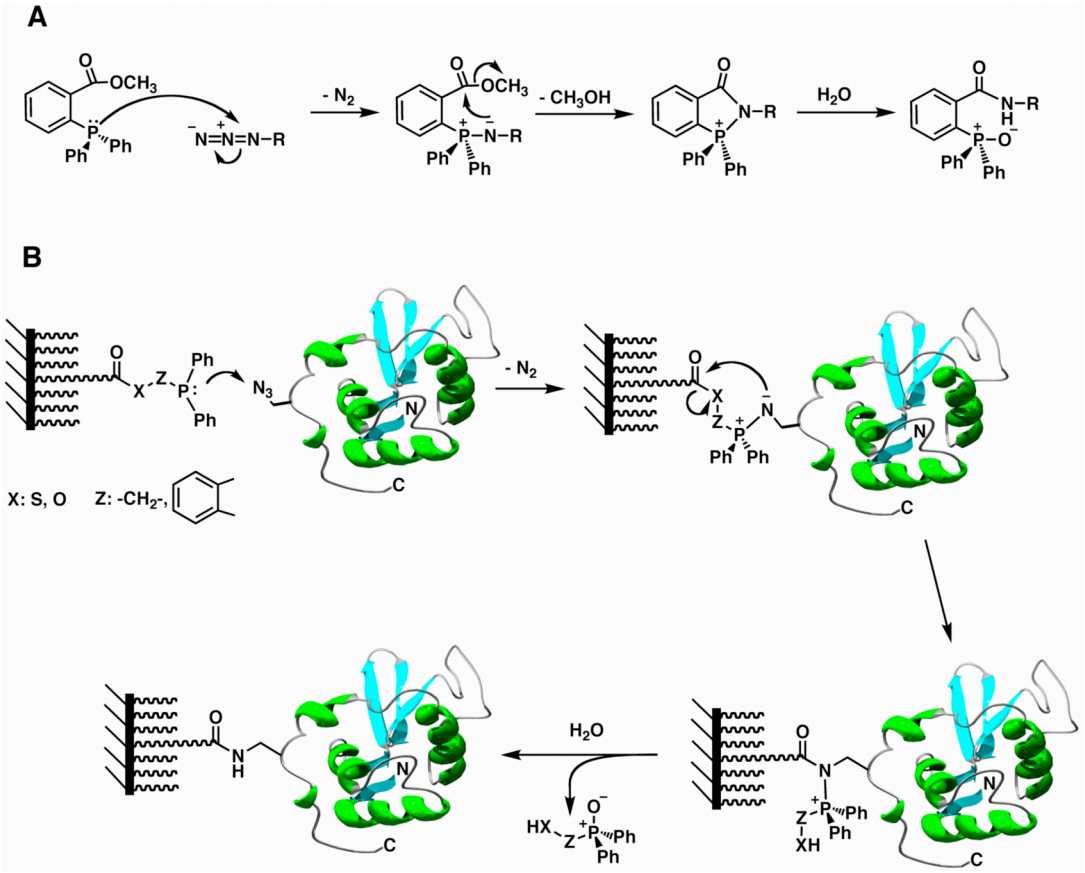

Figure 4. Chemoselective attachment of proteins to surfaces using a modified version of the Staudinger reaction. (A) Proposed mechanism for the Staudinger reaction. (B) Site-specific immobilization of an azide-containing protein onto a solid support using a traceless version of the Staudinger reaction.

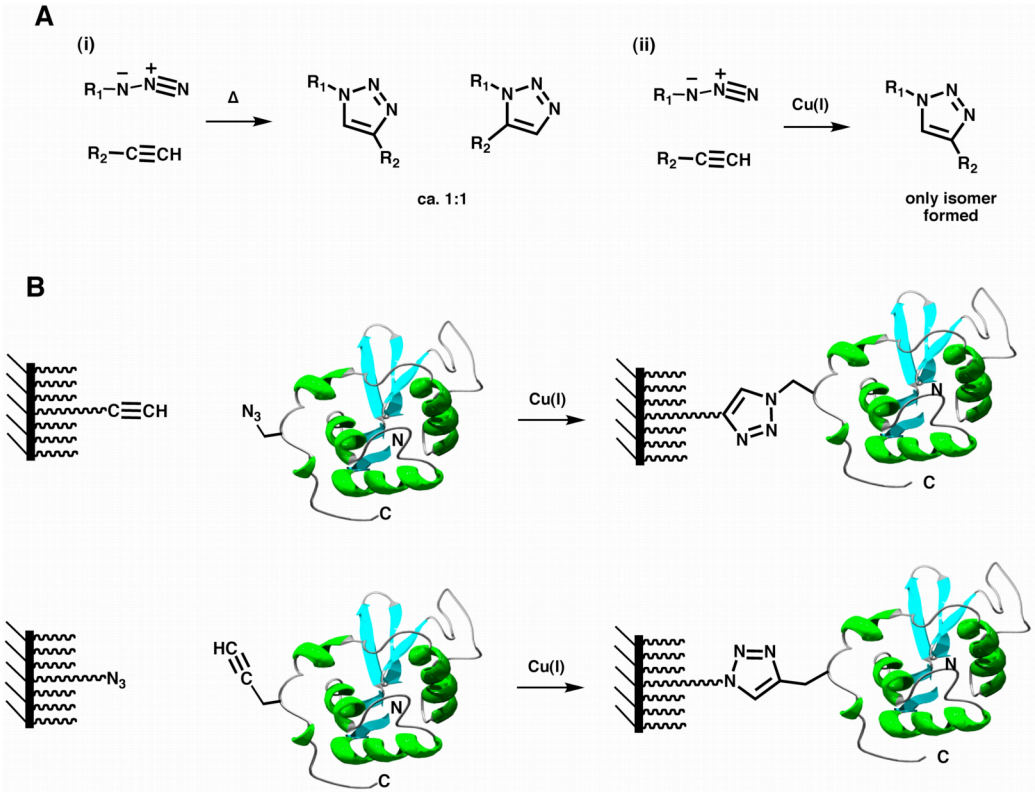

Figure 5. Site-specific immobilization of proteins onto surfaces using the Cu(I) catalyzed Huisgen 1,3-dipolar cycloaddition or "click" chemistry. (A**)** Mechanism and regiospecificity of the Huisgen 1,3-dipolar cycloaddition. In the absence of Cu(I), the reaction requires high temperatures and usually results in a mixture of the 1,4- and 1,5-disubstituted tetrazoles. The addition of Cu(I) as catalyst produces only the 1,5-regiosomer in very mild conditions.[35] **(**B) Immobilization of azide- and alkyne-containing proteins using catalyzed Huisgen cycloaddition.[56-58]

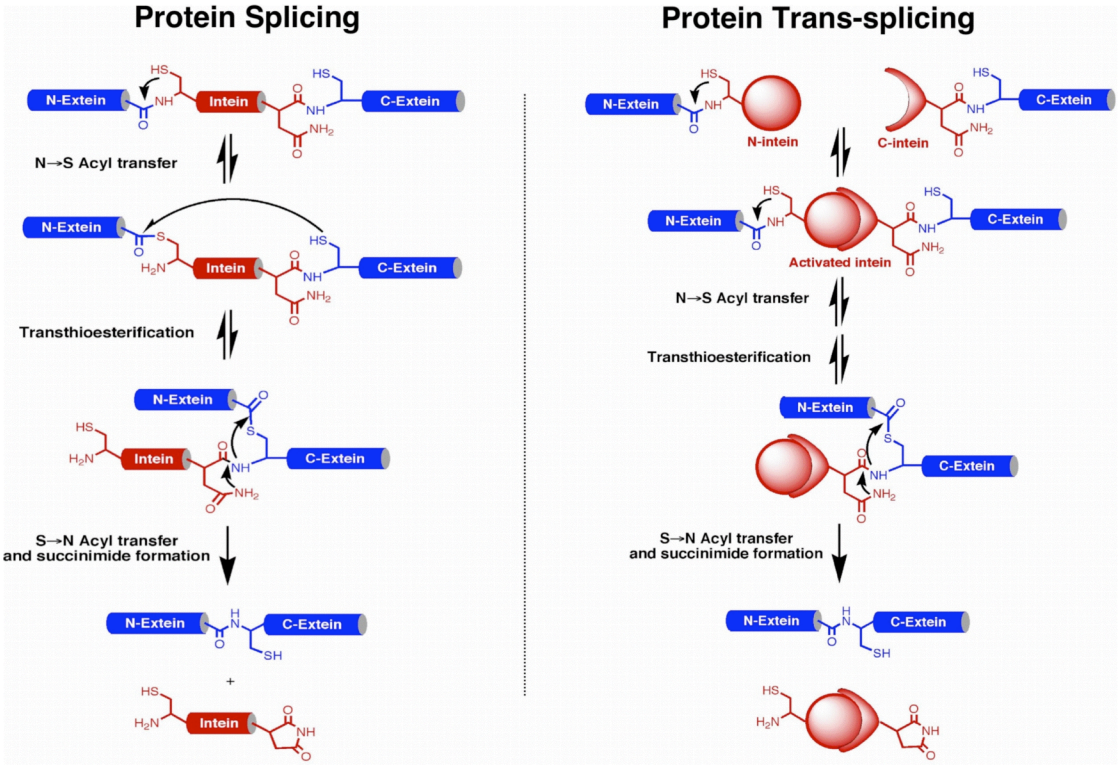

Figure 6. Proposed mechanism for protein splicing in cis (left) and in trans (right).[46,69,82]

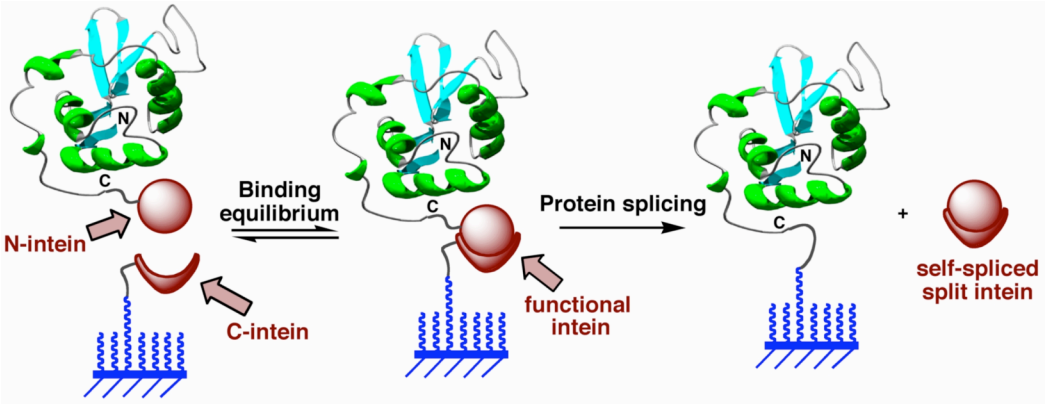

Figure 7. Site-specific immobilization of proteins onto a solid support through protein trans-splicing.[42]

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