Bioconjugate Chemistry

Review

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Protein Engineering For Directed Immobilization

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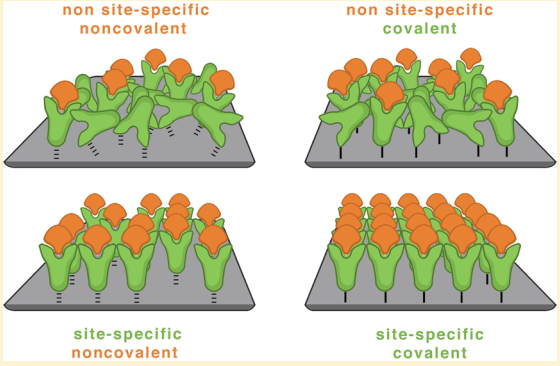
ABSTRACT: Much effort has been put into the optimization of the functional activity of proteins. For biosensors this protein functional optimization will increase the biosensor's sensitivity and/or selectivity. However, the strategy chosen for the immobilization of the proteins to the sensor surface might be equally important for the development of sensor surfaces that are optimally biologically active. Several studies published in recent years show that the oriented immobilization of the bioactive molecules improves the sensor's properties. In this review, we discuss the state of the art of the different protein immobilization strategies that are commonly used today with a special focus on biosensor applications. These strategies include nonspecific immobilization techniques either by physical adsorption, by covalent coupling, or by specific immobilization via site-specifically introduced tags or bio-orthogonal chemistry. The different tags and bio-orthogonal chemistry available and the techniques to site-specifically introduce these groups in proteins are also discussed.



INTRODUCTION

Many biobased applications, including microarray proteome analysis, imaging probes, single molecule studies, biochips, drug screening, biomedical implants, drug delivery systems, and biosensors[1,2] involve protein−protein or protein−ligand interactions. Many of these applications in which biorecognition is desired rely on the immobilization of biologically active molecules.[3] For biohybrid materials, these biomolecules are immobilized on a broad class of substrates ranging from carbon nanotubes,[4] silicon nanowires,[5] semiconducting polymers,[6−8] magnetic nanoparticles,[9] to atomic force microscopy tips.[10,11]

In such materials, the biochemical property or activity of the protein is transferred to the material surface, thereby allowing a selective target interaction. In the context of biosensors, for instance, the aim is to detect target molecules in relevant concentrations and in various matrices, not only in biofluids for medical diagnostics (e.g., blood, plasma, urine, ...) but also in food or environmental matrices (water, soil, air, ...).[12−16] In biosensors, the biochemical receptor molecules are coupled to a transducer surface that translates the target-binding event into a measurable effect such as an electrical signal.[17]

Despite the promising symbiosis between material technology, biochemistry, and biotechnology, it is still not evident to implement biosensor concepts in routine diagnostics. It is crucial that the receptor molecule is bound to the transducer surface in a highly controllable way, so that the orientation is optimal for the specific biomolecule without affecting its activity. This is one of the crucial concerns in order to obtain a selective and sensitive detection in a reproducible way. This optimal orientation is however highly specific for the biomolecule of interest. For the detection of antigens, for example, it is of utmost importance that the binding site of the protein is optimally accessible for the target to be detected (Figure 1). Another example of optimal accessibility is that when subtilisin is immobilized with the active site oriented toward the solution, the enzyme shows a higher catalytic efficiency as compared to subtilisin that is immobilized by a conventional method that leads to a random oriented immobilization.[18] In contrast, other cases require the active center of the protein to be in close contact with the supporting

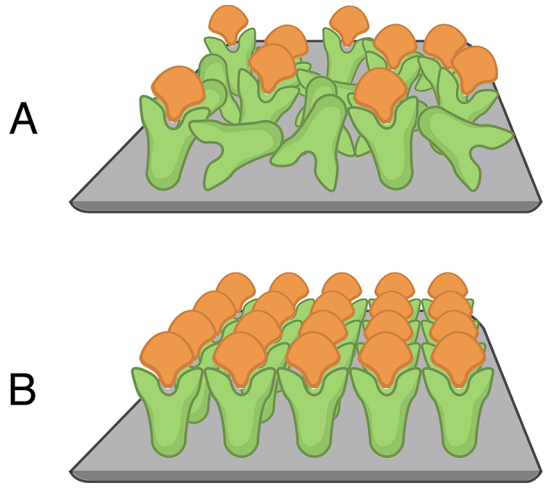


Figure 1. Difference between random (A) and oriented (B) protein immobilization and the influence on antigen binding.

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surface, for example, for optimal electron exchange of redox enzymes with the support. Another example is the immobilization of lipases on hydrophobic substrates. The highly hydrophobic active site of the enzyme will face toward the hydrophobic support which leads to a hyper-activation of the enzyme toward hydrophobic substrates.[19−21]

A second complication for protein immobilization is the need for homogeneously covered substrates. If the surface coating is heterogeneous, the reproducibility and accuracy of the method might be very weak.[4] Heterogeneously covered sensor surfaces may lead to heterogeneous data and can produce false-negatives.[22]

Although this can be averaged out for larger surfaces, it becomes more and more apparent with the current trend to miniaturize bioactive surfaces on the micro- or nanometer scale. With an oriented immobilization of proteins, however, biosensors can be miniaturized without losing sensitivity.[23]

In addition to the protein orientation and homogeneity of the surface, the stability of the coupling of the receptor to the supporting surface may play a crucial role. The chemistry used for the immobilization therefore also has an important role.

In conclusion, controlling the orientation and coupling chemistry of proteins on surfaces is critical for optimal functioning. It is, however, difficult, if not impossible, to formulate a general immobilization strategy, since the method used has to be not only protein, but also application specific.

The coupling of proteins to surfaces can be substantially more difficult than that of their DNA counterparts. This is mainly due to the heterogeneous nature of proteins and the close relationship between spatial structure and functionality and explains the growing need for fundamental knowledge regarding a controlled functionalization and immobilization of proteins onto surfaces in several fields of biotechnology.

The available protein modification strategies can be classified according to various levels of selectivity and difficulty, ranging from nonspecific attachments with low control on protein orientation, reproducibility, and coupling strength (random adsorption), to more sophisticated techniques to create highly structured bioconjugates with high controllability, reproducibility, and stability (site-specific functionalization leading to oriented covalent coupling). The specifications of the final application, i.e., sensitivity (detection limit), stability (shelf life), or cost-efficiency, determine the above-mentioned level that can be employed. In this context, Stephanopoulos and Francis presented an interesting review reporting the influence of experimental circumstances on the selection of an appropriate protein modification strategy, including a simple decision tree that can narrow down the possibilities in many instances.[24]

This review therefore will provide an overview of the currently available techniques and the state of the art of protein immobilization strategies. It is not intended to be exhaustive but rather to guide through the impressive versatility of protein immobilization techniques present today with special focus on the biosensor field.

PROTEIN IMMOBILIZATION STRATEGIES: NON SITE-SPECIFIC

Non Site-Specific, Noncovalent Immobilization. Standard protocols for the immobilization of proteins, including enzymes, are based on noncovalent, nonspecific/selective adsorption protocols.[23,25] The most simple and inexpensive method is by physical adsorption. With this method, proteins are immobilized via weak interactions between the protein and the surface (i.e., hydrogen bonds, electrostatic interactions, hydrophobic interactions, and van der Waals interactions).[26] This technique typically involves simply 'dipping' the substrate in a protein solution. The nonadsorbed proteins are then removed in a washing step.[27] Although physical adsorption offers some advantages (simplicity and possible reusability of the substrate[28]), it however generally leads to a substrate surface coated with proteins in a disordered orientation which can give rise to many disadvantages (conformational changes influencing the proteins activity or reducing the accessibility to its functional sites). The extent of conformational change in the adsorbed state is dependent on the protein (size, intrinsic stability, surface charge, H-bonds, etc.), the type and chemistry of the substrate, the substrate surface hydrophobicity or hydrophilicity, and the coupling conditions such as temperature, pH, and ionic strength of the solution.[29] For example, random physicosorption on hydrophobic polystyrene microplate surfaces is a very common method for protein immobilization for standard enzyme-linked immunosorbent assay (ELISA). Other very frequently used polymer surfaces in protein chips include hydrogels on gold, a feature of Biacore, and other surface plasmon resonance (SPR) technologies.[22]

Besides resulting in a disordered and heterogeneous orientation of the proteins on the substrate surface, an additional drawback is that the adsorbed proteins tend to leach out or be washed away from the substrate[30,31] which poses problems when developing long-life (in situ) biosensors.

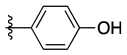
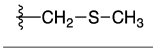
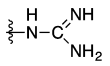
Despite the benefit of simplicity of physical adsorption, the above-mentioned drawbacks may become crucial since the reproducibility of surface orientation and coverage may be very weak. Therefore, although protocols based on physical adsorption work for certain applications, in general they will lead to a partial or even complete loss of activity of the bioactive surface over time.

Non Site-Specific, Covalent Immobilization. A more robust way to create biofunctionalized surfaces is to immobilize the proteins at the surface by means of covalent bonds. This requires the presence of two mutually reactive chemical groups on the protein and on the substrate surface. In addition, the reaction should ideally work under physiological conditions (i.e., aqueous buffers at neutral pH) in order to avoid protein denaturation during the coupling reaction. Most literature methods describing a covalent coupling strategy exploit the reactivity of endogenous functional groups present in the side chains of the amino acids. Table 1 shows a list of some of the reactive groups present in naturally occurring amino acids that can be used for covalent immobilization.

Amines and thiols are both good nucleophiles. Carboxylic acid groups can be activated to make them reactive toward nucleophiles. These chemical groups are therefore most commonly used[31−33] for nonspecific covalent coupling. In these strategies the naturally occurring functional groups are used as such to chemically couple the protein covalently to complementary functional groups present on a surface. Figure 2 shows some commonly used coupling approaches using amine, thiol, and carboxyl chemistry.

Amine Chemistry. The amine groups of proteins are the most used moieties for covalent immobilization. Lysines are present in most proteins, and can make up 6%[34] to over 10%[35] of the overall amino acid sequence and are frequently located on the surface of the protein. Lysines are very reactive toward electrophilic agents without the need to be activated and provide good stability.[30,36,37]

Table 1. Reactive Functional Groups in Naturally Occurring Amino Acids

Reactive group		Amino acid
	Primary amine	N-terminus ε-amino group of Lysine
	Carboxylic acid	C-terminus Glutamic acid Aspartic acid
	Thiol	Cysteine
	Hydroxyl	Serine Threonine
	Phenol	Tyrosine
	Thioether	Methionine
	Imidazole	Histidine
	Guanidino	Arginine

N-Hydroxysuccinimide (NHS) esters are the most commonly used agents to react with protein amine groups, with the formation of stable peptide bonds (Figure 2A). This method has been recently explored by Wang et al.[38] who immobilized ricin (a highly toxic protein that is an inhibitor in protein synthesis) on a gold surface using a NHS ester. Ricin has nine lysine residues on its surface, which, by reacting with NHS, were supposed to determine which side of the ricin structure is in contact with the substrate and which side is exposed to the solution. The authors then visualized single ricin molecules in situ by using an AFM tip modified with an antiricin aptamer (also via NHS chemistry) and observed different conformations due to the covalent coupling of the different lysines. This confirms that variable orientations of the protein on the substrate surface appear when multiple and accessible lysine residues are present in the protein structure.

NHS is also often used as an activating reagent for carboxylic acids. Activated acids (basically esters with a good leaving group) can react with amines to form amides. This approach was examined by Pei et al.[39] In this study, a method for immobilization of antibodies on a carboxyl-functionalized surface has been optimized. To study the effect of activation reagents, the carboxylic acids were activated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), EDC/NHS, or EDC/sulfo-NHS toward the coupling of a monoclonal antimyoglobin antibody at pH 4.0. The results showed that the immobilization was most effective for EDC/sulfo-NHS activation. This difference can be explained by electrostatic attraction versus repulsion forces between the activated substrate and the protein at these reaction conditions. Since the antibody has a pI of 7−8, it will be positively charged at pH 4.0, while the surface charge is positive, neutral, or negative for EDC, NHS, and sulfo-NHS, respectively (Figure 3). EDC esters are however also considered to be rather unstable in aqueous solutions, so deactivation before the introduction of sample is possible (Figure 3A). This study clearly shows that electrostatic attraction can be essential for successful immobilization and that this is highly dependent on the pK_a of the surface functional groups, the protein pI, and the pH of the reaction buffer.

Other chemistries used to immobilize proteins via the exposed amine groups include aldehydes, iso(thio)cyanate, epoxide, sulfonyl chlorides, and cyanogen bromide.[19,26,35,40]

Thiol Chemistry. Cysteines contain a reactive thiol (SH) group. A thiol group (pK_a ∼8 for cysteine) is more nucleophilic than a primary amino group (pK_a ∼10.5 for the side chain amine of lysine), especially at pH below 9.0, at which the amines of lysine are protonated.[41] Free cysteines have a relatively low natural abundance in proteins; they mostly appear in oxidized disulfide bridges. Due to this low abundance, cysteine is an interesting tag for immobilization reactions because 'random' orientations or multiple contact points are less a problem. If no free cysteines are present in the protein, they can relatively easily be inserted at a site of interest by site-directed mutagenesis.

A commonly used chemical group to selectively modify surfaces toward covalent protein coupling via the free thiols is that of maleimides that react stoichiometrically with cysteines (Figure 2B). This coupling reaction is highly specific and efficient. In order to use maleimide-cysteine coupling reactions, the protein must be maintained in a reduced form. To prevent the formation of disulfide bridges and consequent inactivation of the cysteines, reducing agents such as dithiothreitol (DTT) and tris(2-carboxyethyl)phosphine (TCEP) are used. These reducing agents must however be removed before conjugation to avoid competition between the target thiol of the protein and the thiol group of the reducing agent. To prevent reoxidation of the target thiol, the conjugation reaction should be performed immediately after the removal of the reducing agent.[42]

A specific maleimide-cysteine coupling reaction was recently reported by Ménard and co-workers.[43] In this study, a human cytochrome P450 enzyme was immobilized on maleimide functionalized agarose beads and silica microspheres. Using site-directed mutagenesis, 3 of the 4 cysteines were replaced by other amino acids, allowing the enzyme to be site-specifically immobilized on the solid supports through the remaining cysteine. After immobilization, the enzyme was found to still be active.

Another functional group that is used to react with thiols is the vinyl-sulfone group (Figure 2C). This reaction, forming a thioether bond, can be performed in aqueous solutions and under mild conditions.[44] These vinyl-sulfone groups are stable in aqueous solution for extended periods, as they are not subject to hydrolysis at neutral pH, which makes them useful for coupling of thiol-containing proteins in aqueous buffer conditions.[45] However, at higher pH, vinyl-sulfones can also react with amines and hydroxyls, which is a drawback.

Since thiols are known to form disulfide bonds under oxidative conditions, a covalent attachment can also be established to substrates functionalized with disulfide probes via a thiol-disulfide exchange (Figure 2D). This reaction is selective for cysteines, but just like natural disulfide bonds,

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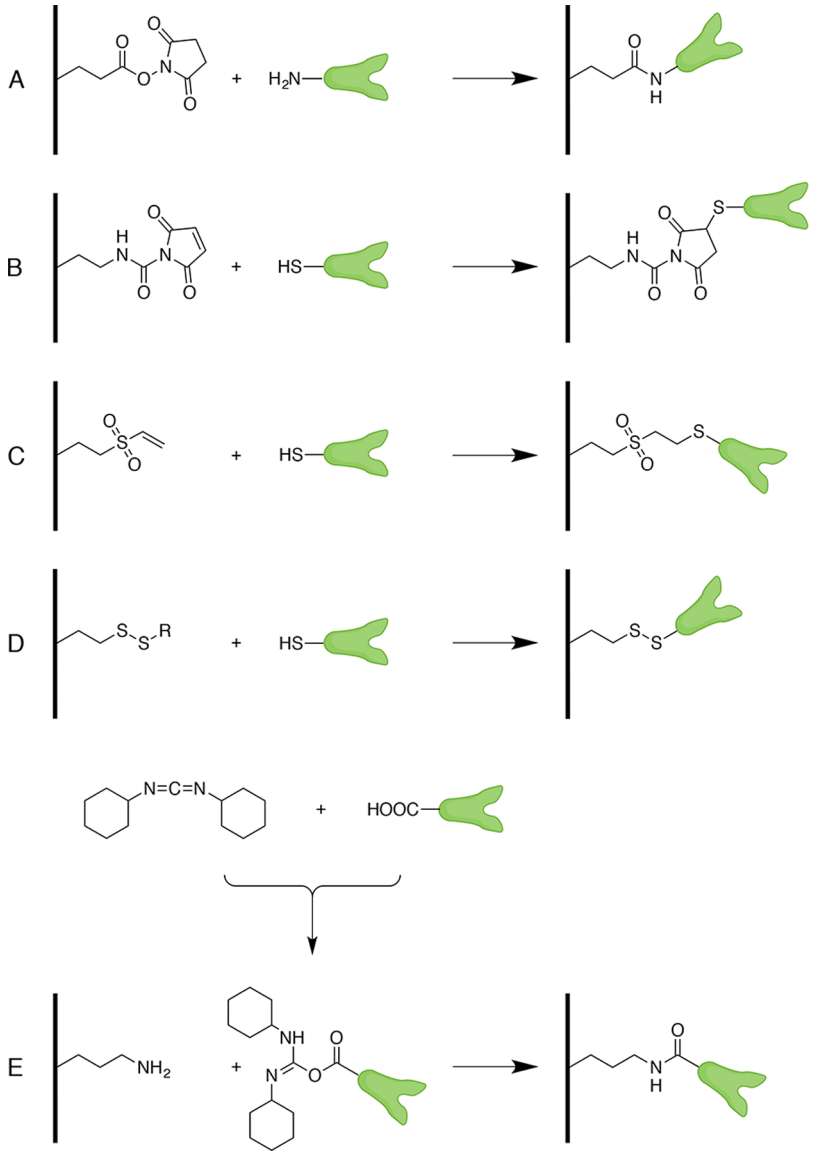

Figure 2. Reactivity of some endogenous amino acid functional groups toward commonly used immobilization chemistry: (A) immobilization via the amine group on N-hydroxysuccinimide (NHS) modified substrates, (B) immobilization via thiols on maleimide, (C) immobilization via vinyl sulfone, (D) immobilization via disulfide-modified substrates, (E) immobilization via *N,N′*-dicyclohexyl carbodiimide-activated (DCC) carboxylic acid groups on amine-modified substrates.

these couplings are not resistant to reducing agents like mercaptoethanol or DTT.[41]

Other strategies that can be used to immobilize proteins via thiol functions include reactions with haloacetamides or with a photochemically promoted thiol−ene reaction (see Thiol−Ene section).[40,41]

Carboxyl Chemistry. The immobilization of proteins using the carboxylic side chains is interesting since the amino acids glutamic and aspartic acid constitute a major fraction of the surface exposed amino acids.[26] The carboxylic acid function of these amino acids, along with the C-terminus, can react with amines using the routine coupling chemistry also used for solid phase peptide synthesis. This coupling reaction is activated by a carbodiimide like *N,N′*-dicyclohexyl carbodiimide (DCC) or EDC and results in a rapid and quantitative formation of a peptide bond (Figure 2E).

Other Chemistries: Tyrosine and Tryptophan. Although amines, thiols, and carboxylic acid groups are the most common targets for protein modification and immobilization, research has also been done for other amino acids. Tyrosines are relatively rare on protein surfaces and can be genetically introduced without changing the overall charge state or redox sensitivity.[46] Tyrosines, however, are often overrepresented near active sites of proteins.[34] Targeting tyrosine for modification or immobilization therefore needs careful consideration.

An interesting tyrosine approach was recently reported by the group of Carlos Barbas.[47] The authors developed a method that uses the reactivity of tyrosine with certain diazodicarboxylate-related molecules to create a rapid aqueous ene-type reaction, named tyrosine-click reaction (Figure 4A). To study the potential of this tyrosine-click reaction for protein modification, it was used to selectively PEGylate chymotrypsinogen and to create a novel antibody−drug conjugate. It was found that the tyrosine click linkage demonstrated stability to extremes of pH and temperature, indicating that this linkage is

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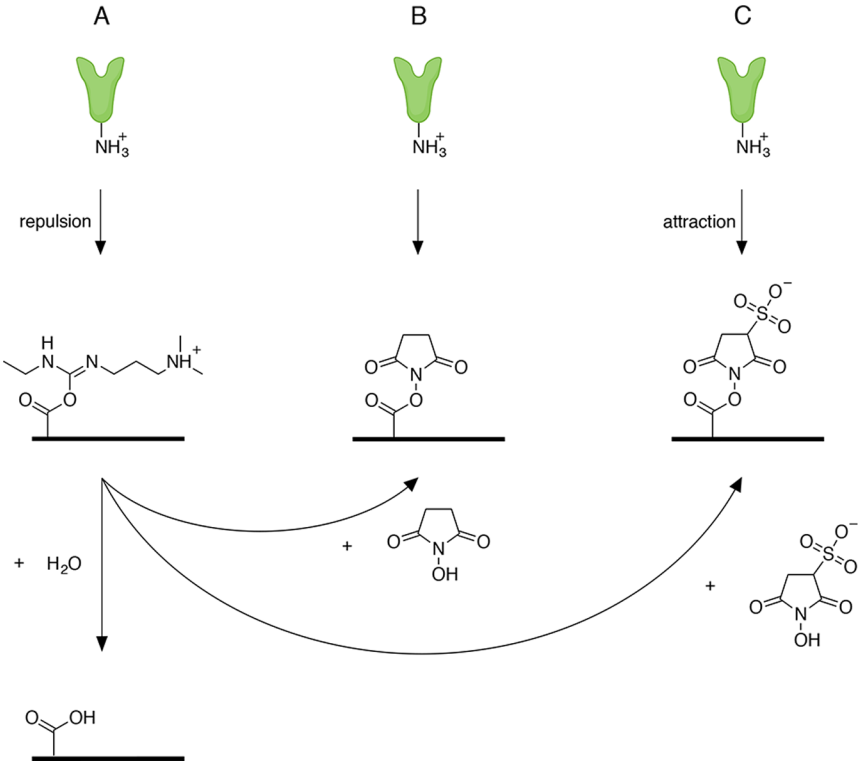

Figure 3. Example of the electrostatic effect for different carboxylic acid activation methods. (A) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), (B) EDC/NHS, or (C) EDC/sulfo-NHS on the binding of a monoclonal antimyoglobin antibody at pH 4.0.[39]

significantly more robust than maleimide-type linkages that are commonly employed in bioconjugations.[48]

Tyrosine has also been targeted for protein conjugation by palladium-catalyzed π-allylation (Figure 4B),[40,49,50] by a three-component Mannich-type coupling reaction with aldehyde and aniline reagents (Figure 4C),[46,51,52] and by a nickel(II)-mediated radical coupling with magnesium monoperoxyphthalate as an oxidant (Figure 4D).[53,54]

Being the rarest amino acid in proteins, tryptophan might also be an attractive target for site-specific modification and immobilization. The residue-specific modification of the indole side-group of tryptophan residues has been done through the in situ generation of rhodium carbenoid reagents resulting in alkylated indoles (Figure 4E).[50,55] This reaction, however, requires acidic (pH 1.5−3.5) conditions which may have undesirable affects on the protein's structure and function.[34,40]

Because proteins usually contain multiple copies of a specific amino acid, immobilization therefore theoretically occurs in multiple orientations resulting in what is classically called random immobilization. This can be circumvented by creating amino acid-specific depletion mutants in which all but one amino acid of a specific type are removed (see in Thiol Chemistry for cysteine depletion). Of course this can have major implications on the protein structure and function, and this approach should be considered on a protein-to-protein basis.

However, immobilization methods based on endogenous amino acid chemistry are not necessarily entirely random but can result in surface coatings in which the majority of the proteins have a similar or even unique orientation. This is illustrated by Fuentes et al.[56] who immobilized horseradish peroxidase (HRP) on agarose which was functionalized with glyoxyl, glutaraldehyde, or cyanogen bromide (BrCN), functional groups that can react with the present amino groups in the protein.

Glyoxyl-agarose has a dense layer of linear aldehyde groups, which reacts with primary amino groups in the protein[36,56] to form a Schiff's base.[57] This Schiff's base has to be reduced to transform them into stable covalent bonds. This immobilization requires a multipoint immobilization at pH 10.0.

In glutaraldehyde-agarose, the primary amine groups react with the aldehydes present on the agarose. When performed at neutral pH values (pH 7.0−8.5) only the terminal amino group will preferentially react and multipoint covalent immobilization may not be very high.[58]

Cyanogen bromide immobilizes proteins at neutral pH values via the amino terminal.[36] When these different HRP-immobilized surfaces were incubated with polyclonal anti-HRP, it was found that different amounts of antibody were bound to the HRP surface. The results showed that, when a protein is immobilized following a certain protocol, the protein could interact with some antibodies, but not with others. If conventional amine immobilization would produce a purely random orientation of HRP on the substrate, the clear difference in antibody binding between the different immobilization protocols would be difficult to explain. This indicates that the different NH_2 immobilization protocols immobilize proteins through specific regions. In other words, the orientation is determined by the reaction procedure and conditions.[19] Although the ε-amino group of lysine is probably the most abundant primary amino group at the surface of a protein, only nonprotonated amino groups will be nucleophilic enough to react. The pK_a of a surface exposed ε-amine group of lysine is typically that of the free amino acid, around 10.5, while the terminal amine group has a pK_a value of 7−8.[19,36] Although there may be other lysine residues with altered pK_a values

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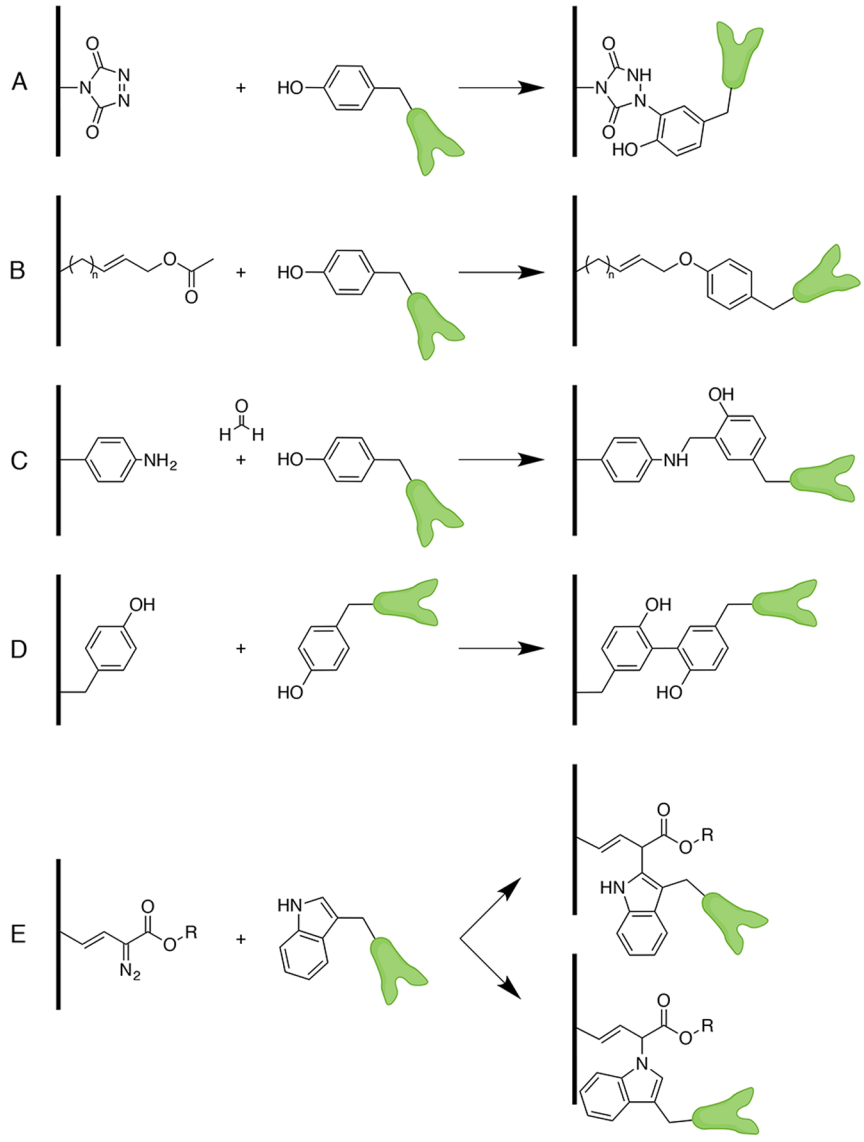

Figure 4. Reactivity of tyrosine and tryptophan toward commonly used immobilization chemistry. Tyrosine can be immobilized by (A) a tyrosine-click reaction between diazodicarboxylate molecules and tyrosine, (B) palladium-catalyzed π-allylation, (C) a three-component Mannich-type coupling reaction with aldehyde and aniline reagents, (D) reaction by a nickel(II)-mediated radical coupling with magnesium monoperoxyphthalate as an oxidant. Tryptophan can be immobilized by (E) a reaction resulting in alkylated indoles via in situ generation of rhodium carbenoid reagents.

present, these are likely to be located at internal pockets in microenvironments not accessible for immobilization reactions.[36] This means that at neutral pH, the reactivity of the terminal amine group will be significantly higher than the reactivity of the protonated ε-amine group and proteins will first be immobilized with this terminal amine group, provided it is accessible.

To conclude, several endogenous chemical groups of the amino acids can be used to covalently immobilize proteins on surfaces. Because proteins in general possess multiple copies of the amino acids, these chemistries are not unique in the protein, and as a result, multiple protein orientations can occur at the substrate surface. Although this is not a site-directed and controlled immobilization, it still can result in a quite homogeneous orientation of the proteins on the substrate that may be sufficient for many applications. This orientation however may be very hard to predict or to alter if it seems not to be the optimal orientation. More site-directed immobilization techniques that result in a controllable and homogeneous orientation of the proteins may therefore be very advantageous for applications in which sensitivity and reproducibility are an important issue.

PROTEIN IMMOBILIZATION STRATEGIES: SITE-SPECIFIC

Because of the difficulty controlling protein immobilization when purely relying on the endogenous functional groups, much attention has gone to the development of alternative strategies to generate homogeneous and reproducible substrate surfaces in a controllable way. This site-specific immobilization approach can also be divided in noncovalent and covalent strategies.

Site-Specific, Noncovalent Immobilization. Several noncovalent approaches exist to immobilize proteins to substrate surfaces in an oriented way. Most of these approaches are based on complementary affinity interactions between biomolecules. Affinity immobilization techniques exploit the selectivity of specific interactions, which occur in almost all

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biological processes: between antibodies and antigens, lectins and free saccharidic chains or glycosylated macromolecules, nucleic acids and nucleic acid-binding proteins, hormones and their receptors, avidin and biotin, polyhistidine and metal ions, etc.[59] Due to the high selectivity of these interactions, it is possible to control the protein alignment on substrates if the latter is functionalized with the respective counterpart and the selective biological tag is site-specifically introduced in the protein. One way of introducing a biological tag in a protein is accomplished by recombinant techniques. This, of course, requires genetic engineering of the protein of interest.

Polyhistidine. One commonly used affinity tag is the polyhistidine or His-tag. This method is based on the interaction of histidine or histidine-rich regions in a protein with divalent metal ions (Ni^{2+}, Cu^{2+}, Zn^{2+}). The strategy is inspired by immobilized metal ion affinity chromatography (IMAC).[60] The polyhistidine tag is recombinantly added to the C- or N-terminus of the protein of interest. When incubated on metal treated substrates (through a chelating agent like nitrilotriacetic acid (NTA)) the polyhistidine-tail of the protein selectively interacts with the metal. This interaction is reversible upon addition of competitive ligands like imidazole or histidine or by metal chelators like ethylenediaminetetraacetic acid (EDTA). This reversibility can be an advantage for certain applications where reusability of the substrate is important, but can be disadvantageous if stability and storage time are an issue. An example of a recent application of a His-tagged protein for the development of biosensors was reported by Ganesana et al.[61] This paper describes site-specific affinity immobilization of $(His)_6$-tagged acetylcholinesterase onto Ni/NiO nanoparticles for the development of an electrochemical screen-printed biosensor for the detection of organophosphate pesticides. Recently this affinity based method has also been applied to immobilize proteins on silicon nanowires for the development of field effect transistor (FET) based biosensors[5] or for immobilization of horse heart Cytochrome c on gold electrode surfaces for biosensing devices where direct enzymatic charge transfer is required between the electrode and enzyme.[62]

Peptide Epitope Tags. An epitope is a portion of an antigen molecule that is recognized by an antibody. Although epitopes can in principle be composed of any type of molecule, most epitope tags are constructed of short peptides. Using recombinant techniques, the genetic sequences coding for the peptide epitopes that are recognized by common antibodies can be fused to the gene of the protein of interest. An epitope tag could be placed anywhere within the protein, but typically they are placed on either the N- or the C-terminus.

Although theoretically any short stretch of amino acids known to bind an antibody could become an epitope tag, there are a few commonly used epitopes.

A FLAG-tag is an affinity tag consisting of the short, highly charged and therefore soluble octapeptide Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys.[63] Commercial vectors are available in which this tag can be genetically fused to the N- or the C-terminus of the target protein.[64] The FLAG peptide includes the binding site to several highly specific anti-FLAG poly- and monoclonal antibodies. Due to its highly hydrophilic character, the FLAG peptide is likely to be located on the surface of the fusion protein and is therefore of interest for oriented immobilization. This tag was for example used to immobilize subtilisin by its C-terminus to nonporous polystyrene and silica beads coated with protein A and conjugated with monoclonal anti-FLAG antibody. It was found that the site-specifically immobilized subtilisin not only had higher enzymatic activity than its randomly immobilized counterpart, but also showed longer storage stability.[65]

The myc-tag, derived from the c-myc gene, is an epitope of eleven amino acids (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-Asn) and an anti-c-myc antibody was developed in 1985.[66] The tag sequence can be genetically fused to either the N- or C-terminus of target proteins without the loss of its affinity toward anti-myc antibody.[67] It has been widely used for protein detection, as immunochemical reagent in cell biology, and in protein engineering as well as for protein purification.[67,68] The c-myc-tag has also been used for protein immobilization. Wingren et al. reported the proof-of-principle for a protein microarray design based on myc-tagged single-chain antibody fragments. The probes were immobilized via engineered C-terminal myc-tags to prearrayed monoclonal antitag antibodies. The results showed that specific and sensitive antibody microarrays were obtained.[69]

Other epitope tags are the HA-tag (a nonapeptide tag Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala derived from an immunogenic peptide from influenza virus hemagglutinin (HA)[70,71]) and the V5-tag (the 14 amino acid tag Gly-Lys-Pro-Ile-Pro-Asn-Pro-Leu-Leu-Gly-Leu-Asp-Ser-Thr derived from the C-terminus of the P and V proteins of Simian virus 5).[72,73]

Biotin−(Strept)Avidin Interaction. Another well-established affinity based immobilization technique is based on the strong interaction between (strept)avidin and biotin, and biotinylated proteins. This method was used to immobilize a biotinylated aldo/keto reductase on streptavidin-coated templates.[74] The activity of the site-specifically immobilized enzyme was comparable to that of the wild-type enzyme in solution and 60- to 300-fold greater than that of the randomly immobilized reductase. Furthermore, the enzyme was found to be surprisingly stable, showing no loss of activity for over a week, and even after 50 days, more than 35% of activity was maintained. Similar observations were recently done by Yu et al.[9] who immobilized a membrane-bound sialyltransferase via a biotin-labeled cysteine at the C-terminus on streptavidin-functionalized magnetic nanoparticles (MNPs). Using the streptavidin−biotin interaction, it was possible to immobilize the enzyme under mild ligation conditions and with an approximately 2-fold increase in activity as compared to other immobilization methods.

DNA-Mediated. Conjugation of proteins with artificial nucleic acids allows them to be modified with a robust tag. Protein−DNA conjugates have been reported for affinity based directed immobilization of proteins.[75,76] For instance, the study performed by Fruk et al. describes the DNA-directed immobilization of horseradish peroxidase (HRP) on electrochemically active surfaces. Kinetic analyses of the peroxidase activity of the HRP-DNA conjugates revealed substantial enzymatic HRP activity, and the electrodes modified by specific DNA hybridization of the HRP conjugates with electrode-bound capture oligomers yielded highly active devices.

The DNA affinity approach is interesting due to the ease of sequence design and preparation in combination with the very high specificity of Watson−Crick base pairing. Although many methods exist to prepare DNA−protein conjugates,[77] there is still a need for site-specific coupling of the DNA to the protein.

Other Affinity Based Methods. Other affinity based immobilization tags include binding of Glutathione-S-transferase (GST) to glutathione, binding of antibodies to protein A or protein G via the Fc-region, maltose-binding protein with

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maltose, chitin-binding protein with chitin, or calmodulin with its phenothiazine ligand.[19,29,59,78]

Some applications can benefit from a noncovalent immobilization due to the possible regeneration and reuse of the sensor surfaces. For other applications though, the noncovalent character of the immobilization approach through affinity tags can be a major disadvantage. However, besides directed noncovalent coupling, affinity tags can also be used in a two-step process in which the protein is first directed to and orientated on the surface by the affinity tag, after which a covalent coupling takes place between the protein and the surface resulting in an oriented and covalent coupling.[79]

Site-Specific, Covalent Immobilization. In order to have an oriented and covalent coupling of a protein to a substrate in a controllable way, a unique chemical group or sequence has to be introduced in the protein at a site-specific location. When relying on endogenous amino acids, this means that the target amino acid for the coupling reaction is uniquely present on the surface of the protein. However, coupling reactions are often performed in complex mixtures of biomolecules, possibly also bearing this amino acid and as a consequence this may lead to unwanted proteins coupled to the surface.

The presence of a single and site-specific functional group in the protein would make it possible to selectively couple the protein to a mutually reactive group on the surface. To be unique in the protein, the functionality should ideally be bioorthogonal, i.e., not appearing in, or cross-reacting with, the endogenous amino acids. When such a bioorthogonal group is introduced at a site-selective position in the protein, i.e., at a strategically chosen location that has a minimal influence on the conformation of the target-binding site, one is able to create a unique chemical 'handle' to covalently bind the protein to the complementary functionalized substrate surface without interfering with the protein's activity.

Several bioorthogonal chemistries are reported that can lead to an oriented covalent immobilization of proteins. In this context, the so-called 'click' reactions have become exceedingly popular in both academic and industrial research. The 'click' chemistry concept[80] consists of an ideal set of efficient and highly selective chemical reactions in organic chemistry. The characteristics of a 'click' reaction in general include mild reaction conditions, insensitivity to oxygen and water, the ability to use water as reaction medium, and the formation of a stable product under physiological conditions. In recent years a 'click' toolbox has been developed with chemistries useful for protein modification. This toolbox includes, for example, copper or ring-strain catalyzed azide–alkyne cycloadditions, Staudinger-ligations, Diels–Alder cycloadditions, thiol–ene additions, and oxime formation[81] (Figure 5).

Cycloaddition. The reaction of terminal or internal alkynes with organic azides, both examples of bioorthogonal groups, results in a 1,2,3-triazole. This reaction has been known for more than a century, being first performed in 1893 by A. Michael between phenyl azide and diethyl acetylene-dicarboxylate.[82] The copper-catalyzed Huisgen 1,3-dipolar cycloaddition of azides and alkynes,[83,84] is an archetypal example of 'click' chemistry (Figure 5A). Several Cu(I) sources can be used directly, but in many protocols the catalyst is prepared in situ by reduction of Cu(II) salts by, for example, TCEP or ascorbic acid.

Unfortunately, the mandatory copper catalyst can be toxic to cells and may cause proteins to precipitate, reducing the overall applicability of this chemistry for protein modification.[85,86]

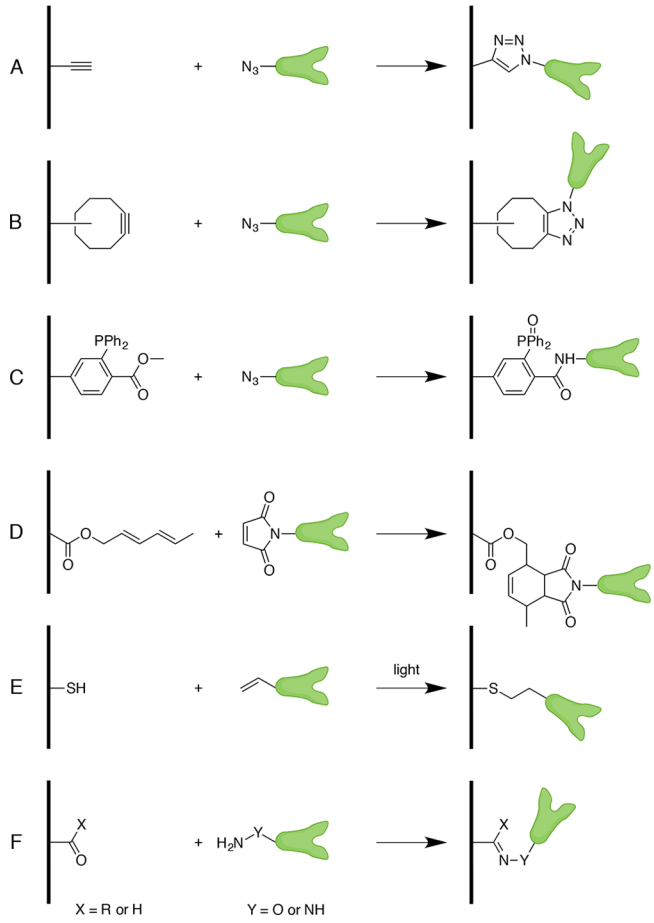

Figure 5. Bioorthogonal chemistries used for protein immobilization: (A) copper catalyzed cycloaddition, (B) ring-strain catalyzed cycloaddition, (C) modified Staudinger ligation, (D) Diels–Alder cycloaddition, (E) thiol–ene additions, (F) oxime ligation.

However, a copper-free Huisgen cycloaddition has been reported by Bertozzi and co-workers.[87,88] This reaction uses the release of ring strain energy of a cyclooctyne group to enable the Huisgen 1,3-dipolar azide–alkyne cycloaddition to proceed rapidly without the need of an additional catalyst (Figure 5B).

Since 'click' chemistry has been shown to work for protein modifications,[89] efficient methods for the controlled introduction of azides or alkynes into biomolecules have become of great interest. Both azides and alkynes are introduced in proteins without affecting the protein structure and function.

Staudinger Ligation. An azide is a particularly interesting chemical group. Besides being bioorthogonal, this functional group is relatively small, explaining why an appropriate reactive partner for azides, beside alkynes, was greatly wanted. In the Staudinger reaction azides react with triphenylphosphines to produce an aza-ylide.[90] In the presence of water, however, this intermediate hydrolyzes spontaneously to yield a primary amine and the corresponding phosphine oxide. A modified Staudinger ligation was introduced by the group of Bertozzi (Figure 5C).[91] In this type of Staudinger reaction, an ester group is strategically placed on one of the phosphine's aryl substituents, resulting in a stable amide bond, rather than the amine product of aza-ylide hydrolysis.

Diels–Alder. Diels–Alder cycloaddition usually takes place between an electronically matched pair of a dienophile and a

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conjugated diene to form a six-membered unsaturated ring structure (Figure 5D). Diels–Alder reactions proceed in water at room temperature with a higher rate and selectivity than in organic solvents. For this reason, this chemistry has been proposed for the immobilization of proteins.[26]

Thiol–Ene. Just like the reaction between alkynes and azides, the highly efficient reactions of thiols with reactive carbon–carbon double bonds were already described in the early 1900s.[92,93] The term 'thiol–ene' denotes the addition of a thiol to an '-ene' group via a free radical mechanism upon initiation either by a radical mechanism or by light (Figure 5E). This type of reaction was predominantly employed in polymer synthesis and materials science, but in recent years, the thiol–ene reaction has attracted researchers from other fields because of the biocompatible 'click' chemistry characteristics.[94] Besides the 'click' characteristics, the photoactivatable character of the reaction offers the possibility to create patterns of immobilized proteins on a surface. This surface patterning by photolithography can be achieved by using photo masks or by laser irradiation.[95−97] A disadvantage of this ligation method is that thiols are not bioorthogonal, resulting in possible cross-reactions with other proteins.

Oxime Ligation. The term 'oxime ligation' refers to the condensation of an oxyamine or hydrazide with an aldehyde or ketone to give a stable oxime linkage (Figure 5F). Reactions between aldehydes or ketones with alkoxy-amines or hydrazides are generally slow[98] and the reaction normally proceeds at pH 4–5, which makes it less attractive for use in biological systems.[99] However, the recent discovery of aniline as a catalyst of this reaction was key to extending the utility of this reaction, even making it possible for conjugating at neutral pH.[100,101]

Neither oxyamine/hydrazide nor aldehyde/ketone functionalities are naturally present in proteins. For this reason, the oxime ligation has been used for the labeling of proteins and for the site-specific immobilization of proteins on biosensor surfaces.[95,102]

Protein Engineering. A number of chemical and biological techniques are available to introduce bioorthogonal functional groups into proteins. The easiest way is by using a short bifunctional cross-linker that can be directly coupled to the side groups of the endogenous amino acids, usually lysines or cysteines. However, this method suffers from the same limitations as described for the covalent immobilization by means of the endogenous functional groups. The multiplicity at which natural amino acids occur in proteins limits the possibility to site-specifically attach the bifunctional linker to the protein. This might result in modifications at unfavorable locations in the protein (i.e., active site or antigen binding site). Moreover, the resulting mixtures of modified proteins are difficult, if not impossible, to purify and characterize biochemically.[103,104] For this reason, improved site-directed protein modification techniques are required.

Enzymatic Modification. An interesting method to introduce specific functionalities into proteins is by means of enzymes that are involved in post-translational modification. These enzymes typically recognize short peptide sequences and covalently modify the protein with small molecules. This notion was explored by Ting and co-workers using an *Escherichia coli* enzyme biotin ligase (BirA).[105,106] BirA catalyzes the biotinylation of a lysine side chain within a 15 amino acid consensus 'acceptor peptide'. BirA recognizes this sequence irrespective of its surrounding and also tolerates subtle modifications of the biotin structure. The biotin ligase from *E. coli* is orthogonal to the peptide recognized by mammalian biotin ligases.[107] Consequently, mammalian proteins tagged with the BirA recognition peptide sequence can be selectively biotinylated and coupled to streptavidin-conjugated surfaces. In addition, Ting and co-workers demonstrated that BirA also accepts a ketone-containing analogue of biotin termed ketobiotin as a substrate.

The tolerance of the *E. coli* BirA for unnatural substrates is limited to conservatively modified biotin isosteres. However, the group of Ting discovered that yeast biotin ligase accepts an alkyne derivative of biotin, while *Pyrococcus horikoshii* biotin ligase utilizes both alkyne and azide biotin analogues.[108]

Another enzyme, sortase A (SrtA), has also been studied extensively for protein ligation applications. SrtA, a transpeptidase from *Staphylococcus aureus*, is present on its plasma membrane and catalyzes a cell wall sorting reaction that attaches surface proteins to the cell wall envelope. SrtA cleaves proteins between a threonine and a glycine residue within the recognition motif LPXTG (with X being any amino acid) near the C-terminus thereby generating a covalent enzyme intermediate. The threonine carbonyl group of the thioester intermediate is then attacked by the N-terminus of an oligoglycine nucleophile, resulting in the formation of a covalent peptide bond.[109] Over recent years, several researchers have exploited this specific reaction for a range of biotechnology applications, including the incorporation of non-native peptides and nonpeptidic molecules into proteins, the generation of nucleic acid–peptide conjugates and neoglycoconjugates, protein cyclization labeling of cell surface proteins on living cells, and covalent protein immobilization on solid supports.[110,111]

Recently, Jiang et al. reported on C-terminal modification and immobilization via SrtA-mediated ligation (SML) of recombinant human thrombomodulin (TM), a cofactor for the activation of the anticoagulant protein C pathway via thrombin. A truncated TM mutant consisting of epidermal growth factor-like domains 4–6 (TM456) with a conserved pentapeptide LPETG motif at its C-terminus was expressed and purified in *E. coli*. The truncated TM456 was successfully immobilized onto an N-terminal diglycine-functionalized glass slide surface via SML as confirmed by fluorescence imaging. Bioactivity testing showed an enhanced activity of the in this way immobilized recombinant TM.[112]

Other examples of enzymes that can be used for site-selective protein modification are: protein farnesyltransferase (PFTase catalyzes the transfer of a farnesyl isoprenoid group from farnesyl diphosphate to the sulfur atom of cysteine in the tetrapeptide sequence CAAX positioned at the C-terminus of a protein or peptide acting as a PFTase substrate. PFTase can be used to modify proteins with substrates that incorporate a bioorthogonal functionality, including azides and alkynes[98]), transglutaminase (TGase catalyzes the attachment of primary amine-containing probes to proteins tagged with polyglutamine sequences), lipoic acid ligase (LplA or a mutated LplA catalyzes the attachment of an alkyl or aryl azido-lipoic acid derivative to proteins containing the appropriate peptide substrate), formylglycine-generating enzyme (FGE catalyzes the transformation of a cysteine to a formylglycine in proteins that contain the motif CXPXR), phosphopantetheinyl transferases (AcpS or Sfp catalyzes the attachment of a coenzyme A (CoA) probe to proteins containing the appropriate protein/peptide substrate; see Sletten and Bertozzi[40] and references therein).

Self-Labeling Protein Tags. The above-described enzymatic modification techniques can be classified as a three-component system containing: the protein of interest (POI) fused to a specific tag (component 1), the modifying enzyme that recognizes this tag (component 2), and the substrate of the enzyme that has to be coupled to the POI (component 3). Enzymatic modification can also be performed using so-called self-labeling protein tags. Self-labeling protein tags, mostly derived from enzymes,[113] react covalently with a substrate. However, in this system the auxiliary enzyme is the tag itself and is linked directly to the POI, making this a two-component system.[114]

One of those self-labeling protein tags that has been used to specifically label proteins with two components is the SNAP-tag. Since this tag was first reported by Kai Johnsson and colleagues,[115] it has been employed as a tool for both labeling and protein immobilization.[116] The tag is a 20 kDa protein based on the human DNA repair protein O^6-alkylguanine-DNA alkyltransferase (hAGT) that transfers the alkyl group from its substrate, O^6-alkylguanine-DNA, to an internal reactive cysteine residue in the hAGT protein.[117,118] The labeling itself is highly specific with respect to hAGT but promiscuous with respect to the substrate; hAGT reacts readily with O^6-benzylguanine (BG). On this basis, a method was developed in which BG derivatives (substituted at the 4-position of the benzyl ring with biotin or fluorescein) were used as substrates for (a mutant of) hAGT.[115,119] Modifications of the hAGT substrate were found to have no significant influence on the reaction with hAGT.[120] When a POI therefore is fused to the SNAP-tag, the result of the reaction is a covalent coupling between the POI-SNAP fusion protein and the labeled substrate by a thioether.[117]

This method was used for the direct surface immobilization of SNAP-tag fusion proteins on benzylguanine modified surfaces. For example, Engin et al. reported the use of this approach to produce self-assembled monolayers (SAMs) on gold surfaces based on mixtures of a benzylguanine-terminated thiol (BGT) and a matrix thiol.[121,122] In this study, a bacterially produced recombinant SNAP-GFP fusion protein was incubated with BGT surface patterns created by microcontact printing and the patterned immobilization of the fusion protein was clearly demonstrated by fluorescence microscopy.

In recent years, similar SNAP-tag approaches were reported to selectively immobilize Sonic hedgehog (a member of the hedgehog family of growth factors) onto benzylguanine modified patterned SAMs,[123] cytokines to benzylguanine-modified beads,[122,124] or different classes of proteins onto modified CdSe/ZnS quantum dot surfaces.[125]

In 2008 the group of Kai Johnson further developed the self-labeling protein approach by the generation of another AGT-based tag, named CLIP-tag which reacts specifically with O^2-benzylcytosine derivatives.[126] SNAP-tag and CLIP-tag possess orthogonal substrate specificities, which means that SNAP and CLIP fusion proteins can react simultaneously and specifically with different molecular probes.

Another enzyme tag, known as HaloTag technology, was developed by Promega. The protein tag is a recombinantly modified haloalkane dehalogenase designed to covalently bind to synthetic ligands (HaloTag ligands).[127−129] Haloalkane dehalogenases are 33 kDa monomeric enzymes that catalyze the net hydrolytic conversion of a chloroalkane or a bromoalkane to the corresponding alcohol and hydrogen halide.[130] As an intermediate during the catalysis, an ester bond is formed between the enzyme and the hydrocarbon substrate. In the wild-type haloalkane dehalogenases the hydrocarbon is subsequently released as an alcohol.[127] In the HaloTag, the modified enzyme contains a critical mutation in the catalytic site that traps the reaction intermediate as a stable covalent adduct.[131] The synthetic ligands comprise a chloroalkane linker attached to a variety of useful molecules, including solid surfaces.[132] Covalent bond formation between the protein tag and the chloroalkane linker is highly specific, occurs rapidly under physiological conditions, and is essentially irreversible.[127]

This method was successfully used by the group of Rao to couple proteins to quantum dots. In an in vitro approach, a mutant of a bioluminescent protein, *Renilla* luciferase, was genetically fused to the N-terminus of the HaloTag protein. The expressed fusion protein was incubated with quantum dots coated with the chloroalkane ligand resulting in self-illuminating quantum dot conjugates by the site-specific coupling of the luciferase to the quantum dots.[133] An in vivo cell labeling method was reported in which the HaloTag protein was expressed at a cell membrane anchoring domain and ligated with quantum dot conjugates that are functionalized with a HaloTag ligand.[134]

Although these two-component self-labeling enzymes simplify the modification system, they use large tags (~20 kDa for SNAP and CLIP-tag and 33 kDa for the HaloTag) which may not be suitable for all proteins, especially smaller ones, and protein immobilization strategies.

Native Chemical Ligation. Although Native Chemical Ligation (NCL) was first discovered by Wieland et al. in 1953,[135] it was only applied in the 1990s as a protein modification technique to ligate unprotected peptide fragments by Kent et al.[136]

Expressed protein ligation (EPL), an extension of NCL, relies on self-splicing proteins. Protein splicing is a post-translational process in which a precursor protein undergoes self-catalyzed intramolecular rearrangements that result in the removal of an internal protein domain, termed an intein, and the ligation of the two flanking polypeptides, referred to as the N- and C-exteins.[137]

In EPL, the protein of interest is recombinantly expressed as a fusion protein with an intein. Intein's catalytic activity will shift the chemical equilibrium from a peptide bond toward a stable C-terminal thioester. EPL-inteins are mutated so that they are unable to undergo S−N acyl transfer.[40] The intein in turn is often fused to a chitin-binding domain (CBD) so that the expressed fusion proteins can be isolated on a chitin column. After washing the column (Figure 6) to remove undesired proteins, the protein of interest can be cleaved from the intein-CBD fusion by NCL with terminal-thiol-containing molecules (Figure 6B). When the splicing from the intein is performed with a thiol-containing peptide or a modified cysteine, a rearrangement spontaneously occurs to produce a stable peptide bond (Figure 6A). When the terminal-cysteine-containing molecules also contain a bioorthogonal group of choice, the EPL results in a protein terminally modified with this bioorthogonal group.

EPL has been used extensively in the areas of biotechnology and chemical biology. The technique was, for example, used by Lin et al. to site-specifically modify maltose binding protein (MBP) and enhanced green fluorescent proteins (EGFP).[138] These proteins were expressed using the commercially available IMPACT (Intein Mediated Purification with an Affinity Chitin-binding Tag) system from New England Biolabs.[139] The proteins were purified with chitin-beads and treated with an

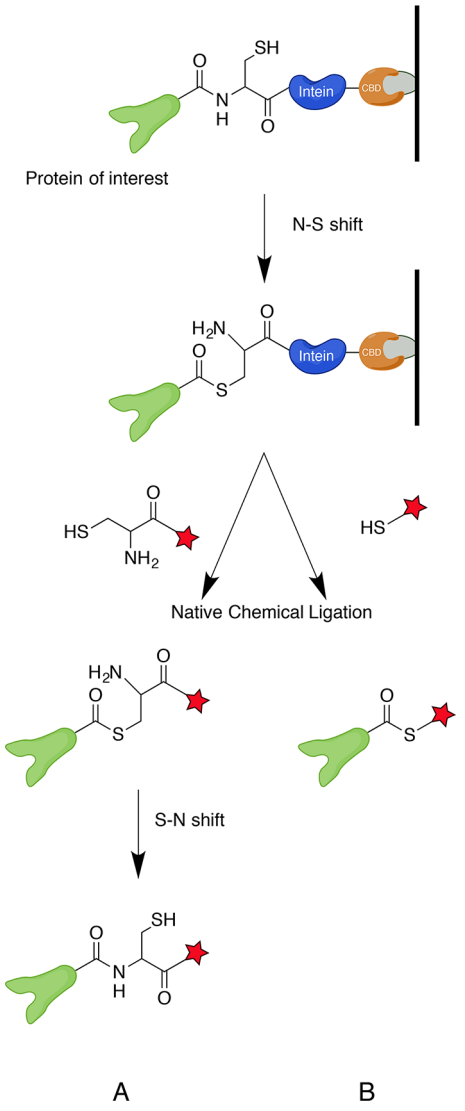

Figure 6. Expressed protein ligation (EPL): the protein of interest is recombinantly fused to an intein and a chitin-binding domain (CBD) to facilitate purification on a chitin column. The recombinant protein of interest is selectively cleaved from the immobilized chitin by native chemical ligation (NCL) with a molecule containing a thiol and a bioorthogonal functionality of choice (red star).

alkyne- and/or an azide-modified cysteine. Alkyne-modified MBP was then conjugated with several target azide-containing molecules (a fluorescein isothiocyanate (FITC), biotin, *N*-acetylglucosamine, a diazide linker, and a glycoprotein) using copper catalyzed azide/alkyne cycloaddition. EGFP was modified with either an alkyne or an azide and coupled to azidated or alkynated surfaces, respectively. It was found that chemoselective attachment to the surface was achieved and that the protein structure was maintained.

More recently, EPL was used to modify several enzymes. Sialyltransferase (PmST1) and cytidine monophosphate (CMP)-sialic acid synthetase (CSS) were site-specifically and covalently immobilized on PEGylated magnetic nanoparticles (MNPs) with an N-terminal cysteine through NCL. The resulting site-specifically immobilized PmST1 showed increased activity compared to the native free enzyme. However, this increase may be influenced by nonspecific interaction between the substrate and the surface of the MNP. Increasing the length of the PEG-linker between the enzyme and the MNP-surface increased the activity of the immobilized enzymes. In addition the authors immobilized a membrane-bound sialyltransferase via a biotin-labeled cysteine at the C-terminus to streptavidin-functionalized MNPs using EPL. Using a streptavidin−biotin interaction, it was possible to immobilize the enzyme under mild ligation conditions and provided an approximately 2-fold increase in activity compared to other immobilization methods on MNPs.[9]

A drawback of EPL is that modifications are restricted to the N- or C-terminus of the protein. This limits its applicability to proteins where at least one of the termini is surface exposed and not near or part of the active site of the protein. In case the termini are not available or that modification interferes with the protein's activity, other strategies are needed.

Auxotrophic Expression. Another strategy to incorporate bioorthogonal chemistries in a protein involves the replacement of one of the 20 natural amino acids by a structural analogue. This method exploits the promiscuity of the wild-type tRNA synthetases by 'highjacking' the organism's native translational system.[86,140] The incorporation of amino acids is controlled by aminoacyl-tRNA synthetases (aaRS). These enzymes are responsible for the coupling of amino acids to tRNA. As a consequence of the substrate tolerance of aaRS, an amino acid analogue can be coupled to the corresponding tRNA. If the original is not present in the growth medium and the host organism is auxotrophic (i.e., incapable of producing the original amino acid), the amino acid analogue will be incorporated into the protein at specific places where the natural amino acid is genetically encoded.[86]

Using this technique, several methionine analogues are used in protein biosynthesis with high efficiency. In the group of Bertozzi, for example, two azide-functionalized amino acids, azidoalanine and azidohomoalanine were tested as in vitro substrates for methionine-tRNA synthetase. The results of both in vitro and in vivo assays confirmed azidohomoalanine as an excellent methionine surrogate. A target protein (murine dihydrofolate reductase, mDHFR) containing azidohomoalanine was modified by Staudinger ligation with an appropriately engineered phosphine bearing a FLAG peptide.[141]

Zhang et al. used this technique to create an artificial protein scaffold that was used to immobilize a recombinant leucine zipper component (the acidic component ZE) on a surface. The ZE component was fused to an elastin mimetic domain (ELF) for surface anchorage due to its strong adhesion to hydrophobic surfaces. ELF consists of 25 amino acids, including 1 phenylalanine. ELF was expressed in a phenylalanine auxotroph bacterial host harboring a mutant *E. coli* phenylalanyl-tRNA synthetase (an Ala294Gly mutation known to broaden PheRS substrate specificity[142]). Expression was performed in media supplemented with the phenylalanine analogue *para*-azidophenylalanine resulting in azido-functionalized proteins. Typical yields were 50 mg/L, and the amount of incorporation of *para*-azidophenylalanine was approximately 45%. Irradiation of the proteins, spin-coated on a hydrophobic octyltrichlorosilanized surface, covalently cross-linked the protein to the substrate through photodecomposition of the aryl azide groups of *para*-azidophenylalanine.[143]

Using this modification technique, it is possible to incorporate functionalities in a protein, orthogonal to the endogenous amino acids. This incorporation is however at a residue-specific level rather than a site-specific level. When a natural amino acid is encoded multiple times in the protein,

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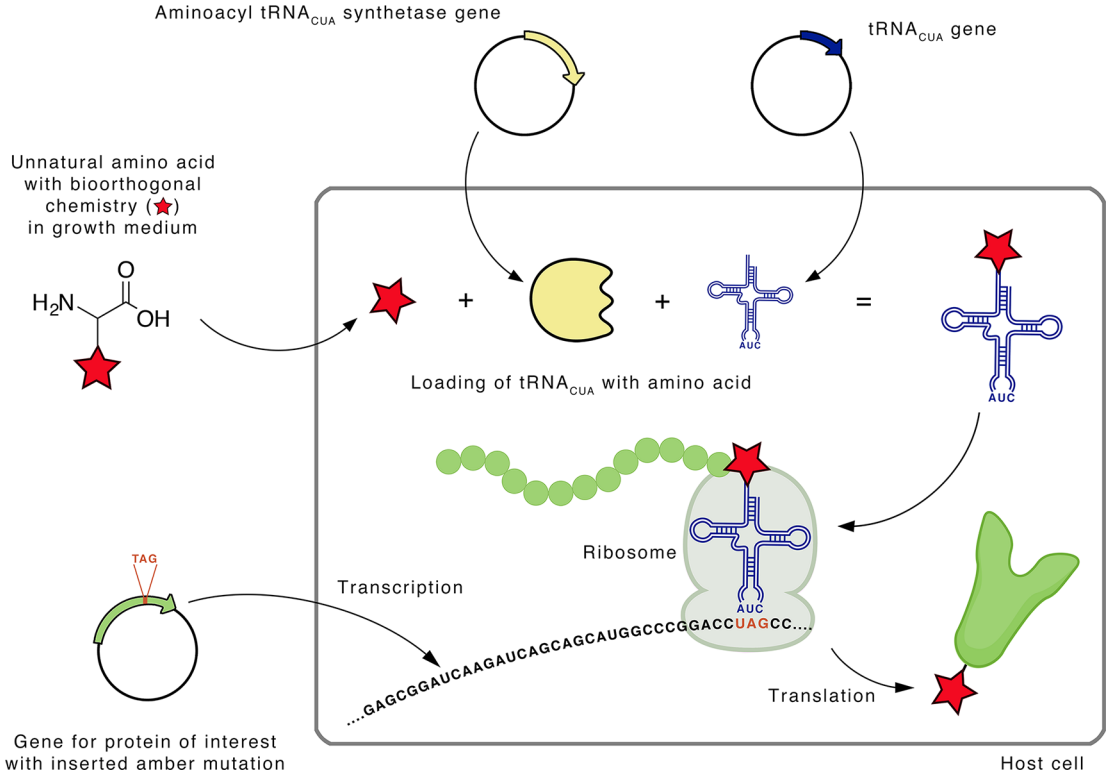

Figure 7. Schematic representation of the site-specific incorporation of unnatural amino acids into proteins by amber suppression. In this method, the gene for the protein of interest is mutated with an amber codon at the desired site of modification. A mutant aminoacyl-tRNA$_{CUA}$ synthetase recognizes the unnatural amino acid bearing the desired functionality and loads it to the corresponding tRNA$_{CUA}$. This leads to the expression of the protein of interest with the desired functionality at the genetically encoded site.

multiple copies of the amino acid analogue can be incorporated. When the incorporation efficiency is below 100%, the result will be a heterogeneous modification.

Nonsense Suppression. The most elegant and controllable way to site-specifically incorporate bioorthogonal functionalities in proteins is during the actual protein translation, using the so-called 'genetic code expansion'. An in vitro method for the introduction of unnatural amino acids was first independently reported by Chamberlin[144] and Schultz[145] in the late 1980s[40,146] but has since been extended to in vivo methods. In theory, to genetically encode an unnatural amino acid, one needs different components of the biological translation machinery: a unique codon and corresponding tRNA recognizing this codon, an unnatural amino acid, and a new aminoacyl-tRNA synthetase that recognizes the unnatural amino acid and loads it to the corresponding tRNA. Moreover, these components must meet the following criteria: the unnatural amino acid must be metabolically stable and biologically available to be imported in the host cell. Second, the unnatural amino acid should not be a substrate for the endogenous synthetases. Third, the unique codon must be recognized by the new tRNA but not by any endogenous tRNA and fourth, the aminoacyl-tRNA synthetase/tRNA pair must be specific for the unnatural amino acid. It further has to be functional in the host organism and should not crosstalk with the endogenous translational system in the host organism.[147]

The natural genetic code consists of 64 unique triplet codons from the possible permutations of the 4 nucleotides. The 20 natural amino acids are encoded by 61 codons, leaving 3 codons, called stop (nonsense) codons, as termination factors: TAG (amber), TAA (ochre), and TGA (opal).[148] One method for the site-specific introduction of unnatural amino acids is the so-called 'nonsense' suppression that uses stop (nonsense) codons and suppressor tRNA, which specifically recognizes one of the three stop codons. The method is based on the fact that only one stop codon is needed for the termination of protein translation, leaving the other two to be reassigned to an additional unnatural amino acid.[146] Since the amber stop codon is the least used in both *E. coli* and yeast, this codon is the most frequently used stop codon in nonsense suppression. The use of the amber codon to code for an unnatural amino acid requires a suppressor tRNA$_{CUA}$ and the corresponding aminoacyl-tRNA$_{CUA}$ synthetase, which has been evolved to selectively bind the unnatural amino acid to the suppressor tRNA$_{CUA}$ (Figure 7). Besides methods based on stop codons, additional methods have been developed based on quadruplet codons (four nucleotides) to incorporate unnatural amino acids into proteins.[149,150] The main drawback of all suppressor-based approaches is the relatively limited capacity of suppressor tRNA loaded with unnatural amino acids to fully decode (suppress, read-through) nonsense (triplet or quadruplet) codons.[151]

This 'genetic code expansion' has been used for the site-specific introduction of unnatural amino acids in bacteria, eukaryotic cells, and animals.[152,153] To date, more than 70 unnatural amino acids with different chemistries have been incorporated into proteins.[154,155] Moreover, it is possible to produce proteins containing various unnatural amino acids at defined positions in milligram amounts from bacterial systems.[113]

An in vitro approach of this method was recently reported by Yoshimura et al.,[4] who incorporated an azide group site-specifically in calmodulin (at a site known not to interfere with

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the calmodulin activity[156]) and coupled this protein at the end of a carbon nanotube. For this, a cDNA encoding the calmodulin was mutated so that the codon for the target amino acid was replaced by the amber codon. Using this cDNA as a template, transcription and translation were carried out in vitro. The translation reaction mixture contained a suppressor $tRNA_{CUA}$ carrying the CUA anti-codon, the unnatural amino acid azidotyrosine and the corresponding aminoacyl-tRNA synthetase, so that the azidotyrosine could be incorporated specifically at the position of the amber codon. Calmodulin is a calcium-binding protein that regulates the activities of a variety of cellular proteins. The protein undergoes a conformational change when bound to Ca^{2+}, which then facilitates an interaction with its substrate calmodulin-binding protein (CBP). The biological activity of calmodulin was assayed by formation of a complex with enhanced cyan fluorescent protein conjugated with CBP. In the presence of Ca^{2+}, a fluorescent signal was visible at the tip of the nanotube, indicating a site-specific coupling of the calmodulin. In the absence of Ca^{2+}, the signal was drastically reduced. No signal was detected for the wild-type calmodulin (no azide group), demonstrating the specificity of the ligation technique.

The group of Schultz recently reported an in vivo use of the amber suppression technique.[157] In this study the authors used an aminoacyl-tRNA synthetase/tRNA pair to site-specifically incorporate *p*-acetylphenylalanine at defined sites in each of the two Fab-fragments in response to an amber nonsense codon. The mutant Fab-fragments were then selectively coupled via a stable oxime bond to bifunctional linkers bearing an alkoxy-amine on one terminus and an azide or cyclooctyne group at the other. In a second step, the Fab conjugates were linked to obtain the heterodimer through a copper-free cycloaddition. With this technique, a rapid construction of bispecific antibodies with excellent in vitro activity was demonstrated.

CONCLUSIONS

In order to develop sensitive and reproducible biosensor surfaces, especially with miniaturization in mind, it becomes increasingly important to immobilize proteins covalently and with a controllable and homogeneous orientation to complementary functionalized surfaces. Despite the fact that a large number of chemical or chemical biology tools have been developed, still more research is needed to mature the present techniques. These efforts finally pave the way to the implementation of biosensor concepts into daily routine diagnostics.

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Notes

The authors declare no competing financial interest.

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