Selective Covalent Protein Immobilization: Strategies and Applications

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1. Introduction

The development and applications of site-selective protein immobilization have undergone significant advances in recent years. Notably the recent fusion of synthetic chemistry and molecular biology (chemical biology) has seen the development of some very powerful and efficient site-specific, covalent protein attachment methods. In addition, many important applications of anchoring proteins onto supports have begun to emerge, including the fabrication of functional protein microarrays, biosensors, and continuous flow reactor systems, as well as finding applications in single-molecule enzymology and nanotechnology.[1−5] Protein microarrays are particularly sought after for functional proteomics, including high-throughput analysis of protein−protein and protein−nucleic acid interactions as well as protein−small molecule interactions, including drug screening.[1,6,7] It was anticipated for some time that protein arrays would have a profound impact on biological and medical science. However, the lack of suitable high-throughput methods for immobilizing large numbers of functionally intact proteins to surfaces has hindered the widespread development of the protein microarray.

Together with this increasing emphasis on high-throughput applications, there has been demand for highly specific yet facile and generally applicable methods for the immobilization of a wide range of proteins. Ideally the methods that are developed should enable the capture of proteins from cell lysates to generate homogeneous, stable, and high-density protein surfaces with retention of maximum protein function, circumventing the need for laborious protein

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Farid Khan obtained his Ph.D. from the University of Cambridge (2004), where he studied the folding of GFP using fluorescence and NMR. Previously, he has worked for a number of years at GlaxoSmithKline in fluorescence assay development for drug discovery. He spent two years as a postdoctoral researcher at the Babraham Institute in Cambridge, where he codeveloped novel protein arrays from DNA arrays using cell free synthesis and characterized robust protein immobilization methods. He is currently employed at the University of Manchester as a Systems Biologist at the Manchester Centre of Integrative Systems Biology. His primary role is on the characterizing of enzymes in metabolic pathways, and he is a M.Sc. lecturer in Biotechnology and Enterprise. He is a founder of Lumophore Ltd., a consulting company specializing in applications of protein array technology.

Jason Micklefield graduated from the University of Cambridge in 1993 with a Ph.D. in Organic Chemistry, working with Prof. Sir Alan R. Battersby to complete the first total synthesis of Haem d1. He then moved to the University of Washington, U.S.A., as a NATO postdoctoral fellow with Prof. Heinz G. Floss, investigating various biosynthetic pathways and enzyme mechanisms. In 1995 he began his independent research career as a Lecturer in Chemistry at Birkbeck College, University of London, before moving to Manchester in 1998, where he is now Chair of Chemical Biology. Prof. Micklefield's research interests are at the chemistry−biology interface and include the redesign of nucleic acids, small-molecule control of gene expression, biosynthesis and biosynthetic engineering, nonribosomal peptides, biocatalysis, and enzyme mechanism.

purification. To this end, attention has focused on oriented (site-specific) protein immobilization methodology. Site-selective methods allow the defined attachment of proteins with uniform orientation where the bioactive site (binding epitope or catalytic site) is freely accessible for further analysis or application.[8−10] This in turn maximizes the biological activity of the proteins and, therefore, the signal-to-noise ratio and resolution of the array or biosensor. In contrast, random attachment (covalent or otherwise) to surfaces can reduce protein function by steric hindrance of available bioactive sites and by reduction of protein stability due to modification of critical residues in proteins that are essential for structure and activity. In the case of nanotechnology-based applications, this is a particularly significant issue since only a relatively small number of proteins will be present at each location and uniformly oriented proteins with maximal biological activity will be crucial. In light of these issues, a number of new site-specific biologically mediated methods of protein immobilization have been developed that offer a potential solution to some of the earlier problems encountered with the use of non-covalent immobilization or nonselective chemical protein ligation methods.

This review will cover the recent developments and applications of site-selective protein immobilization with a focus on the biologically mediated methodology. This includes enzyme-mediated attachment to a small-molecule immobilizing "ligand" and the development of genetically encoded protein tags for immobilization present in the literature up until late 2008. The earlier methods for non-covalent and nonselective protein immobilization will be dealt with briefly to put this work into context. The reader is also directed to reviews covering earlier methodologies and related topics including applications such as protein immobilization for microarray fabrication or biocatalysis.[7,11−14] In addition, the review will also illustrate some high-profile applications where protein immobilization is a crucial component.

2. Current Applications of Protein Immobilization Technologies

2.1. Protein Microarrays

The advent of high-density array printing technology and improved methods for high-throughput production and purification of large numbers of proteins have in recent years allowed the preparation and exploitation of protein microarrays.[1,6,13,15−20] However, many of the applications demonstrated thus far have relied on protein attachment methods that result in nonsite-specific immobilization, either covalently through amine, aldehyde, and epoxy-derivatized surfaces[21,22] or through adsorption on nitrocellulose, hydrogel, or polylysine coated slides (see section 4.1).[9,23−25] A number of more selective approaches have been demonstrated and employ affinity reagents that bind specific epitopes or tags on proteins and render them in a correctly orientated manner such as nickel nitrilotriacetic acid (NTA) coated slides, which are used to bind His_6-tagged proteins[26,27] and streptavidin (or avidin) coated slides.[28,29] However, these only provide non-covalent attachment.

Since the landmark papers describing large-scale protein arrays of 10 800 features by Schreiber in 2000[22] and of 5 800 unique features from the yeast proteome by Snyder in 2001[30] (Figure 1), arrays of 1 000−10 000 proteins have typically been achieved, which is relatively low compared to DNA arrays.[20] Nevertheless, the application of protein arrays is becoming more widespread with the introduction of commercially available products such as the Yeast ProtoArray from Invitrogen, which contains 4 088 unique *S. cerevisiae* protein fusions with *N*-terminal glutathione-*S*-transferase (GST) spotted on nitrocellulose coated glass slides.[31] Similarly, targeted arrays of human proteins such as the Human ProtoArray for kinases enable rapid screening of approximately 8 000 kinase targets against potential human protein substrates in a simple and robust format.

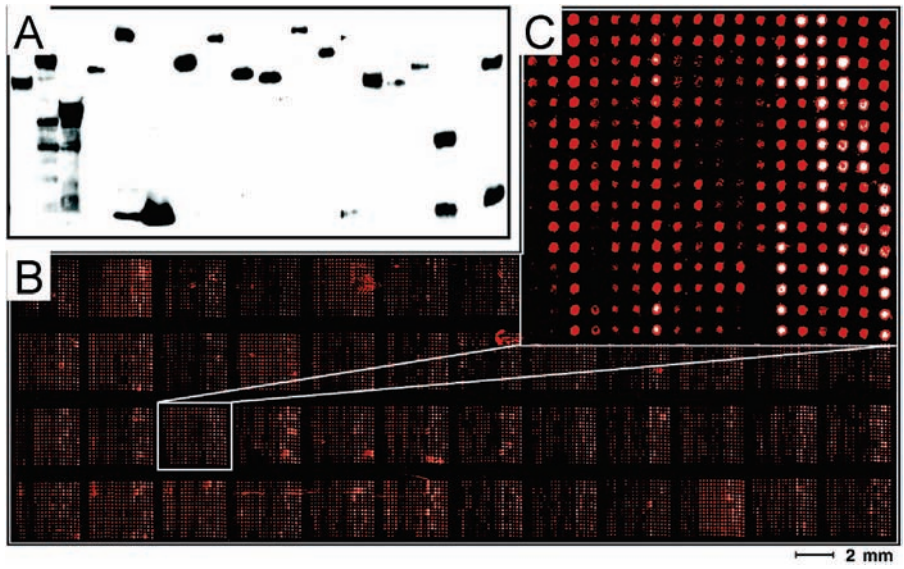

Figure 1. Representative images from the first reported yeast proteome array. (A) Immunoblot analysis of 19 representative proteins demonstrating their purity after high-throughput expression and purification; (B) a fluorescence micrograph of 6 566 protein samples representing 5 800 unique proteins spotted on a single nickel-coated microscope slide and treated with fluorescently labeled antibodies; (C) an enlarged image of one of the 16 × 16 blocks of spots. (Reproduced with permission from ref 30. Copyright 2001 AAAS.)

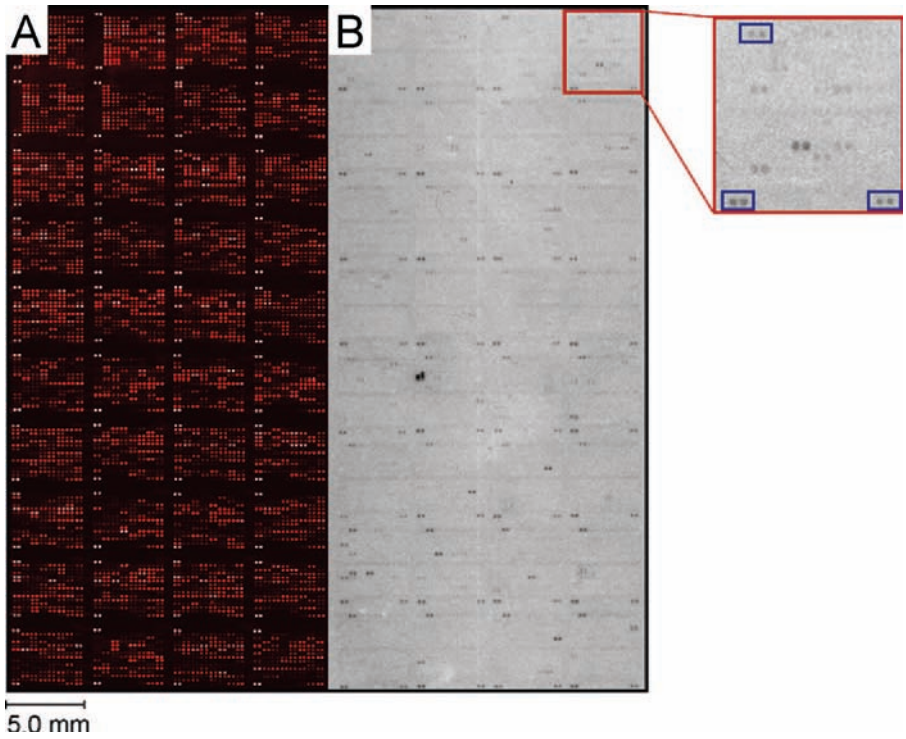

Figure 2. Representative images from the yeast proteome microarray used for mapping kinase activity. (A) A fluorescence micrograph of array after labeling with fluorescently tagged antibodies and (B) radiomicrograph of the array after a kinase assay employing ^{33}P-radiolabeled adenosine triphosphate. The dark spots indicate labeled phosphorylated substrates on the array that darken the X-ray film used for imaging. (Reproduced with permission from ref 35. Copyright 2005 Nature.)

2.1.1. Research and Discovery

Using protein microarrays, it is possible for the functions of thousands of proteins to be explored in a single experiment, and this has allowed the discovery and mapping of novel protein−protein, protein−DNA, protein−drug, and protein−lipid interactions.[18,32,33] In one example, 119 predicted yeast kinases were immobilized and challenged with 17 different substrates.[34] This study detected 27 novel tyrosine kinase catalytic activities, a 3-fold increase in the number of known kinases, and identified a number of novel features in some of these proteins. In another example, protein arrays were used in the global analysis of yeast protein phosphorylation[35] (Figure 2). Here, chips with 4 400 proteins were used to identify 1 325 proteins involved in 4 200 phosphorylation events in an in vitro kinase assay. Novel protein−protein interactions have also been identified using calmodulin binding proteins with yeast proteome chips.[18] In addition to the six known calmodulin binding partners, 33 new binding proteins were identified, including one with a novel consensus binding motif. In terms of DNA−protein interactions, yeast proteome arrays have been interrogated with single- and double-stranded yeast genomic

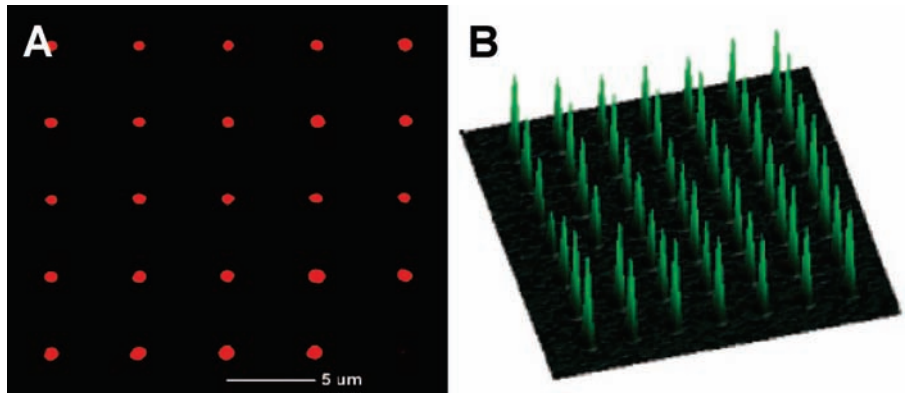

Figure 3. Examples of fluorescence images from protein nanoarrays; (A) produced with an atomic force microscope (AFM) tip-based "nanoarrayer" where a 565-spot array of mouse IgG was deposited in 700 nm diameter spots with a 5 μm pitch and then interrogated with a Cy3-labeled antimouse IgG and (B) a three-dimensional representation of a "nanopipette"-generated 49-spot array with 60−90 nm spot diameter after treatment with the solutions of IgG labeled with AlexaFluor 488. (Figure 3A reproduced with permission from ref 45. Copyright 2004 Wiley. Figure 3B reproduced with permission from ref 44. Copyright 2004 American Chemical Society.)

DNA.[32] This led to the identification of more than 200 DNA binding proteins of which one protein, Arg5,6, a mitochondrial enzyme involved in arginine biosynthesis, was found to have a dual role in regulating gene expression as well as functioning as a metabolic enzyme. Protein microarrays consisting of whole proteomes have been used to identify protein−protein interactions and protein−drug interactions.[6] More recently, *E. coli* proteome chips have been created bearing 4 256 proteins, and these chips have been used to develop assays for identifying proteins involved in the recognition of potential base damage in DNA.[36] These experiments identified two novel proteins, YbcN and YbaZ, that were found to be base-flipping proteins. In using protein arrays for the interrogation of whole proteomes for the discovery of drug targets, yeast proteome arrays have been probed with a library of small molecules that suppress the activity of rapamycin in yeast in order to find new proteins involved in the target-of-rapamycin (TOR) signaling network.[33]

2.1.2. Proteomic Profiling and Diagnostics

The most successful applications of protein microarray technology have been with antibody microarrays that have been used in antibody profiling, identification of microbial antigens, and biomarker detection.[37] In the latter case, dedicated arrays for detecting cancer and disease biomarkers are particularly notable and hold much promise for the screening and diagnosis of disease.[1,38−40] In this respect, there have been reports of the use of arrays for the screening of patient serum samples against autoantibodies present in rheumatoid arthritis[41] and systemic lupus erythematosus.[42] The detection of antibodies against the severe acute respiratory syndrome (SARS)-CoV coronavirus using a coronavirus protein microarray as a rapid diagnostic test has also been reported.[43] These arrays were able to accurately detect viral autoantibodies in greater than 90% of the serum samples from patients who showed symptoms of SARS infection.

Overall, one of the current limitations of protein microarray technology relates to the number of features that are detectable on the surface. In order to create very high density formats of >100 000 proteins, the spot size and morphology are important factors for the resolution of individual spot features and subsequent data analysis,[20] which are in turn partly dictated by the immobilization method employed. Recently, prototype nanoscale arrays have been fabricated using "nanoarrayer" and "nanopipette" instruments with features from 60 nm with the latter (Figure 3).[44−46] However, in order to fully realize the development of "superdensity" protein nanoarrays for the profiling of whole organisms or individual patients, site-specific covalent methods of protein immobilization will also be required. As noted in the introductory section, such site-specific immobilization would maximize the biological activity of the attached proteins by allowing the uniform and unhindered presentation of the protein active sites. The resulting increased signal strength and quality from any assays would thus allow higher-density arrays and resolution. From a practical aspect, protein microarrays are processed using several wash steps and therefore covalent attachment as this would minimize protein loss through desorption from the array substrate. The maximal resolution of individual microarray spots is thus dependent on available sites that are used for functional detection (site-specific attachment increases functional detection) and the nature of the linkage of protein to the surface (covalent attachments are more stable during processing of microarrays).

2.2. Biosensors from Immobilized Proteins

Proteins can also act as recognition elements in sensor devices where the protein is attached to a solid support and the combined unit acts as a transducer of an optical or electronic signal. There has been long-standing interest in the development of such sensors in the environmental, chemical, and medical sciences for the detection of a variety of analytes, as well as more recently for the detection of chemical and biological warfare agents. Ideally such sensor devices should be portable, rapid, and straightforward to use. Where proteins are employed in the sensing elements, there continues to be the need for attachment methods that ideally preserve or increase the stability of the immobilized protein, since such devices may be stored for prolonged periods before deployment and must be sufficiently robust for field use.[2,47,48] Research in this area can be generally divided into three themes: the development of sensors for new analytes, new modes of sensing, and miniaturization.

One recent example encompassing the former two themes is the use of microcantilevers functionalized with antibodies against prostate-specific antigen (PSA), a disease marker commonly measured in the diagnosis of prostate cancer.[49]

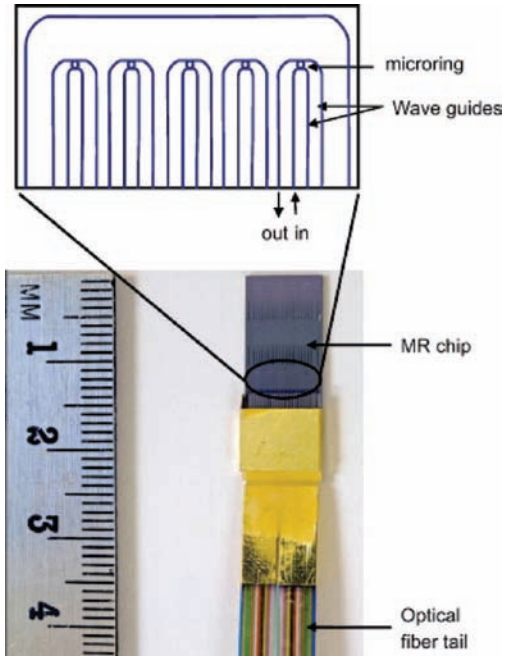

Figure 4. Photograph of the five-ringed microring resonator chip used as a sensor for bacteriophage M13. The magnified view illustrates the five microrings and the individual input and output ports. (Reproduced with permission from ref 52. Copyright 2008 Elsevier.)

This system relies on measuring the nanometer-scale deflection of the cantilevers upon binding of PSA to the immobilized antibodies. It was demonstrated that clinically relevant quantifications of both free and complexed PSA over a range of 0.2−60.0 ng/mL could be made in a background of human serum albumin and human plasminogen at 1 mg/mL. Furthermore, sensors based on this technology are particularly appealing since they require no additional labeling reagents for maximum performance (i.e., they are "label-free").

A related method using resonating microcantilevers[50] has also been described for the detection of prion proteins that are responsible for bovine spongiform encephalopathy (BSE) in cows, scrapie in sheep, and Creutzfeldt−Jakob disease (CJD) in humans.[51] This system relies on antiprion protein antibodies anchored to the cantilevers as the recognition element. The presence of prion proteins bound to the antibodies resulted in a change in the resonance frequency that was measured. It was demonstrated that an array of such cantilevers allowed the detection of 20 μg/mL of prion proteins in label-free operation, while if antibody-conjugated nanoparticles were applied subsequently in a sandwich assay, the sensitivity of these sensors dramatically increased to 2 ng/mL.

Another notable mode of sensing relies on the use of proteins immobilized on optical microring resonators. These devices can be employed label-free, are highly sensitive (<50 ng/mL for protein detection), and are straightforward to operate, and miniaturized designs such as one containing five sensors in less than 1 cm have been reported (Figure 4).[52] In one recent example,[53] antibodies immobilized within the microrings were used to quantify bacteriophage M13 virus. These sensors had a detection limit of $\sim 2.3 \times 10^3$ pfu/mL, superior to other reported sensors, and the detection dynamic range was shown to span 7 orders of magnitude.

In working toward even greater levels of miniaturization and high-specification sensors, researchers are now exploiting

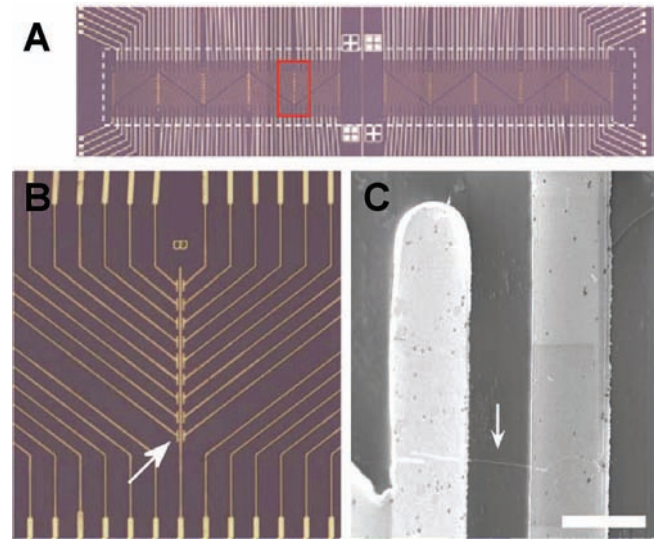

Figure 5. (A) Image of nanowire sensor array; (B) magnified image of the area highlighted in the red box with the arrow highlighting the position of a single device (size of image = 350 μm × 400 μm); and (C) a scanning electron microscopy image of one device where the nanowire horizontally spans the electrodes and is highlighted with the arrow (the scale bar represents 2 μm). (Reproduced with permission from ref 4. Copyright 2005 Nature.)

"bottom-up" nanotechnology-derived platforms such as self-assembled monolayers (SAMs) and Langmuir−Blodgett films, which promise a greater degree of sensitivity and miniaturization. In one example, a SAM-based sensor for B-type natriuretic peptide, a biomarker for heart failure, was reported to detect as little as 40 pg/mL of the peptide. The total assay time from blood samples to yield the final result was reported to be 70 min.[54]

Another nanotechnology-inspired sensor technology involves the use of semiconducting nanowires that act as field effect transistors. Such devices are able to alter their electrical conductance in response to the binding of various molecules to their surfaces, and thus, nanowires that are functionalized with recognition elements such as antibodies can act as specific biosensors.[4,55] Examples where antibodies toward free and complexed PSA, carcinoembryonic antigen, and mucin-1 attached to the surfaces of nanowires have been produced, and sensitivities of up to 0.9 pg/mL have been reported. Perhaps most importantly, multiple functionalized nanowires may be incorporated into a single device allowing internal controls with unfunctionalized nanowires as well as parallel sensing of multiple analytes (Figure 5).

2.3. Immobilized Enzymes in Biotechnology and Chemical Manufacturing Processes

On a larger scale, enzymes are employed in a wide range of industrial processes that would be impossible or uneconomical by conventional chemical methods.[56] While living organisms constantly regenerate enzymes that are degraded through usage, when used in manufacturing, these relatively valuable proteins must be recovered, preserved, and recycled. One means of addressing these issues is to immobilize these enzymes on an insoluble material to enable facile recovery of the enzyme and purification of the product.[57,58] Research in this area has been ongoing for several decades, and since the first report of racemic amino acid resolution with immobilized *Aspergillus oryzae* aminoacylase by Chibata in 1967, a great number of examples have been reported.[59−61]

One of the major trends in this area continues to be the development of new supports. These include mesoporous materials (materials containing highly uniform pores between 2 and 50 nm), such as mesoporous silica[62,63] and nanoparticles,[64] that provide very large surface areas for the biotransformation to take place as well as improved stability in some cases. Another development has been the increasing interest in the use of continuous-flow systems to streamline production workflows, such as the PASS*flow* system.[65] Such in-line bioreactors have been shown to allow automated purification and immobilization of His_6-tagged proteins that could subsequently be used as highly active biocatalysts in benzoin reactions and ester hydrolysis.[5]

2.4. Nanotechnology and Single-Molecule Enzymology

Apart from the applications noted above in arrays, sensors, and nanoarchitectured support materials, protein immobilization in other nanotechnological platforms has been used in fundamental research such as single-molecule enzymology. Here, immobilization is employed to entrap and isolate single biomolecules on a surface where they can be individually interrogated.

A general approach for the immobilization of single proteins on pegylated gold-thiol SAMs and subsequent interrogation of single molecules by atomic force microscopy (AFM) has been described.[66] Using this combination, AFM could be used to confirm the isolation of single proteins on the surface as well as the measurement of nanomechanical properties of the proteins such as mechanical unfolding.

In another example of single-molecule enzymology,[3] confocal fluorescence microscopy was used to study the kinetics of a single molecule of biotin-labeled β-galactosidase immobilized on a streptavidin-coated polystyrene bead, which itself was immobilized on a glass slide (Figure 6). This study demonstrated that the enzyme exhibited molecular memory at high substrate concentrations, characterized by clusters of turnover events separated by periods of low activity.

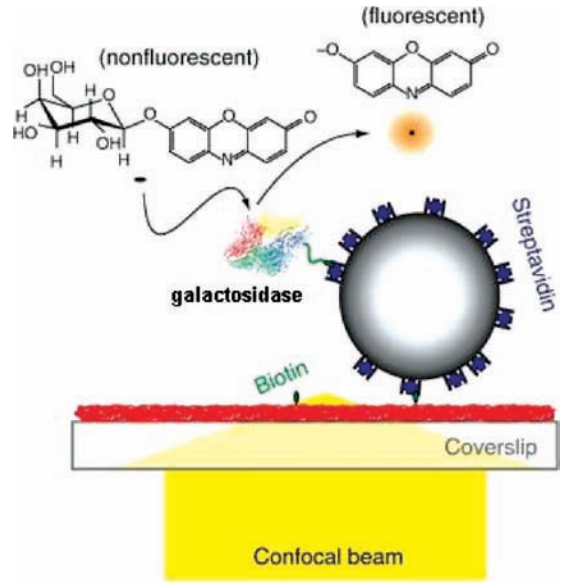

Figure 6. Schematic representation of enzyme immobilization for single-molecule fluorescence measurements and enzyme kinetics by confocal microscopy. A single β-galactosidase molecule is linked to a streptavidin coated polystyrene bead, which is then bound to a glass coverslip functionalized with biotin. The pro-fluorophore resorufin-β-D-galactopyranoside substrate is added and converted to the fluorescent resorufin by the enzyme molecule. The individual reactions are detected before the resorufin rapidly diffuses out of the confocal detection volume. (Reproduced with permission from ref 3. Copyright 2006 Nature.)

3. Chemical and Physical Methods of Protein Immobilization

Bioconjugation, where biomolecules are linked together or to solid supports, is an important aspect of the biological sciences and critical to the applications discussed above, as a result, many such methods have been described through the years.[2,11,12] Of these, a small number have become widely applied due to their ease of use, flexibility, and familiarity in the wider research community. In general, these classical methods rely on physicochemical adsorption phenomena or on functional groups that are naturally present in proteins. They are therefore extremely straightforward to employ and applicable to all proteins, both native and modified.

3.1. Classical Non-Specific Immobilization Methods

3.1.1. Non-Covalent Protein Adsorption

Non-covalent methods of protein immobilization are widely employed and involve either passive adsorption onto hydrophobic surfaces or electrostatic interactions with charged surfaces. Here, the use of nitrocellulose membranes or polystyrene microtiter plates for hydrophobic adsorption and polylysine coated slides for electrostatic binding are perhaps the most widely familiar. As noted above, the major advantage of immobilization in this manner is that neither additional coupling reagents nor modification to the protein of interest is required. However, non-covalent immobilization typically involves relatively weak and reversible interactions. As a result, proteins can leach out from the support, which in turn results in loss of activity over time and contamination of the surrounding media.[26] This has implications in the overall robustness and recyclability of systems, particularly when used in analytical assays and sensor devices. It is also well-known that adsorption of protein onto surfaces often results in conformational changes and denaturation of proteins[67] that can result in massive losses in protein activity.[8] Furthermore, since there is no control over the packing density of the immobilized proteins, their activity may be further reduced by steric congestion.[21,68]

3.1.2. Classical Covalent Immobilization Methods

For more stable attachment, the formation of covalent bonds is required, and these are generally formed through reaction with functional groups present on the protein surface. In common with non-covalent adsorption, these methods can be used on unmodified proteins since they rely only on naturally present functional groups. For example, the exposed amine groups of Lys residues readily react with supports bearing active esters, with the most common being *N*-hydroxysuccinimide (NHS, also abbreviated HOSu) esters, to form stable amide bonds (Figure 7). However, one disadvantage of using NHS esters is that they are unstable in aqueous conditions, and thus, the attachment of proteins in aqueous buffers will compete with ester hydrolysis, resulting in only modest immobilization yields. As an alternative, aldehyde groups can be coupled with exposed

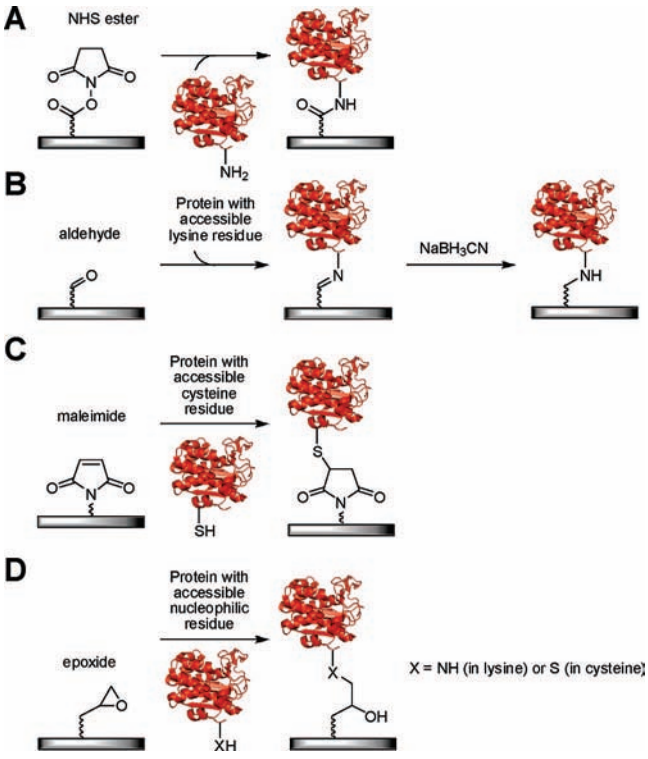

Figure 7. Widely used conventional chemoligation methods for immobilization with nucleophilic residues of proteins. The reaction of lysine residues to NHS esters (A) or aldehydes (B) and cysteine residue bonding to maleimide groups (C). Epoxides may react with either of the nucleophilic residues (D).

amines on proteins to produce an imine[22,30] that can be reduced using sodium cyanoborohydride or other reagent to form a stable secondary amine linkage (Figure 7). The nucleophilicity of the amine group also allows reaction with epoxide-functionalized materials (Figure 7). Epoxides have the advantage of being relatively stable to hydrolysis at neutral pH, which allows easy handling of the materials but can result in slow or incomplete coupling.[12]

The Cys residue bearing the thiol group is also often employed for protein immobilization and readily undergoes conjugate addition with α,β-unsaturated carbonyls (e.g., maleimides) to form stable thioether bonds (Figure 7). It has been shown that maleimide groups strongly favor conjugate addition with thiols at physiological pH (6.5−7.5) since under these conditions amines are predominantly protonated. As proteins generally have very few surface-exposed Cys residues, it is possible to achieve site-selective immobilization, especially if the protein of interest can be engineered to remove all but one surface Cys residue or to insert a single Cys on the surface where none previously existed.[69,70] The nucleophilicity of the thiol group also means that it can react with epoxides and NHS esters, although in practical terms this latter reaction is relatively slow and the resultant thioester moiety is susceptible to degradation. The presence of a Cys unit is also a crucial component of immobilization methods derived from native peptide ligation (NPL) and related methods employing protein splicing. These are described in further detail in section 4.4.

For the acidic Asp and Glu residues, the generic method in which they can be used for immobilization is by conversion to their corresponding active esters in situ with a carbodiimide coupling agent and an auxiliary nucleophile. The most commonly used example of the former is 1-ethyl-

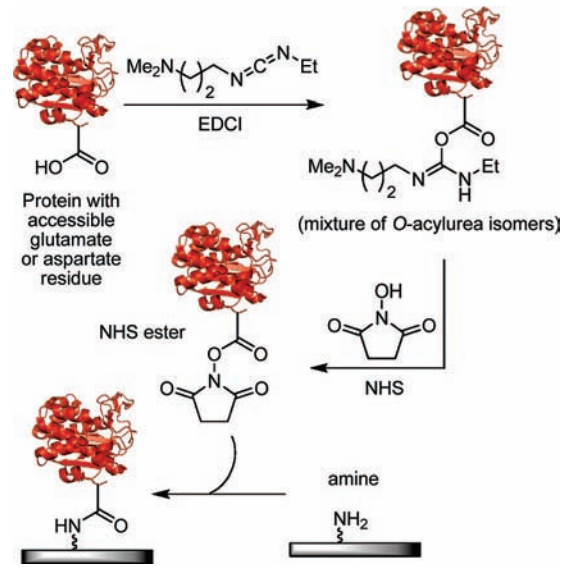

Figure 8. Carbodiimide-mediated chemoligation of Glu and Asp residues on proteins to amine-functionalized materials via the in situ generation of an NHS active ester.

3-(3-dimethylaminopropyl)carbodiimide (EDCI, but also abbreviated EDC or EDAC; and also known as "water-soluble carbodiimide", WSCI, or WSC), while NHS is widely used as the auxiliary to generate the NHS ester on the protein. This active ester can then react with amine-bearing supports (Figure 8). The advantage of this combination of reagents is that both are water-soluble and may be used in aqueous media, although the instability of carbodiimides and the subsequently generated active esters under these conditions means that the reaction yields are rather low. There is also the risk that the NHS esters formed on the protein molecule may then couple to other protein molecules to give poorly defined polymers.

In cases where the protein of interest has been subjected to a post-translational modification, the components in the modification may be used to immobilize the protein. The classical example of this is the immobilization of antibodies through the glycosides present on the C_H^2 domain of the F_C region. Oxidative cleavage of the 1,2-diols on the oligosaccharides (usually with periodate) generates aldehyde moieties that can then be used for attachment in a semispecific manner to hydrazine or hydroxylamine functionalized supports via their respective hydrazone or oxime (Figure 9).[71] In addition to antibodies, this strategy has also been applied to a range of other proteins that feature post-translational glycosylation, including several protease and oxidase enzymes.[72,73] However, it should be noted that, apart from the multiple potential attachment points on a polysaccharide chain, random orientation may also occur if the desired protein has more than one site of glycosylation on its surface.

Thus, other than in a few specific cases, conventional immobilization methods suffer from the general disadvantage of generating a heterogeneous population of presented proteins due to the random orientation of the immobilized biomolecules, a significant number of which will be inactive.[8,10] In cases where exposed carboxy- or amino-bearing residues are used for immobilization, another disadvantage is the coincidental alteration of protein stability. The surface electrostatic charges conferred by carboxy- or amino-bearing surface residues are important in maintaining protein folding and segregation of hydrophobic and hydrophilic domains.

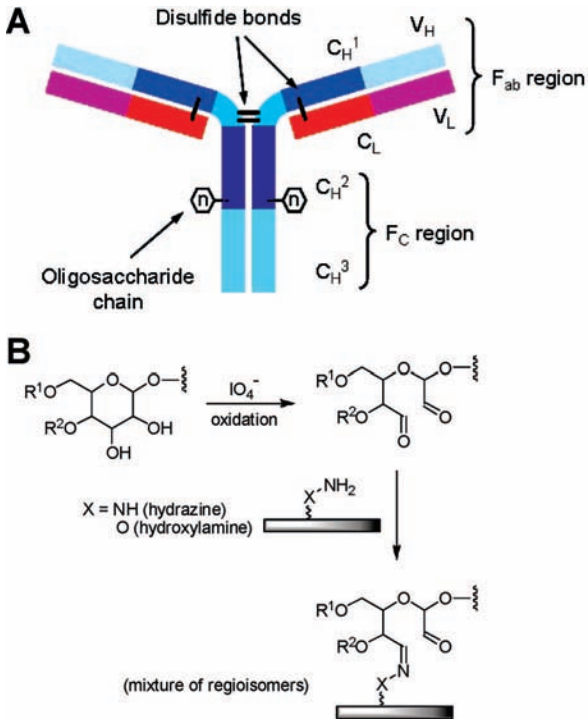

Figure 9. (A) Schematic structure of antibodies illustrating the site of glycosylation on the C_H^2 domains and (B) oxidation of the F_c region oligosaccharides with subsequent attachment to hydrazine or oxime-derivatized supports.

Thus, any immobilization chemistries that result in the loss of these charged groups may have a detrimental effect on protein stability and activity. Furthermore, such methods demand the use of purified proteins since the reactivity and nonspecificity of these methods will also result in the coimmobilization of other impurities that may be present in the mixture.[68] In cases where large numbers of proteins are required for screening purposes, the large number of concomitant purifications needed is extremely costly in terms of resources and time. For these reasons, approaches that enable covalent site-specific immobilization of selected proteins are highly desirable.

3.2. Site-Specific Chemoligation Methods for Immobilization

In recent years, several selective immobilization methods that are bioorthogonal (i.e., where the attachment reaction is unaffected by the regular cellular biochemistry) and able to proceed under mild physiological conditions have received increasing attention. Typically these methods rely on the labeling of the protein of interest with an azide moiety since it is not present in biological systems. In the Staudinger ligation, the reaction of an azide with a phosphine forms an intermediate iminophosphorane (aza-ylide) that can then react with electrophiles to give a variety of products (Figure 10). In the context of chemoligation, the generation of an iminophosphorane is typically followed by reaction with an ester to form a stable amide bond. In the first version of this approach, the electrophilic ester is incorporated into the phosphine to give a final product with the phosphine oxide attached to the linkage. "Traceless" variants of this reaction have also been developed that result in the formation of a native amide bond and extrusion of the phosphine oxide byproduct (Figure 10).[74]

Since its first description by Bertozzi in 2000,[75] the Staudinger ligation has been exploited in a large number of applications from the labeling of proteins and cell-surface glycans to the chemical synthesis (and semisynthesis) of proteins.[74,76] More recently, the Staudinger ligation has also been applied for the immobilization of peptides and proteins. In the first example of this, RNase S was immobilized on microarray slides.[77] RNase S consists of two tightly bound fragments, S-peptide and S-protein, neither of which have any independent enzymatic activity. A truncated form of S-peptide consisting of 15 amino acid residues was chemically synthesized where the ε-amino group of Lys1 was replaced with an azido group. This was then immobilized on to pegylated microarray surfaces bearing diphenylphosphine moieties by the Staudinger ligation (Figure 11). Subsequently, the anchored S-peptide was found to efficiently bind S-protein (103 amino acids in size), and RNase activity was also shown to be reconstituted, indicating that the correct conformation of the complex was achieved. There have also been a number of examples where this chemoligation method has been used to immobilize full-length proteins, although in all cases a two-step process was required: labeling of the protein of interest with the relevant moiety (e.g., the azide) followed by the actual immobilization reaction. Thus, in order to achieve site-selective immobilization, a method for site-selectively introducing the required azide moiety must first be employed, such as an enzymatic reaction that recognizes specific protein sequences. These enzymatically mediated reactions will be described in the following sections.

Another method of selective chemoligation is derived from the Huisgen 1,3-dipolar cycloaddition of azides with an alkyne, where the covalent link is formed through the formation of a 1,4-disubstituted 1,2,3-triazole. This reaction has been popularized as "click" chemistry by Sharpless, and in the most well-known version, a terminal alkyne and azide are reacted with Cu(I) catalysis to give near-quantitative conversions to the triazole.[78,79] (Figure 12) Here, the alkyne moiety is also rarely present in biological pathways and adds further versatility to this reaction since the alkyne may be introduced to the biomolecule instead of the azide. This type of "click" chemistry has been used in a variety of applications to enable the attachment of biomolecules bearing either the azide or alkyne with various polymers, fluorophores, or biochemical labels functionalized with their counterpart moieties. Notably, this has also been used to label the surfaces of living cells.[80−82] Similar to the Staudinger ligation, the site-selective attachment of proteins also requires its use in tandem with an enzymatic site-selective labeling method.

In recent years, a number of copper-free variants of the original "click" reaction have been developed in order to circumvent the cytotoxicity of the Cu(I) catalyst. These employ various functionalized cyclooctynes where the reaction is promoted by strain relief within the eight-membered ring.[80,83−85] Another related family of reactions is "photoclick chemistry", which relies on photoirradiation to trigger pyrazoline formation between tetrazoles and alkenes.[86,87] An added advantage of this method is that the newly formed heterocycle is fluorescent, enabling the monitoring of the reaction progress. Although these are significant developments, to date these have not yet been applied for the purpose of protein immobilization.

A bioorthogonal attachment method based on the Diels−Alder cycloaddition between alkenes and dienes has also been reported.[88] Here, quinones presented on a gold-thiol SAM

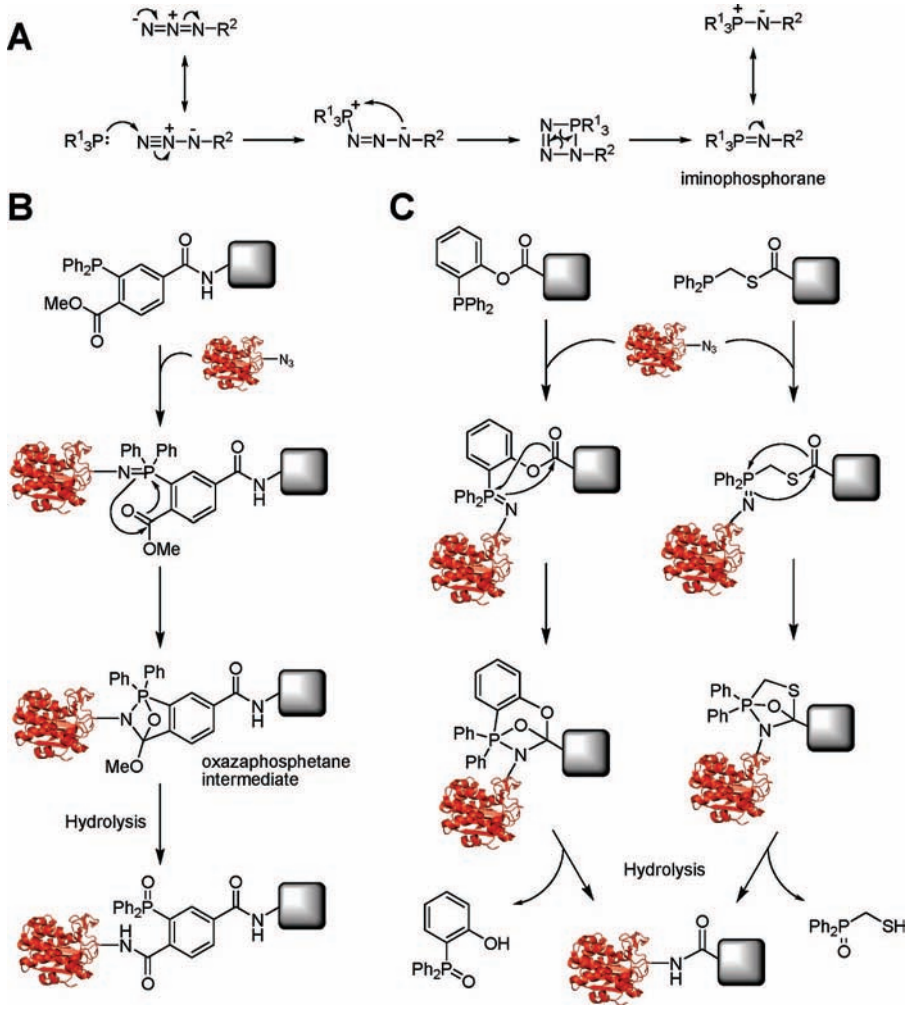

Figure 10. Chemistry of the Staudinger ligation outlining: (A) the formation of the nucleophilic iminophosphorane intermediate; (B) the Staudinger ligation with incorporation of the triarylphosphine oxide; and (C) the "traceless" versions of the ligation where the phosphine oxide moiety is extruded during the reaction, leaving a "native" amide bond.

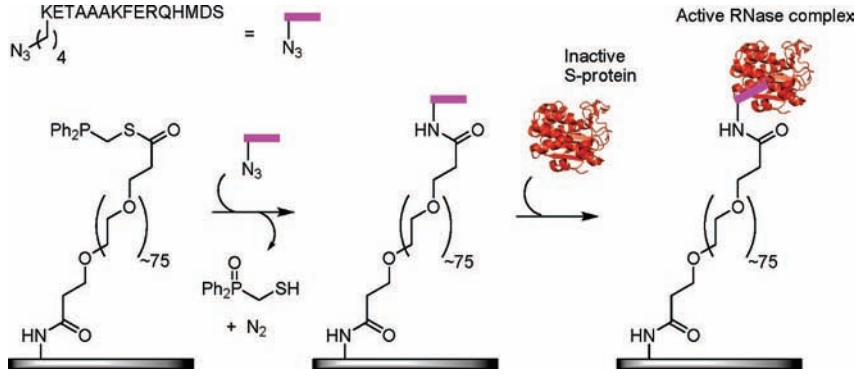

Figure 11. Immobilization of the (N_3)Lys1 S-peptide on to a pegylated phosphine-functionalized microarray slide by Staudinger ligation followed by association of S-protein to form the enzymatically active complex.

were used to immobilize short chemically synthesized cell adhesion peptides, which were appended with a cyclopentadiene moiety (Figure 13). The immobilized peptides were then shown to enable the control of cell adhesion to only the labeled areas of the SAMs. However, this reaction has not yet been applied to full-length biologically active proteins. Recently, another Diels−Alder derived protein labeling method between *trans*-cyclooctene and *s*-tetrazine has been reported.[89] This reaction was shown to be rapid (k_2 = 2000 M^{-1} s^{-1}); although in common with the newer "click" chemistry derivatives, it has not yet been applied specifically toward protein immobilization. These reactions are appealing for protein immobilization since they do not require the addition of any catalyst and both binding partners are stable to ambient and physiological conditions.

4. Biologically Mediated Immobilization Methods

Although classical chemoligation is widely used for protein immobilization in the applications described above, these reactions possess a number of drawbacks related to the nonspecific nature of the underlying attachment chemistry.

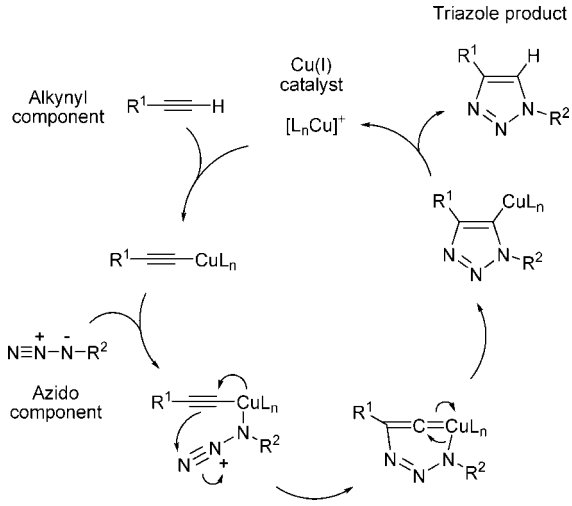

Figure 12. Proposed mechanism of Cu(I)-catalyzed "click" chemistry between a terminal alkyne and azide. This cycloaddition reaction forms the triazole product with the consequent ligation of the R^1 group to R^2.

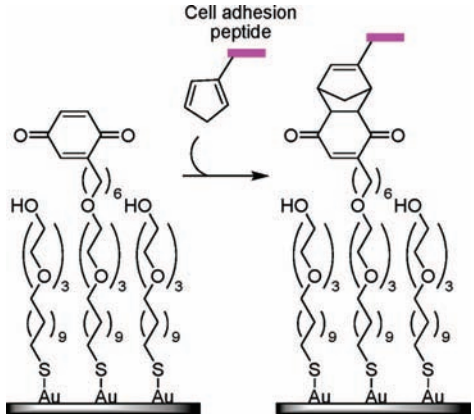

Figure 13. Immobilization of cell adhesion peptides bearing a cyclopentadiene to a SAM presenting quinones through the Diels−Alder cycloaddition.

As a means to address the need for site-specific immobilization, methods based on biological reactions have received increasing attention since these can be highly selective and proceed under mild conditions, thus reducing the risk of protein degradation. Accordingly, the strategies described in this section can be characterized by three main features: (1) The attachment is specific relative to the protein sequence and, therefore, to the location on the protein where the bond is formed. (2) The conditions under which immobilization occurs are mild and have a reduced risk of protein degradation or denaturation. (3) Since the protein is genetically encoded, the attachment site (sequence) can be engineered into the protein using standard molecular biology techniques. The general strategy is, therefore, first to introduce the DNA sequence coding for the tag adjacent to the gene encoding for the protein of interest. Subsequently, expression of the engineered synthetic gene then yields a fusion protein of the original protein of interest attached to the tagging protein or peptide containing the attachment site. This fusion protein is then used for the immobilization procedure.

4.1. Non-Covalent Biologically-Mediated Immobilization Methods

Over the years, a number of protein−protein and protein−small molecule binding interactions have been harnessed for immobilization.[7,12,90−92] These strategies exploit the selectivity of such interactions and are, therefore, highly specific with respect to the identity of binding partners as well as the location on the molecules at which binding occurs. Historically, many of the tags that have been described were developed for protein purification by affinity chromatography, but few have been widely co-opted for immobilization in other applications.

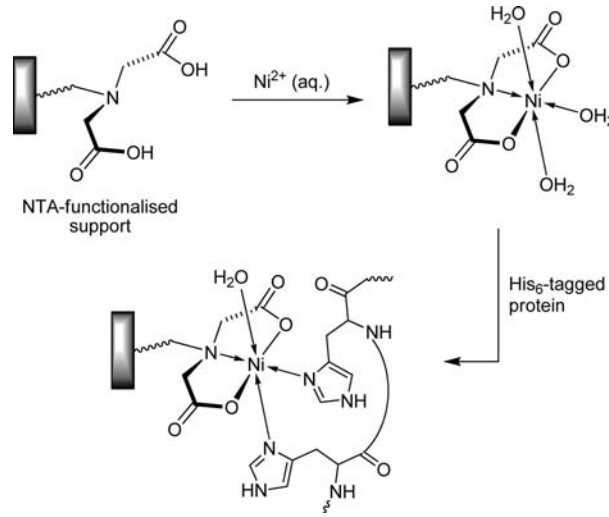

Figure 14. Immobilization of His_6-tagged proteins on to an NTA-functionalized support. The chelating NTA moiety is first "activated" with the addition of a hexacoordinate metal ion (in this example Ni^{2+}) followed by addition of the tagged protein. The protein is immobilized on the support through chelation of the metal ion by the imidazole moieties of the His residues.

Perhaps the most well-known genetically encoded affinity tag is the polyhistidine tag, which was first fully realized by Hochuli in 1988.[93,94] This small tag, usually consisting of six sequential His residues, chelates transition metals including Cu(II), Co(II), Zn(II), or Ni(II), although the latter is most commonly employed. Here, a support bearing a chelating moiety such as nitrilotriacetic acid (NTA) or iminodiacetic acid is treated with a solution of the relevant metal salt to produce a support presenting the metal ions. This metal-activated support is then used for protein immobilization through chelation with the His residues of the tag (Figure 14). This method of immobilization is widely used for the temporary capture of proteins during purification and is often termed immobilized metal affinity chromatography (IMAC).[94,95] The tag may be located at either the *N*- or *C*-termini as well as exposed loops,[96] and its small size means that there is a good degree of flexibility in terms of experimental design. The tag will also continue to function even if the protein is denatured since the complexation to metal ions is not dependent on its secondary structure. All these, coupled with the fact that many commercially available protein expression plasmid vectors include this tag, make this method of immobilization widely used. The ability of these His_6-tags to allow site-selective immobilization has been very well demonstrated in their use to immobilize the 20S proteasome, a multiprotein complex responsible for the degradation of cellular proteins and the generation of peptide epitopes for antigen presentation.[97,98] Here, positioning of the tags on the externally facing H0 helices of the α-subunits gave axially oriented proteasomes, while tagging at the *C*-termini of the *β*-subunits allowed side-on immobilization. (Figure 15) This exquisite example of oriented immobilization has in turn allowed structural information about the

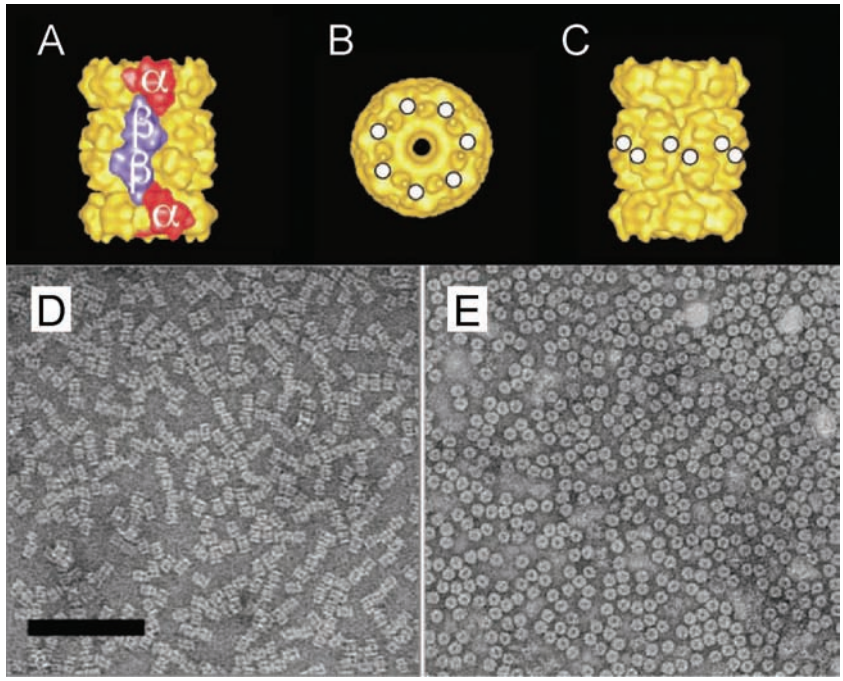

Figure 15. Schematic image of the heptameric 20S proteasome from *T. acidophilum* with (A) representative α- and *β*-subunits highlighted in red and blue, respectively; (B) the location of the His_6-tags included in the H0 helices the α-subunits; and (C) the location of the tags when attached to the *C*-termini of the *β*-subunits. Transmission electron micrographs of the His_6-tagged proteasomes immobilized on metal-chelating lipid films where (D) the proteasomes tagged at the H0 helices of the α-subunits show exclusively head-on orientations and (E) proteasomes tagged at the *C*-termini of the *β*-subunits show only side-on orientations. (Scale bar represents 100 nm.) (Reproduced with permission from ref 98. Copyright 2002 ASBMB.)

proteasome to be deduced. Apart from its application in microarrays and purification, this metal affinity binding has also been applied on nanofabricated SAMs.[99−101]

However, the general level of selectivity of this method is relatively low since several endogenous proteins have been identified that are also able to bind to metal ions, thus competing with the desired His_6-fused protein.[102] As a result, for most applications, such as the proteasome example above, the desired His_6-fusion protein must be purified prior to use. The strength of the binding interaction is also relatively weak ($K_d \approx 1-10\ \mu M$), although proteins bearing tags with 10−12 His residues or two separate His_6-tags have been shown to give improvements of up to 1 order of magnitude, enabling in situ immobilization of the target protein on Ni-NTA slides directly from cell lysates.[26,27]

Antibodies, apart from being the target of immobilization for use in microarrays and biosensors, may also be used as a means of immobilizing other proteins due to the selectivity of their binding interactions. This concept is regularly applied for purification through the use of columns with immobilized antibodies acting to trap their epitope target and is known as immunoaffinity chromatography.[91,103] The method is, however, rarely used for other applications for several reasons. In order to achieve uniform immobilization, a well-defined monoclonal antibody is needed; polyclonal antibodies are unsuitable since they are not a single species but a heterogeneous population of antibodies that bind their epitope in a variety of conformations. Indeed, without in-depth structural knowledge of the binding interaction, it is impossible to determine strength of the binding or if the binding may block the active site of the protein of interest. Furthermore, the antibodies themselves are often attached to the support through nonspecific methods, so well-defined orientations cannot be achieved through this means.

In general, because these selective non-covalent strategies are derived from the area of protein purification, the binding interactions are necessarily reversible and the nature of the reversibility often means that they are often inappropriate for subsequent immobilization in the end application. For example, proteins fused to glutathione-*S*-transferase (GST) may be trapped on a glutathione-agarose gel column during purification and subsequently eluted with a solution of glutathione.[104,105] As a result, the GST-tag on the purified protein that is eluted is already bound to glutathione and cannot easily be used for a subsequent immobilization. Further, the relatively weak nature of the binding means that they are often unsuitable for many applications that require the longer-term or more robust immobilization of proteins. Indeed, in one of the original protein microarray papers by Snyder,[30] even though the library members were all expressed as fusions to GST, this was employed only for affinity purification and the proteins. The purified proteins were later immobilized by covalent binding to aldehyde groups on the slides, or onto Ni-coated slides through their His_6-tags.

Undoubtedly the most well-known and extensively researched protein-mediated immobilization technique relies on the non-covalent interaction of either avidin or streptavidin to proteins functionalized with biotin.[106−109] The protein that bound to biotin was first identified and named "avidin" by Eakin in 1941,[110] but it was not until 1968 that the application of this interaction for selective capture of avidin onto biotin-functionalized sepharose was demonstrated by Wilchek.[111] The converse, the use of avidin immobilized on sepharose for the capture of biotin-bearing proteins, was finally reported in 1975 by Berger.[112] The interaction between biotin and (strept)avidin, is extremely strong ($K_d \approx 10^{-15}$ M), and this combined with the fact that these proteins are unusually stable to heat, denaturants, extremes of pH, and proteolysis

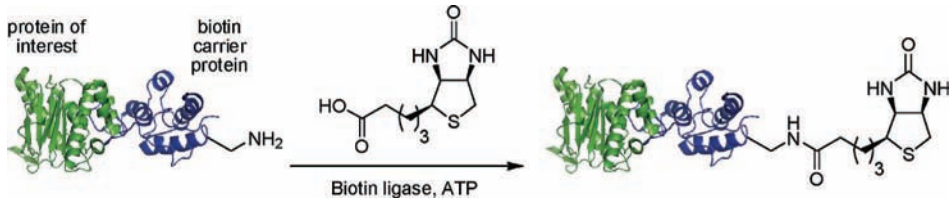

Figure 16. Ligation of biotin to the protein of interest fused to BCCP. This reaction is mediated by the enzyme biotin ligase.

means that the binding is essentially irreversible. The widespread availability of supports such as microtiter plates, microarray substrates, and magnetic particles that are coated with these proteins has also greatly contributed to the popularity of this method as a means of protein immobilization. However, in order to exploit this attachment for protein immobilization, the protein of interest must first be labeled with biotin. Classically, this can be achieved with a number of nonselective chemical biotinylation reagents[107] such as biotin NHS ester, but in common with the bioorthogonal chemoligation methods described above, a method for selective biotinylation of the target protein must still be employed if site-selective attachment (with respect to the location on the target protein) is desired.

It has been known for many years that, in *E. coli* and the yeast *S. cerevisiae*, proteins fused at the *C*-terminal with the 75 amino acid biotin carboxyl carrier protein (BCCP) module from *E. coli* acetyl CoA carboxylase are efficiently tagged by the cells' native biotinylation machinery in vivo. Such BCCP fusions have been harnessed for the site-specific biotinylation of a variety of proteins where the biotin is attached at its carboxylic acid terminal to a conserved BCCP Lys residue via an amide bond.[113] (Figure 16) The enzyme responsible for this ligation in *E. coli*, biotin ligase/synthetase (BirA), has also been used in vitro to label proteins fused with BCCP. This BirAcatalyzed ligation may also be applied to a number of other tags, some as small as 14 amino acids, which may be located at the *C*- or *N*-termini.[114,115] In particular, the 15 amino acid "AviTag" with the sequence GLNDIFEAQKIEWHE (the underlined residue indicating the site of ligation) is rapidly biotinylated by BirA. Such fusion proteins may also be tagged in vivo in cells belonging to other organisms by coexpression of BirA with the desired protein.[116]

However, it should be noted that other endogenous biotinylated proteins, although relatively few, still exist in these organisms, and this lack of complete bioorthogonality necessitates isolation and purification of the desired protein, either pre- or postbiotinylation, prior to immobilization on (strept)avidin-coated supports. In order to address this, other methods of attaching the biotin moiety can be employed by harnessing other heterologous post-translational systems. These are described in later sections. Even so, it should be noted that protein immobilization by this strategy is not strictly homogeneous since avidin (or streptavidin) molecules are themselves usually nonsite specifically bound to the support surfaces. Furthermore, both are functionally active as large tetrameric complexes,[106] and it is unclear if such multivalent attachment together with the potential steric congestion may affect the activity of the immobilized protein of interest.

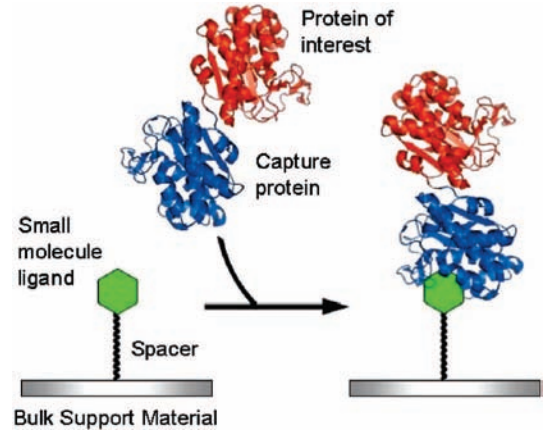

Figure 17. Schematic diagram outlining protein immobilization through an enzymatically active fusion protein that covalently binds the immobilized capture ligand. The protein of interest is thus covalently immobilized onto the support via the fused tagging protein and its ligand.

4.2. Covalet Immobilization via Enzymatically Active Fusion Proteins

One conceptually straightforward method of protein immobilization is through the use of enzymatically active fusion proteins. In this case, a protein of interest is fused to an enzyme (capture protein) that reacts selectively with an immobilized substrate analogue or inhibitor. This reaction forms a covalent bond between the enzyme and the substrate on the surface (Figure 17). Perhaps the first example of this strategy was reported in 2002 by Mrksich, using the serine esterase cutinase from the filamentous fungus *Fusarium solani pisi*.[117] Cutinase is selectively inhibited by alkylphosphonate *para*-nitrophenol esters through esterification of the active site Ser residue, resulting in the formation of a covalent bond. This protein and ligand were chosen because they possessed a number of desirable characteristics for an immobilization technique. The enzyme was relatively small (210 amino acid residues), globular, and monomeric, which would minimize any steric interactions with the fused protein. Both the termini are opposite to the active site and hence would be amenable to the generation of both *N*- and *C*-terminal fusions in which the fused protein would be oriented away from the support surface. Furthermore, the phosphonate diester inhibitor was relatively stable toward hydrolysis, and when bound in the cutinase active site, the alkyl tail of the phosphonate protruded out of the enzyme and offered an accessible location for attachment to the support. In the reported example,[117] the phosphonate inhibitor was immobilized on a gold-thiolate SAM (Figure 18) while the cutinase was fused to calmodulin. Exposure of the surface functionalized with the inhibitor to the fusion protein resulted in immobilization of fusion protein with retention of calmodulin activity. This was demonstrated by its Ca^{2+} dependent binding to calcineurin. Furthermore, selective capture of the desired protein from the periplasmic fraction of a cell

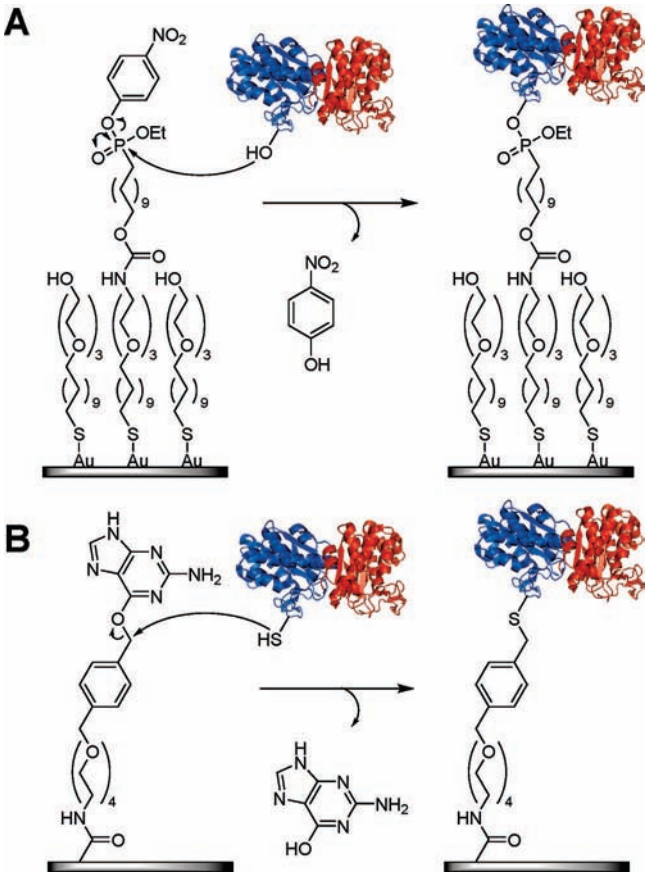

Figure 18. Immobilization of proteins onto surfaces bearing small molecule ligands: (A) capture of cutinase fusion proteins by a SAM displaying 4-nitrophenylphosphonate ester and (B) capture of AGT fusion proteins by O^6-benzylguanine on carboxymethylated dextran surfaces.

lysate was shown, demonstrating that the immobilization was selective between cutinase and its ligand.

This strategy has since been demonstrated for the immobilization of the 10th domain of fibronectin III (FN3), which was subsequently used to direct the adhesion of Swiss 3T3 fibroblast cells and Chinese hamster ovary cells on SAM surfaces.[118] This same surface was also used to immobilize cutinase fusions of antibody fragments including a single-domain fragment (V_{HH}) against lysozyme, single-chain fragments of the variable region (scF_V) against the SH3 domain of human c-Src and the EH domain of frog intersectin, and FN3 domains that recognize the Src SH3 domain and streptavidin.[119] In order to facilitate independent folding of the fused proteins, these fusions incorporated a flexible 15-mer $(GGGGS)_3$ sequence between cutinase and the protein of interest. In all cases, the proteins retained some biological activity as evidenced by their ability to capture their specific antigens.

Another example of using enzymatically active fusion proteins was the use of human O^6-alkylguanine transferase (AGT). In nature, this protein functions in DNA repair by transferring an alkyl group from its substrate, O^6-alkylguanine-DNA, to a Cys residue in its active site. It had already been demonstrated that AGT fusions were site-selectively labeled with O^6-benzylguanine derivatives bearing various fluorophores or biotin,[120] and this strategy was then extended to the immobilization of AGT fusions directly onto carboxymethylated dextran surfaces bearing a modified benzynguanine (Figure 18).[121] This reaction between the fusion protein and the ligand was found to be selective, and the capture of the desired fusion protein from a cell lysate mixture was demonstrated.

The use of AGT fusions was also found to be applicable for the immobilization of proteins for biophysical studies of protein−small molecule interactions.[122] In the first report of this, AGT was fused to cyclophilin D (TCypD), a protein known to bind to the immunosuppressant cyclosporin. The TCypD-AGT fusion protein was immobilized onto gold surfaces presenting the benzylguanine ligand, and the binding of cyclosporin to the immobilized TCypD was studied by surface plasmon resonance (SPR) spectrometry. The results of these experiments showed that the kinetic data on the cyclosporin-TCypD binding were in excellent agreement with the data derived from solution-based experiments with unmodified TCypD, indicating that the formation of the fusion protein and the attachment to the support did not affect the proteins biological activity. In another report,[123] green fluorescent protein (GFP)-tintin and GST fusions to AGT (GFP-tintin-AGT and GST-AGT, respectively) were immobilized on either SAMs or glass slides bearing the benzylguanine ligand. SPR was then used to determine the thickness of the resultant protein layers, and atomic force microscopy was used to measure single-molecule pull-off forces. These experiments showed that the presence of the fused AGT and the immobilization on a surface did not influence the unfolding behavior of either GFP-tintin or GST. The AGT fusion approach has subsequently been applied for the immobilization of proteins on other platforms. In one report, AGT-fusions of acyl carrier proteins (ACPs) from *E. coli* immobilized on microarrays were used to investigate their post-translational modifications by different phosphopantetheine transferase (PPTase) enzymes, yielding insights into the ACP−PPTase interaction.[124] This report thus demonstrated the usefulness of this strategy in the microarray-based profiling of small molecule−protein and protein−protein interactions in post-translational modifications. Notably, much effort has been made to engineer AGT mutants with improved properties. The latest AGT mutants have been optimized to possess increased activity and specificity toward benzylguanine derivatives, reduced size (182 residues), no activity toward DNA, resistance to oxidation, and improved expression properties.[125] The plasmids coding for this fusion protein are also now commercially available under the name "SNAP-tag" by New England Biolabs.

4.3. Enzyme-Catalyzed Protein Labeling and Immobilization

An alternative to the capture protein approach is to use a separate enzyme, typically involved in post-translational modification, to catalyze the transfer of a ligand or affinity label, to a protein or peptide tag fused to the protein of interest (Figure 19). Conceptually, the site-selective labeling of proteins with biotin by BirA[115] can be considered to be an example of this category (see section 4.1). This externally catalyzed strategy offers two potential benefits over the capture protein approach. First, since the enzyme is not incorporated into the final linkage, it may be used in substoichiometric amounts and removed prior to subsequent analysis. Second, the tagging component, or enzyme-recognition site, of the fusion protein may be much smaller in comparison to the capture proteins (e.g., cutinase or AGT) and is much less likely to effect protein folding or otherwise perturb the normal functionality of the protein of interest.

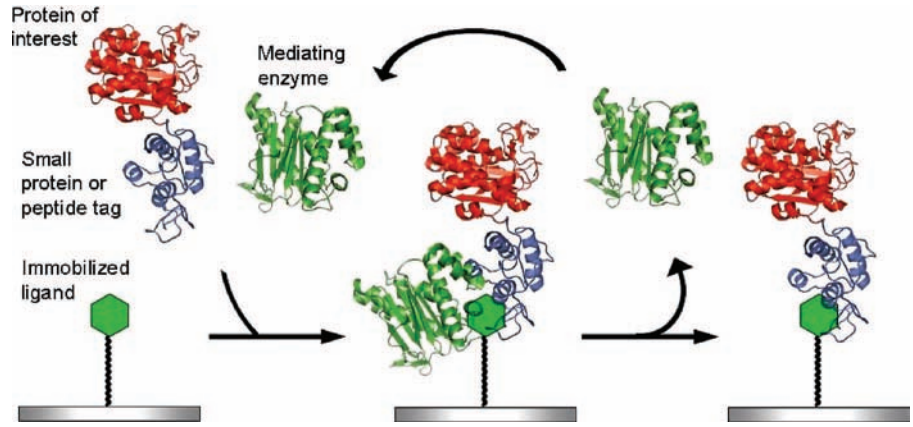

Figure 19. Schematic diagram outlining immobilization through a fusion protein tag catalyzed by a separate enzyme. Here, the tagging protein or peptide does not independently form a bond to the immobilized ligand but depends on a separate mediating enzyme to perform the attachment. Since the mediating enzyme is not also immobilized in this process, a single enzyme may catalyze many immobilization reactions.

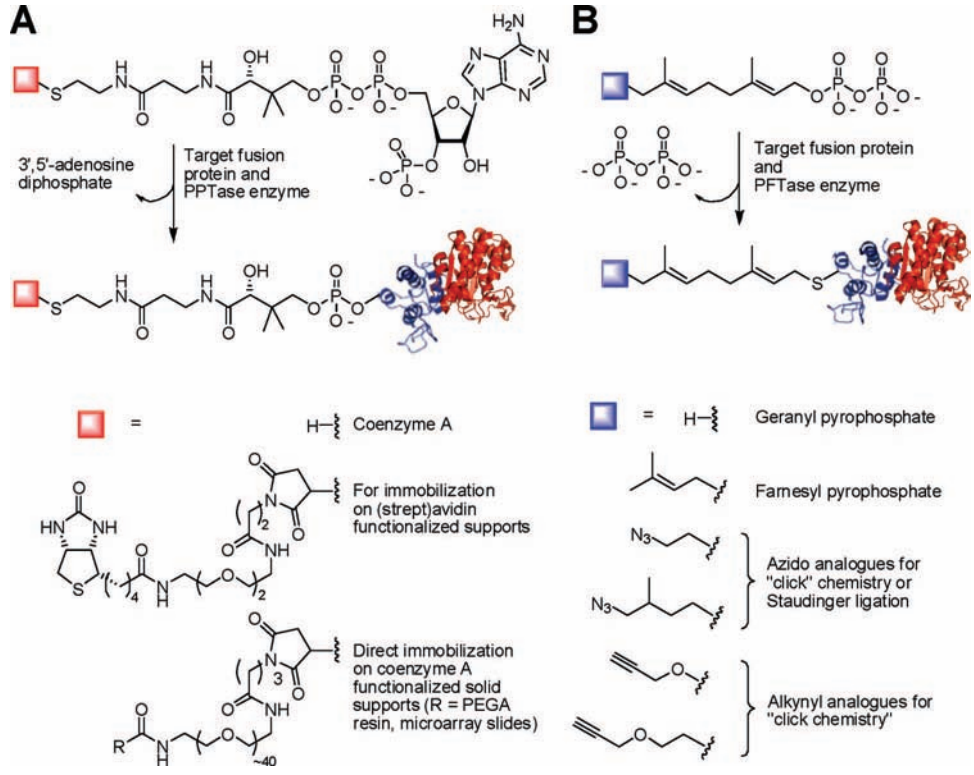

Figure 20. Protein tagging and immobilization through modified post-translational modifications. (A) PPTases catalyze bonding to CoA-ligated supports or biotin through a conserved Ser residue on the fusion protein or tag. (B) PFTase catalyzes bonding to farnesyl or geranyl pyrophosphate analogues bearing azides (for "click" or Staudinger ligations) or alkynes (for "click" chemistry) through the Cys residue of the CAAX box fusion tag.

The first non-biotin example illustrating this concept was reported in 2004 and involved the transfer of phosphopantetheine, derived from coenzyme A (CoA), to peptidyl carrier protein (PCP) domains from nonribosomal peptide synthetase enzymes. In nature, such post-translational modifications of PCPs are mediated by phosphopantetheinyl transferase (PPTase) enzymes (Figure 20). Using Sfp, a relatively promiscuous PPTase from *B. circulans*, it is possible to phosphopantetheinylate a variety of PCPs with CoA analogues bearing various labels such as fluorophores or biotin attached via the *S*-atom of CoA.[126] In the first example of protein immobilization employing this post-translational modification by Walsh,[29] two separate PCPs (each consisting of either 98 or 80 amino acids) were fused to the *N*-termini of three test proteins: enhanced GFP (eGFP), GST, and maltose binding protein (MBP). These were then labeled with CoA-biotin under Sfp catalysis and the biotin-labeled fusion proteins immobilized onto avidin-functionalized microarray slides. Furthermore, it was shown that fusions with β-galactosidase (β-Gal) and luciferase (Luc) that were immobilized on streptavidin microtiter plates maintained their activity. In addition, it was also shown that selective capture of the PCP-fusions was possible from the cell lysate.

While PCPs are relatively small compared to the fusion protein tags described in the previous section, these were still large compared to the affinity tags commonly used in purification such as His_6, Strep-tag (8 residues), or FLAG-tag (8 residues).[90,91] Efforts were therefore made toward developing a minimal recognition sequence that would be phosphopantetheinylated by PPTases such as Sfp. Thus, using

a phage display library of the *B. subtilis* genome, a small 11-mer peptide was identified by Walsh in 2005.[127,128] This "ybbR tag", possessing the sequence DSLEFIASKLA is efficiently phosphopantetheinylated by Sfp at the Ser residue (underlined), with a variety of modified CoA derivatives. Moreover, the ybbR tag was shown to be post-translationally modified when appended to the *N*- or *C*-terminus, as well as when inserted in a flexible loop within the engineered protein of interest. Immobilization was then demonstrated by post-translational modification with CoA−biotin and capture onto streptavidin−agarose beads. Selective labeling of the ybbR-tagged proteins from a cell lysate mixture has also been demonstrated, although in this case a large excess of up to 200 μM of CoA−biotin was used. In a subsequent report, this concept of a minimal tag has been further developed to yield two orthogonal 12-mer peptide tags, "S6" and "A1", which are preferentially labeled by Sfp or AcpS (a PPTase of *E. coli* origin) respectively.[129]

Despite this, all the PPTase-mediated immobilization methods described required two steps, enzymatic tagging of the proteins with biotin followed by the attachment of the tagged protein to the support. In light of this, a direct single-step method for immobilization of ybbR-fusion was then developed (Figure 20).[130] This single-step immobilization was demonstrated on PEGA resin and microarray slides derivatized with CoA for a variety of proteins including the carrier protein BtrI, and ybbR-fused thioredoxin (Trx), Luc, and GST. Under the conditions described in this report, the Sfp-catalyzed immobilization was rapid and the ybbR-GST immobilized site-specifically in this manner exhibited superior activity when compared to nonspecifically immobilized protein on maleimide-derivatized supports. One-step capture of proteins from a cell lysate was also demonstrated. In a more recent report, this type of one-step immobilization has also been described for the attachment of S6- and A1-tagged proteins directly to CoA-functionalized quantum dots.[131] Here, the labeling of tagged cell surface proteins with the quantum dots was also demonstrated.

Another example of a post-translational modification being harnessed for immobilization is the use of protein farnesyltransferase (PFTase). This protein is known to catalyze the attachment of a farnesyl moiety, from farnesyl pyrophosphate, to the thiol group of a Cys residue in a conserved *C*-terminal "CAAX box" farnesylation motif possessing just four amino acid residues. Here, the "C" represents the requisite Cys residue, the "X" is usually Ser, Met, Glu, Ala, or Thr and the "A" positions may be any aliphatic residue.[132] By employing modified farnesyl pyrophosphates bearing suitable bioorthogonal chemical tags, it is possible to selectively modify a protein of interest fused to the "CAAX box" tag and enable its immobilization.

The first two reports of this strategy were simultaneously published in 2006 by Poulter and Distefano. In one, the proteins GST and GFP were fused at their *C*-terminal to the tetrapeptide CVIA motif. These were then post-translationally modified with farnesyl pyrophosphate derivatives bearing azide and alkyne groups under the action of PFTase (Figure 20).[133] The alkyne-tagged proteins were covalently attached to azide-functionalized microarray slides by Cu(I)-mediated "click" chemistry, while the azide-tagged proteins were attached to diphenylphosphinothioester-functionalized slides by a Staudinger ligation. In both cases, a long polyethylene glycol (PEG) spacer was incorporated into the slides between the functional groups and the surface to compensate for the steric hindrance presented by the bulk glass surface. Additionally, the functionalized chains on the slides were presented in a 1:5 ratio relative to nonreactive hydroxy-terminated PEG chains to address any lateral steric hindrance. In the second report,[134] a different azide-functionalized farnesyl pyrophosphate was employed to modify eGFP-CVIA. The protein was then immobilized onto agarose beads bearing alkyne groups through click chemistry. Here, selective immobilization of eGFP from a crude cell lysate mixture was also demonstrated. Similar to the phosphopantethienylation of the PCP fusions described above, an excess of the modified farnesyl pyrophosphate was needed in order to overcome any endogenous ligands present in the lysate. Subsequent reports have also shown that PFTase will also attach geranyl pyrophosphate derivatives onto CVIA-bearing proteins.[135,136] In the reported example, eGFP-CVIA was derivatized with an alkynyl geranyl pyrophosphate and immobilized on to azide-functionalized agarose beads.

Although in principle this "CAAX box" tag allows a high degree of experimental flexibility because a wide variety of amino acids could be employed to construct this motif, the final scope and, therefore, utility of this strategy is unclear since the mechanism by which this tag is recognized by PFTase is not yet well-understood. A variety of proteins ending with this terminal CAAX sequence are predicted in many genome sequences, yet not all proteins containing this motif are farnesylated.[132] There is thus a risk that the desired protein may not be tagged by this method and that undesired proteins may be immobilized instead, or coimmobilized with the desired protein. In the case of the Staudinger ligations to the diphenylphosphine-functionalized microarray slides, a 50:1 DMF/water wash was required to remove any nonspecifically bound proteins. This harsh wash would denature many immobilized proteins and render them inactive. In both the strategies involving phosphopantetheinylation and farnesylation, a significant issue is the possible competition from endogenous CoA or farnesyl (or geranyl) pyrophosphates when tagging is attempted from cell lysates. In cases where a two-step immobilization was employed, this was addressed by the use of a large excess of the desired ligand.

Another strategy reported by Goto that also allows one-step protein immobilization employs microbial transglutaminase (MTG) from *Streptomyces mobaraensis*. MTG catalyzes the transamidation between the amide of a Gln residue on one protein to the ε-amine of a Lys residue of another protein, resulting in a covalent amide bond. Accordingly, by fusing a peptide sequence bearing a Lys-donor recognized by MTG to a protein of interest, it would be possible to covalently link the protein to a support bearing a suitable Gln-donor site. In the first report of MTG-mediated immobilization,[137] the 6-mer Lys-donor tag MKHKGS was fused to the *N*-terminal of alkaline phosphatase (AP). Under MTG catalysis, this protein was then immobilized onto polyacrylic resin (PAR) that had been covalently bonded with casein, a milk-derived protein known to be a substrate for MTG. (Figure 21) Notably, AP activity measurements showed that AP immobilized in this way possessed a higher activity than AP immobilized onto the PAR by nonselective covalent immobilization with amide bonds via NHS esters, indicating a site-selective immobilization where the active sites of the AP were presented favorably. Furthermore, in comparison to immobilization by nonselective physisorption, the MTG-immobilized AP displayed no loss of activity after 10 cycles of assays while the physisorbed AP retained less than 10%

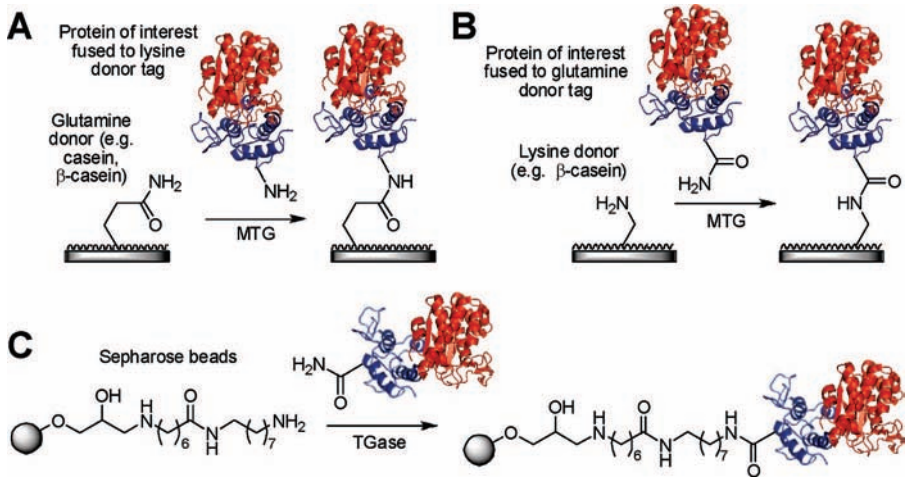

Figure 21. Protein immobilization catalyzed by transglutaminases. (A) MTG catalyzes the attachment of proteins with a lysine donor tag onto supports coated with casein, which acts as a glutamine donor. (B) Alternatively, proteins fused to a tag acting as the glutamine donor can be immobilized to supports with a lysine donor. (C) TGase catalyzes the immobilization of fusion proteins with a glutamine donor onto sepharose beads bearing *n*-octylamine groups.

of its original activity. This strategy was then applied to casein-coated microtiter plates.[138] Further studies into refining this strategy demonstrated that fusions of the tag at the *C*-terminus of AP also allowed immobilization of the protein to agarose beads derivatized with bovine β-casein.[139] The same study also demonstrated that the insertion of a flexible $(GGGS)_2$ spacer between the tag and the protein at the *N*-terminus (i.e., MKHKGS-$(GGGS)_2$-AP) improved the efficiency of the immobilization. Indeed, despite the steric constraints of accessing the polymer, incubation at 25 °C for 3 h with an excess of casein−agarose beads resulted in immobilization of 71% of the protein from the solution. Conversely, it has more recently been shown that it is also possible to use a tag containing the Gln-donor and a support bearing the Lys-donor (Figure 21).[140] Here, it was shown that the previously known Gln-donor sequence LLQG, when fused to the *C*-terminus of either eGFP or GST, enabled the immobilization of these proteins onto β-casein-coated polystyrene microtiter plates. Further optimization experiments on conditions for MTG-catalyzed immobilization indicated that the optimum pH was 5, although the reactions still proceeded at pH 7. This was a notable result since it was previously known that, in solution, this enzyme operated optimally at pH 7 and suggested that the presence of the bulk surface resulted in an increase in the apparent pH at the interface compared to the bulk solution. It was also observed that the immobilization efficiency was inversely related to the ionic strength of the buffers, with the optimum level found to be 5 mM of buffer with no additional salts.

Apart from the direct immobilization of proteins without the need for prior small-molecule labeling and the small size of the tag, this MTG-mediated strategy also offered a number of other practical advantages. MTG is used in industrial food processing and is, therefore, commercially available in bulk quantities (Activa TG by Ajinomoto). In addition, MTG does not require additional cofactors or metal ions for activity. The advantage of using casein as the Gln-donating substrate is that casein itself presents a coating that blocks nonspecific protein adsorption, avoiding the need to use surfactants or other harsh washing reagents, which may denature the proteins of interest. Much research has also been conducted into the structure and mechanism of MTG, and several other Gln- and Lys-donor sequences are known[141,142] that may be used as tags, offering some flexibility in terms of fusion protein design.

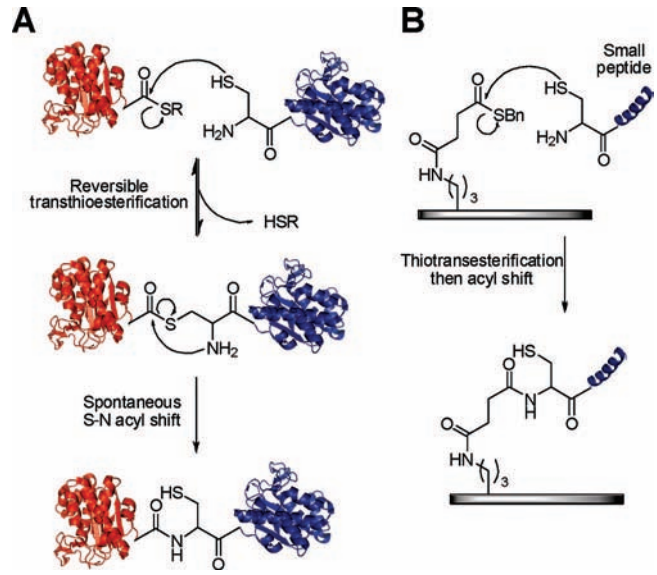

Figure 22. (A) The mechanism of native peptide ligation between a peptide or protein bearing a *C*-terminal thioester (in red) and a peptide or protein with a free *N*-terminal Cys residue (in blue). (B) The use of benzylthioester-derivatized microarray slides for the immobilization of small peptides (in blue) through this strategy.

More recently a similar enzyme from humans, transglutaminase 2 (TGase), has been used for the immobilization of proteins. Previous work with phage-display peptide libraries had identified the 12-residue "T26" peptide (HQSYVDPWMLDH) as a potent Gln-donor. Notably, TGase is promiscuous in relation to its Lys-donor and will accept a variety primary alkylamines. Hence, by fusing the T26 tag to a protein of interest, it would be possible to immobilize the protein onto supports bearing primary amines. This was first demonstrated with cross-linked agarose (Sepharose) gel beads that were functionalized with aminooctyl groups.[143] (Figure 21) Here, the T26 tag was fused to either the *N*-terminus of GST, or the *C*-terminus of the scF_V antibody fragment against bovine serum albumin (BSA) via a $(GGGS)_3$ spacer. Incubation for 30 min at 37 °C with an excess of gel resulted in the capture of 62 and 48% of T26-GST and scF_V-$(GGGS)_3$-T26, respectively, from the reaction solution. It was subsequently shown that the biological activity of the TGase-immobilized GST was

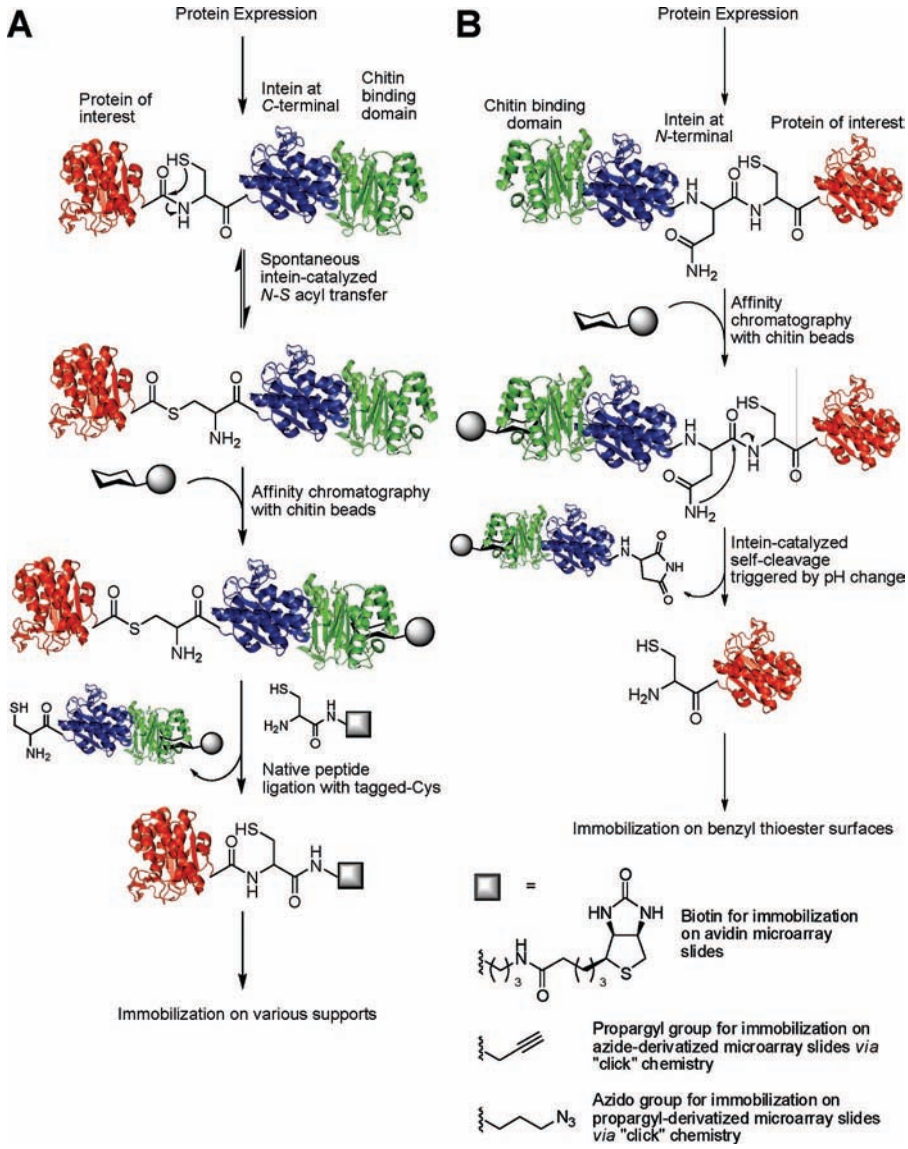

Figure 23. Strategies for employing EPL in the immobilization of intein-fused proteins at the (A) *C*-terminal and (B) *N*-terminal. In the former, the final product is a site-specifically tagged protein, which is then immobilized using strategies specific to the tag, while the latter version results in a protein bearing an *N*-terminal Cys residue, which is then immobilized onto thioester-derivatized supports.

almost double that of the equivalent amount of protein nonsite-specifically immobilized by NHS esters. Similarly, the TGase-immobilized scFv displayed 2-fold higher binding activity for BSA than the antibody-immobilized gel obtained by the conventional NHS ester method.

However, one major drawback of using either of the transglutaminase enzymes is their relatively broad substrate specificity, and any proteins containing surface Lys and Gln residues may potentially react. The extent of this problem is currently unclear but may be significant if a mixture of proteins is used. Indeed, the selective immobilization of a tagged protein from a mixture of other proteins or cell lysate has not yet been demonstrated for this strategy. Furthermore, the target proteins may also present more than one suitable linkage site, resulting in the formation of dimers or polymers of proteins.

4.4. Immobilization Methods Derived from Native Peptide Ligation

Of the peptide ligation chemistries that have been developed to date, possibly the most straightforward and effective is native peptide ligation (NPL) or native thiol ligation. Developed in 1994 by Kent,[144] this method essentially relies on the reaction between a thioester with any Cys derivative where the amino and thiol groups of the Cys residue are free, including proteins with an *N*-terminal Cys. This reaction proceeds through a transthioesterification between the Cys thiol and thioester followed by a spontaneous intramolecular *S*→*N* acyl shift to form an amide bond (Figure 22). The advantages of this method include selectivity for *N*-terminal Cys, the formation of a "native" peptide bond, and the fact that the reaction proceeds under mild physiological conditions without additional coupling reagents. For these reasons, this strategy is widely used in the chemical synthesis and semisynthesis of proteins.[145]

A number of elaborations on this basic theme have been reported in relation to protein immobilization. The first examples of this were demonstrated in the attachment of small synthetic peptides bearing an *N*-terminal Cys to benzyl thioester-functionalized glass microarray slides (Figure 22).[146] However, later versions harnessed the intein-mediated expressed protein ligation (EPL) technique expounded by

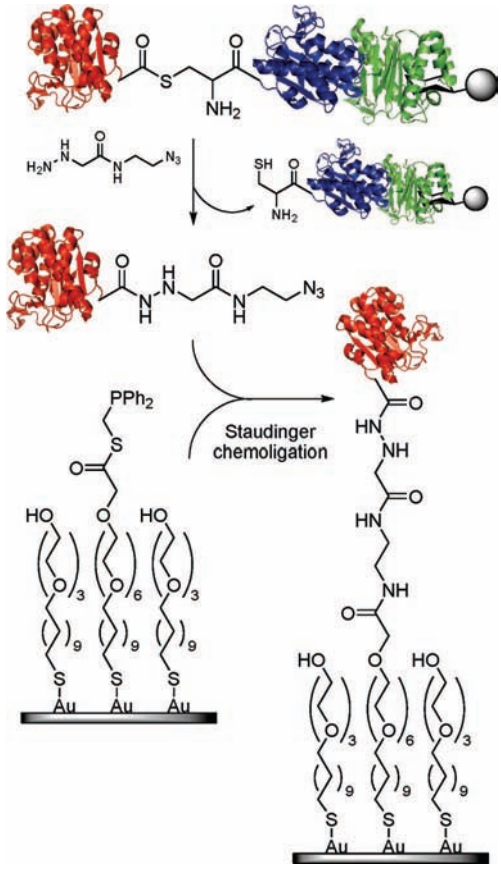

Figure 24. Strategy for the tagging of azides to proteins via EPL with azide-linked hydrazines and subsequent immobilization of the tagged protein onto a diphenylphosphinothioester-functionalized SAM. The intermediate thioester formed in EPL is intercepted by the hydrazine to liberate the protein tagged at the *C*-terminal with an azide group. This azido-protein is then immobilized by a Staudinger ligation to the support.

Muir[147,148] to immobilize recombinant proteins.[28,149−151] In one example, proteins bearing an intein at the *C*-terminal fused to a chitin-binding domain (CBD) were expressed and captured on a chitin affinity column using standard EPL procedures (Figure 23). The column-immobilized fusion protein was treated with Cys-biotin, which displaces the desired protein from the column-bound intein-CBD with the concomitant *C*-terminal labeling of the protein with Cys-Biotin. This purified protein could then be site-specifically immobilized on avidin-functionalized microarray slides.[28,149] Alternatively, it has also been reported that, instead of Cys-Biotin, the internal Cys thioester could be transthioesterified with 2-mercaptoethanesulfonic acid (MESNA).[151] This protein−MESNA thioester could then be immobilized by NPL onto microarray substrates that had been functionalized to present cysteine with free *N*- and *S*-atoms.

As a further variation, the expressed protein with the CBD-intein at the *N*-terminal could be employed. This fusion could be immobilized on a chitin column where a pH change results in the intein-mediated self-cleavage at the Asn-Cys site between the intein and desired protein, resulting in the release of the protein with an *N*-terminal Cys (Figure 23). This purified protein can then be immobilized on thioester-functionalized slides.[149,150] Using these methods, proteins can be site-specifically attached at either the *N*- or *C*-termini by EPL.

Apart from biotin, other tagging moieties may also be employed, with the two most common being azides and alkynes for participation in click chemistry. In the reported example,[152] EPL is used to site-specifically label GFP at the *C*-terminal with either a terminal alkyne or azide. These may then be reacted under click chemistry conditions with the complementary alkyne or azide on microarray surfaces to immobilize the protein. Furthermore, this report compared the site-specific click chemistry immobilization of MBP against nonspecific immobilization through NHS ester-activated slides. The immobilized MPB was subsequently treated with maltose−biotin followed by Cy3-labeled streptavidin. The fluorescence measurements post-treatment demonstrated that the MPB immobilized site-specifically gave a superior signal compared to the nonspecifically labeled protein. In another report it was also shown that proteins that have undergone the *N*→S acyl transfer may be directly reacted with a hydrazide linked to an azide, and these in turn may participate in Staudinger ligations with an immobilized phosphine (Figure 24).[153] Here, RNase A was used as the example protein, and the intermediate thioester was cleaved with a hydrazino−azide to give the azide-tagged protein. This protein was then immobilized on a pegylated SAM bearing diphenylphosphinomethanethioesters.

These EPL-based techniques are particularly elegant since they are "traceless", i.e., it is impossible to determine the method of site-specific attachment based on the examination of the final structure, since it bears none of the protein domains or reagents originally required for the immobilization process. The loss of the mediating intein domains also means that there should be minimal perturbation to the final function of the protein of interest. However, the main issue with these methods is the relatively large size of the intein (454 amino acids), which may interfere with the post-translational processing of the protein of interest (e.g., appropriate folding or post-translational modifications). The large size of the intein also poses a heavy metabolic burden to the expressing organism, potentially lowering protein yields. In fact, in many cases, the intein may be larger than the protein of interest. Another drawback is the slow rate of attack of the incoming reagent and cleavage of the *C*-intein, with the reported procedures requiring incubation periods ranging from overnight to several days.

Another intein-related strategy relies on protein *trans*-splicing, a naturally occurring self-splicing process where the intein domain is split into two fragments (the *N*-intein and *C*-intein). These two fragments are inactive individually but can bind to each other with high specificity to form a functional protein-splicing domain. To harness this for protein immobilization, a strategy has been described utilizing the naturally split DnaE intein from *Synechocystis sp.* PCC6803.[154] Here, the fusion of an *N*-intein (123 amino acid residues) with the protein to be immobilized (in this case, MBP or eGFP) was produced while the *C*-intein (36 residues) was separately generated with the corresponding *C*-extein sequence CFNK and immobilized on to a maleimide-functionalized glass surface. Incubation of the *N*-intein fusion with the *C*-intein glass surface resulted in the immobilization of the MPB, which was subsequently detected by fluorescence (Figure 25). Similar to EPL above, the overall immobilization is "traceless" since all the immobilization machinery is removed from the final product. However, this method is only applicable to immobilization of the target protein at the *C*-terminus. In common with EPL, the immobilization also requires long incubation times (16 h in

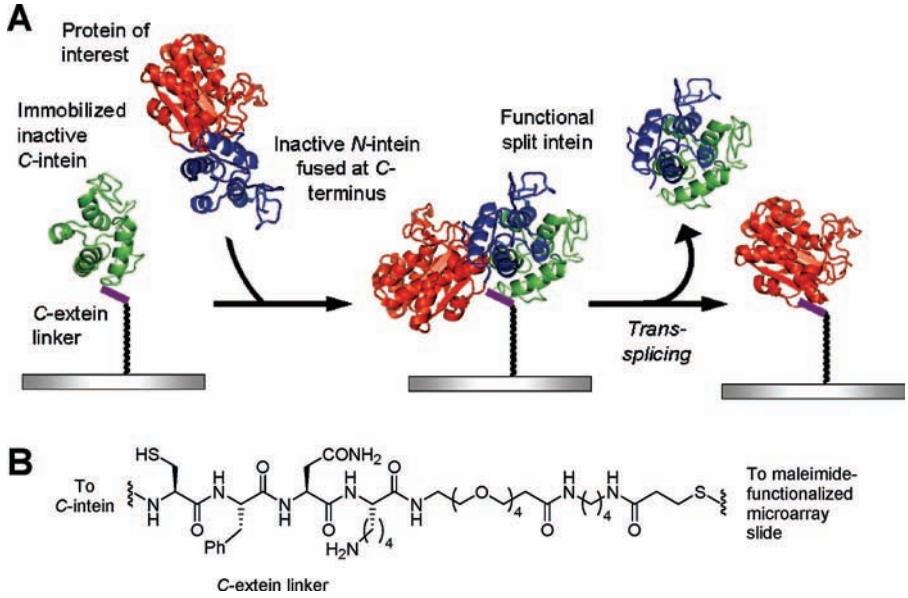

Figure 25. (A) Schematic diagram showing the use of split inteins in the immobilization of proteins with an *N*-intein fused to its *C*-terminus and surfaces functionalized with the *C*-intein and -extein. The *C*- and *N*-inteins are individually inactive but upon binding form an active complex that catalyzes the *trans*-splicing of the protein of interest onto the *C*-extein portion, thereby immobilizing the target protein. (B) Structure of the CFNK-bearing *C*-extein acceptor (represented in A as the purple bar attached to the support material).

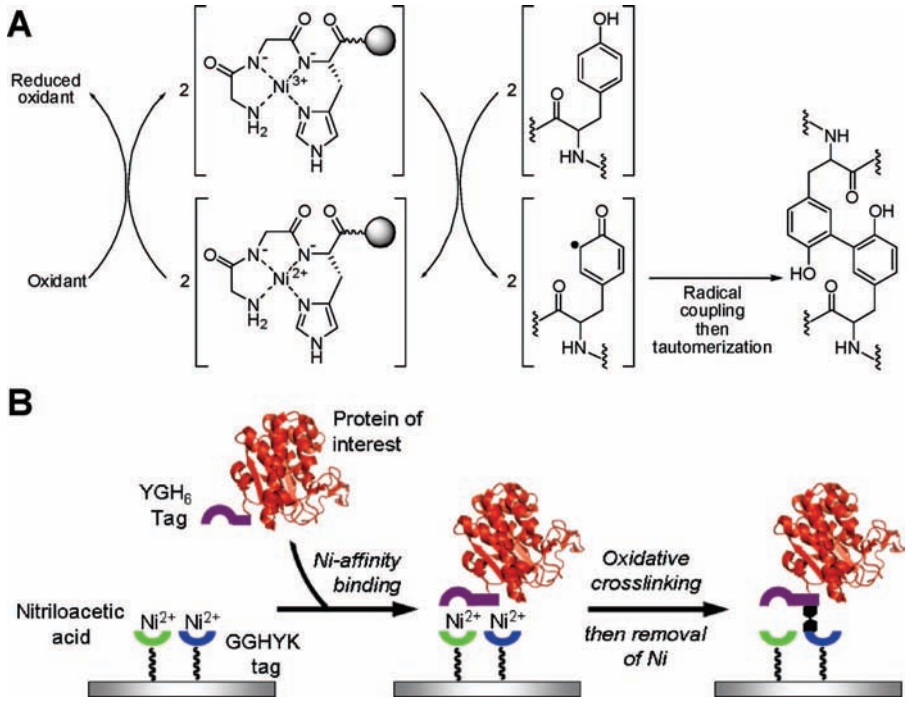

Figure 26. (A) Mechanism of oxidative phenolic cross-linking catalyzed by Ni(II) complexes and (B) a schematic diagram outlining its application to the immobilization of proteins bearing a tyrosine near the polyhistidine tag and supports bearing the GGHYK tag near the Ni-NTA groups. The tagged proteins are first immobilized by metal chelation, but addition of an oxidant initiates covalent cross-linking, after which the Ni(II) may be removed.

a humidified chamber at 37 °C) due the slow rate of trans-splicing, a limitation inherent in all self-splicing proteins.

4.5. Peptide Tags for Non-Enzymatic Site-Selective Immobilization

There are also a number of other site-selective immobilization methods that do not require enzymatically mediated attachment. Similar to NPL, these rely on the recognition of specific functional groups present on amino acids in the protein or an associated peptide tag. One example of this employs phenolic oxidative cross-linking, a phenomenon that is widely observed in nature, which includes protein cross-linking through Tyr residues. Such dityrosine cross-links occur in structural proteins such as elastin and silk and are catalyzed by metalloenzymes, although a simple complex of Ni(II) with the tripeptide GGH can also catalyze these reactions. Since IMAC and immobilization with Ni(II) via His_6 tags and NTA-functionalized supports are already widely used (see section 4.1), it was rationalized that this complex could be used as the cross-linking catalyst instead. By bringing two Tyr residues, one from the protein to be immobilized and another from the support, into close

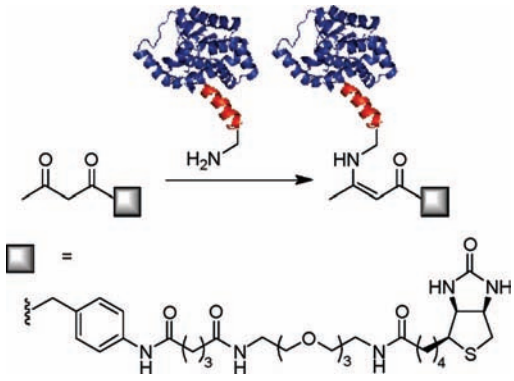

Figure 27. Biotin tagging of recombinant proteins bearing the "rpf1368" tag with 1,2-diketone-linked biotin for subsequent immobilization of avidin microtiter plates. The protein is attached to the biotin tag through an eneamine moiety, which is stabilized by the adjacent ketone.

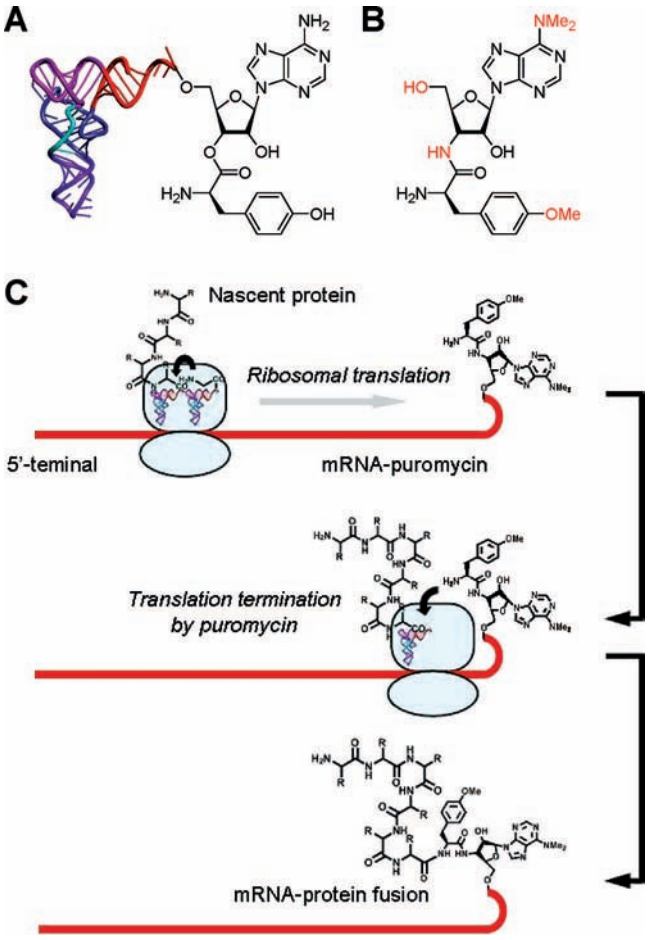

Figure 28. Schematic diagrams of (A) tyrosinyl-tRNA and (B) puromycin, with the main chemical differences highlighted in red. (C) An overview of the application of mRNA-puromycin constructs in the generation of mRNA-protein fusions. Here, at the end of ribosomal translation of the mRNA-puromycin construct, the puromycin is covalently incorporated at the *C*-terminal of the protein resulting in the covalent attachment of mRNA.

proximity to the Ni(II) complex, it would be possible to cross-link the two and form a covalent bond between the support and a specific location on the protein. This principle was first reported using an octapeptide $\underline{Y}GH_6$ tag fused to the *C*-terminus of GFP and PAR microbeads bearing NTA and the peptide GGH$\underline{Y}$K.[155] The immobilization was therefore conducted in two steps: initial non-covalent binding of GFP-YGH_6 to the beads by Ni(II) complexation followed by covalent bond formation by the addition of the oxidant ammonium persulfate (Figure 26). Once the cross-linking had been achieved, the Ni was removed. This method was found to be selective and dependent on the presence of the immobilized NTA and GGHYK groups, as well as Ni(II) ions. The extent of dityrosine cross-linkage could also be easily assessed since this moiety displays a characteristic fluorescence emission at 420 nm. One advantage of this strategy is that any excess or loosely bound proteins and impurities may be eluted from the beads prior to oxidative cross-linking, thus offering in situ purification. In principle, this could therefore be utilized on complex protein mixtures such as cell lysates, although this has not yet been demonstrated.

Because this method relies on His_6-tag binding for the initial immobilization, it is therefore subject to all the advantages and drawbacks associated with their use (see section 4.1). The most significant disadvantage of this strategy, however, is the use of oxidants. Although it was shown that ammonium persulfate did not affect the function of GFP, it is unclear if this reagent may damage other more sensitive proteins such as those with redox functions. It is also possible that other Tyr residues, or other oxidizable residues such as Trp, near the tag will also be attacked by the Tyr radical intermediate, resulting in cross-linking at other locations.

Another example of nonenzymatic immobilization uses a 21-mer tag recognizes and covalently binds to 1,3-diketones.[156] This tag was identified after several rounds of phage display library selection and catalyzes the formation of an enaminone with one of its Lys residues. In a specific example, this "rpf1368" tag (sequence CHNHQKATCRRMRSRETSVKK) was fused to the *N*-terminal of MBP and allowed the labeling of the protein with a biotin derivative bearing 2,4-pentanedione (Figure 27). The biotin-labeled MBP could then be immobilized on avidin-coated microtiter plates. Selective capture of the tagged protein from an unpurified cell lysate was also demonstrated. The biochemistry of this tag was partially characterized, showing that a disulfide bond between the two Cys residues was needed for activity. It was also shown that only one of the Lys residues reacted with the diketone, although its exact location was not reported.

4.6. Translational Level Insertion of Bioorthogonal Tags

The key to the high degree of selectivity displayed by many of the techniques described so far is the introduction of bioorthogonal functional groups, such as azides, alkynes, and ketones. Ideally, such groups would be added directly during ribosomal translation of the mRNA and would, therefore, not be dependent on subsequent post-translational steps. Over the years, a number of strategies have been reported for incorporating "non-canonical" amino acids, i.e., amino acids that are not coded or miscoded on standard tRNA, into ribosomal translation.[157,158] In principle, any of these strategies could be used for the sequence-specific introduction of bioorthogonal tags into a protein of interest, although only a few reports have specifically addressed their application toward protein immobilization. One relatively straightforward means by which this can be achieved is with the use of methionine auxotrophic strains of *E. coli* grown in methionine-free media that is supplemented with an analogue bearing the desired functional group (e.g., azi-

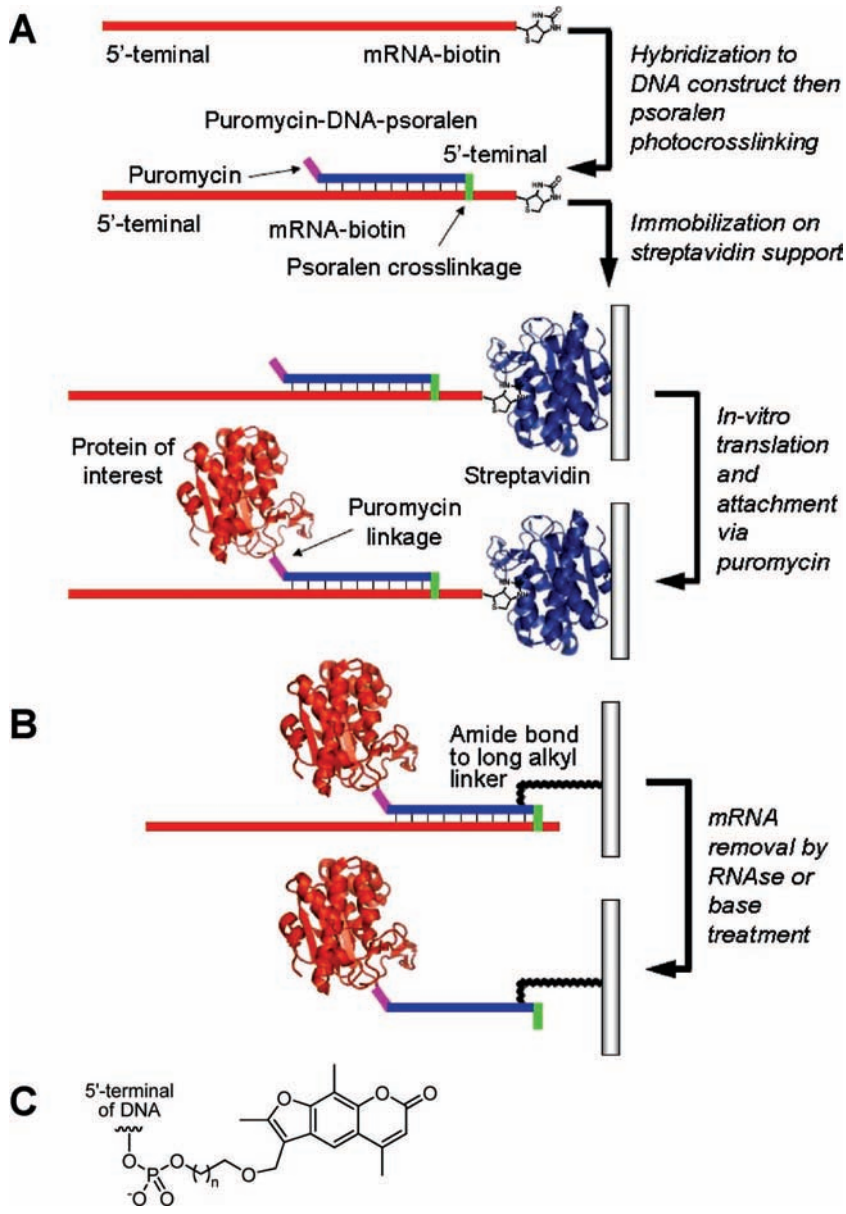

Figure 29. (A) Use of mRNA display libraries for the immobilization of proteins generated through in vitro translation. The proteins are attached to the array surface indirectly via a protein-puromycin-DNA fusion, psoralen cross-linking of the DNA component to the mRNA labeled with biotin, and the interaction of biotin to streptavidin surfaces. (B) A modified version of the strategy involving the covalent attachment of the protein-DNA component to the support, avoiding linkage through mRNA or streptavidin. (C) Structure of the psoralen attached to 5′-terminal of DNA strand.

doalanine). Thus, in the absence of methionine, the analogue is incorporated into its proteins at sites that would have originally been occupied by methionine, giving rise to a protein site-selectively labeled with the unnatural functional group.[159] This strategy will, however, result in multiple incorporation in proteins that possess more than one Met site, as well as incorporation of the synthetic amino acids into other cellular proteins. The degree of incorporation is also incomplete, giving rise to a mixed population of labeled and unlabeled proteins.

A more elegant and stringent method of introducing unnatural amino acid residues would be to engineer an expansion in the RNA codons of the cell translation machinery to accept unnatural amino acids bearing bioorthogonal groups. By incorporating tRNA carrying the synthetic amino acid that recognize the "amber" UAG codon, Schultz has shown that it would be possible to genetically encode for the desired unnatural amino acid to be installed at a specific location on proteins with very high fidelity.[160] The application of this to the immobilization of proteins was first illustrated by the translational incorporation of the *p*-acetylphenylalanine into dihydrofolate reductase (DHFR) and its covalent immobilization via oxime formation between the keto group of the modified Phe residue and hydroxylamine-functionalized microarray slides.[161] The incorporation of other amino acids bearing groups such as azides, alkynes, and thioesters has already been demonstrated, and these may potentially also be applied to protein immobilization. More recently, this basic concept has also been used for the introduction of amino acids bearing a photocleavable biotin group.[162] This enabled the biotin-tagged protein to be trapped onto Neutravidin agarose beads but later released upon photoirradiation.

Although powerful, this method does have a number of disadvantages inherent to the way in which the genetic code is expanded. It relies on the use of the amber nonsense codon

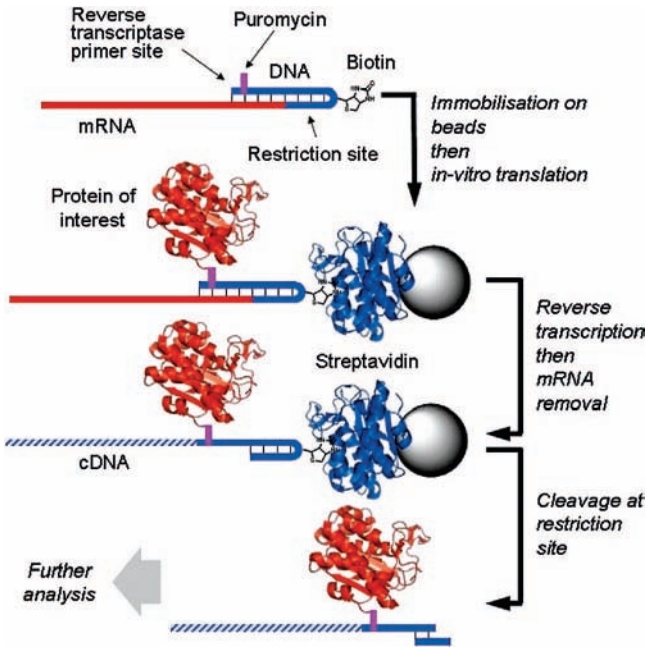

Figure 30. Generation of "cDNA display" libraries. Initially mRNA−DNA constructs are attached via biotin-tagged DNA on to streptavidin-functionalized magnetic beads. In vitro translation followed by termination and attachment to the puromycin results in the immobilization of the protein to the mRNA−DNA construct. Reverse transcription of the mRNA component yields the cDNA that is "displayed".

to code for the unnatural amino acid. Although rarely used by the host organism, it may still result in the incorporation of the amino acid into other proteins, which would be subsequently coimmobilized with the desired protein. This method also requires the availability of the appropriate tRNA bearing the unnatural amino acid, either by direct injection of the synthetically prepared acyl-tRNA or by the use of host strains that have been engineered to produce the tRNA and its cognate amino acyl tRNA synthetase, as well as requiring supplementation with the appropriate unnatural amino acid.

4.7. Protein Immobilization in RNA and DNA Display Systems

Another approach for direct post-translational immobilization of proteins utilizes modified versions of the "mRNA display" technique.[163−165] In this technique, the 3′-end of the mRNA encoding the protein of interest is linked to the antibiotic puromycin via a suitable spacer. The antibiotic is a mimic of tyrosinyl-tRNA, but the Tyr-like component is covalently attached to the ribose moiety of the antibiotic, and thus to the rest of the mRNA chain, through a stable amide bond. During in vitro translation, the ribosome travels down the mRNA while assembling the protein in the usual manner until the end of the mRNA is reached. At this point, the attached puromycin is incorporated into the end of the nascent protein and terminates the translation process. Since puromycin is covalently incorporated into the translated protein through an amide bond with its tyrosinyl amine, the final protein product is covalently attached to the 3′-end of the mRNA at its *C*-terminus (Figure 28). These mRNA−protein fusions can then be hybridized with its complementary DNA (cDNA) strand on a microarray slide, thus allowing the addressing, identification, and assays in standard DNA-microarray format.[166] In order to achieve a covalently immobilized version of this technique, the mRNA-puromycin construct is first immobilized on the support.[167] For example, the mRNA is conjugated with biotin at its 3′-terminus and hybridized with a DNA strand bearing a 3′ puromycin and a 5′ psoralen that allows photoactivatable covalent cross-linking of the mRNA and DNA. This construct can then be immobilized onto a streptavidin microarray slide (Figure 29). Subsequent translation of the immobilized mRNA resulted in the immobilization of the target protein via the 3′-end of the DNA strand. In the same paper, an alternative technique was also described where covalent immobilization through the DNA component of this construct was achieved. This variation, therefore, did not rely on immobilization via RNA, which is more prone to degradation than DNA. Indeed the RNA component could be removed altogether, by RNase digestion or base treatment, prior to further analysis. In addition, the direct covalent DNA linkage to the support circumvented the need for streptavidin (Figure 29). This concept has been even further elaborated with the incorporation of a reverse transcription primer to allow identification of the hybridized mRNA post-translation.[168] These improved DNA-RNA constructs were immobilized on streptavidin magnetic beads to enable high-throughput screening of the translated proteins. Reverse transcription of the mRNA was then used to generate a cDNA tag for each protein, which can be identified by DNA sequencing (Figure 30).

These display methods are particularly applicable to high-throughput screening applications since the facilities for the production of large libraries of RNA and DNA are widely available. The linking of the mRNA (genotype) to the protein that it encodes (phenotype) means that the sequence of any protein of interest may be rapidly decoded. Furthermore, this technology avoids the need to acquire the desired proteins through synthesis or isolation from natural sources. However, it also suffers from the drawbacks associated with in vitro translation including the possibility that the expressed protein may lack essential post-translational modifications or may not be correctly folded because of the absence of the correct cellular environment.

5. Conclusions and Outlook

In this review, a wide range of biologically mediated methods and important applications of site-specific protein immobilization have been discussed. This area of research is notable because it is the culmination of a major multidisciplinary research effort spanning molecular biology, protein engineering, synthetic chemistry, and materials science. Because of the differences in the methodology and underlying biochemistry, it is difficult to draw comparisons on the relative efficiency of the different methods of protein immobilization. Nevertheless, it is likely that no single method will suffice for all applications. In this respect, the plethora of new methodologies that have been developed, and described here, provide researchers with a powerful new "tool box" with which to further develop related applications and devices. A summary of the techniques discussed in this review has been condensed in Table 1.

The development of proteome chip technology, which is used to probe an entire collection of proteins for specific function or biochemistry, has provided a major impetus to this field. While protein arrays offer potential solutions to many fundamental biological questions, the development of robust, reproducible, and fully functional protein arrays has proved a major challenge. The more recent introduction of

Table 1. Summary of Protein Immobilization Strategies Discussed in This Review

Immobilization method	Description	Advantages (+) and disadvantages (−)	
Non-covalent Adsorption (section 3.1.1)			
	The immobilization of proteins on to surfaces due to physicochemical phenomena such as hydrophobic adsorption on hydrophobic materials and electrostatic interactions on charged surfaces.	(+) Universally applicable to any protein, including native proteins	
		Single step immobilization, no labeling of protein required beforehand	
		No coupling reagents required, "reagent free"	
		(−) Reversible immobilization, possible protein leaching from support	
		Immobilization denatures proteins	} Reduced or altered protein activity
		Random protein orientation	
		No control over packing density	
Classical Chemoligation (section 3.1.2)			
NHS ester-functionalized supports	Amide bond formation between protein amino groups and NHS active ester on support. (Figure 7)	(+) Covalent attachment	
		Universally applicable to any protein, including native proteins	
		Single-step immobilization, no labeling of protein required beforehand	
		No coupling reagents required, "reagent-free"	
		(−) NHS esters susceptible to hydrolysis, supports must be carefully stored or freshly prepared prior to use	
		Modest immobilization yields	
		Random protein orientation	
Imine formation on aldehyde-functionalized supports	Imine formation between protein amino groups and aldehyde groups on support (Figure 7).	(+) Universally applicable to any protein, including native proteins	
		Single-step immobilization, no labeling of protein required beforehand	
		Reagent-free (unless reduced amine bond desired)	
		Wide variety of supports	
		No coupling reagents required for imine formation	
		(−) Reversible covalent bond, unless converted to amine with reducing agent	
		Random protein orientation	
Epoxide-functionalized supports	Nucleophilic ring-opening of epoxide by protein amino or thiol groups (Figure 7).	(+) Covalent attachment	
		Universally applicable to any protein, including native proteins	
		Single-step immobilization, no labeling of protein required beforehand	
		No coupling reagents required, "reagent-free"	
		(−) Modest immobilization yields	
		Slow reaction, long immobilization times required	
		Random protein orientation	
Maleimide-functionalized supports	Conjugate addition of thiol on to α,β-unsaturated carbonyls by protein thiol or amino groups (Figure 7).	(+) Covalent attachment	
		Universally applicable to any protein, including native proteins	
		Single-step immobilization, no labeling of protein required beforehand	
		No coupling reagents required, "reagent-free"	
		Selective for thiol groups (amines react slowly)	
		(−) Random protein orientation (unless only one exposed Cys residue)	
Carbodiimide/NHS-mediated coupling	In situ formation of NHS esters on protein carboxyl sites by carbodiimide followed by amide bond formation with supported amine groups (Figure 8).	(+) Covalent attachment	
		Universally applicable to any protein, including native proteins	
		(−) Carbodiimides and NHS esters susceptible to hydrolysis, competing hydrolysis gives poor yields	
		Coupling between proteins gives polymers	
		Random protein orientation	
Periodate oxidation and immobilization of glycopeptides	Oxidation of diols on oligosaccharide chains of glycosylated proteins to dialdehydes that are then immobilized on amino-functionalized supports by imine formation (Figure 9).	(+) Selective only for glycosylated proteins	
		Specific attachment only at glycosylation site	
		(−) Only applicable to glycosylated proteins	
		No control of site of oxidation or subsequent site of attachment on oligosaccharide chain	
		Reversible covalent bond unless converted to amine with reducing agent	
Bioorthogonal Site-specific Chemoligation (section 3.2)			
Staudinger ligation	Reaction between azide-tagged protein and immobilized phosphine generates intermediate iminophosphorane which couples the protein to an immobilized electrophile (Figures 10 and 11).	(+) Site-specific labeling (if azide site-specifically attached)	
		(−) Covalent attachment	
		Immobilized phosphine susceptible to oxidation, supports must be carefully stored or freshly prepared prior to use	
		Not all components commercially available	
		Protein must be labeled with azide prior to immobilization	
"Click" chemistry	Cu(I)-catalyzed triazole formation between azide- and alkyne-tagged partners (Figure 12).	(+) Site-specific labeling (if azide or alkyne site-specifically attached	
		Covalent attachment	
		Either azide or alkyne may be attached to protein, experimental flexibility	
		(−) Protein must be labeled with azide or alkyne prior to immobilization	
		Cu(I) is cytotoxic	

Table 1. Continued

immobilization method	description	advantages (+) and disadvantages (−)
Diels−Alder ligation	Diels−Alder cycloaddition between cyclopentadiene tagged peptide and immobilized quinone (Figure 13).	(+) Site-specific labeling (if cyclopentadiene site-specifically attached) Covalent attachment Electrochemically switchable between unreactive hydroquinone and quinone (−) Method has not been demonstrated for full-length proteins Peptide must be labeled with diene prior to immobilization Not all components commercially available
Non-Covalent Biologically Mediated Immobilization (section 4.1)		
Polyhistidine-tags and His_6-tags	Chelation to immobilized metal-NTA complex by imidazole moieties on His residues (Figure 14).	(+) Site-specific labeling Tag may be at *C*- or *N*-terminus, or inserted in exposed loop of protein Tag widely incorporated into commercial expression vectors Wide variety of supports commercially available (−) Weak non-covalent binding, protein leaching possible, $K_d \approx 1-10\ \mu M$ (although sequential His_6-tags may yield improved results) Relatively poor selectivity compared to other biological methods
Biotin-(Strept)avidin system	Binding between biotin-labeled protein and immobilized (strept)avidin protein.	(+) Site-specific labeling (if biotin site-specifically attached) Very strong non-covalent binding, $K_d \approx 10^{-15}$ M Wide variety of supports commercially available (−) Protein must be labeled with biotin prior to immobilization Large tetrameric (strept)avidin may effect with target protein
Covalent Immobilization via Enzymatically Active Fusion Proteins (section 4.2 and Figure 17)		
Cutinase fusion proteins	Covalent bonding between cutinase and immobilized nitrophenyl phosphonate ester ligand (Figure 18).	(+) Site-specific labeling Covalent attachment Protein may be at *C*- or *N*-terminus Single-step immobilization, no labeling of protein required after expression Immobilization directly from cell lysate, no purification needed (−) Large fusion protein (210 amino acids), high metabolic burden during expression and may perturb target protein activity Ligand susceptible to hydrolysis; supports must be carefully stored or freshly prepared prior to use
AGT fusion proteins	Covalent bonding between engineered AGT and immobilized benzylguanine ligand (Figure 18).	(+) Site-specific labeling Covalent attachment Protein can be at *C*- or *N*-terminus Single-step immobilization, no labeling of protein required after expression Immobilization directly from cell lysate, no purification needed Completely orthogonal to endogenous AGT proteins and DNA Expression vectors and supports commercially available (−) Large fusion protein (182 amino acids), high metabolic burden during expression and may perturb target protein activity
Enzyme Catalyzed Labeling and Immobilization (section 4.3 and Figure 19)		
Biotin ligase (BirA) catalyzed biotin labeling	Protein of interest fused to BCCP or AviTag is site-selectively acylated with biotin by endogenous cellular biotinylation machinery or BirA (Figure 16).	(+) Tag can be at *C*- or *N*-terminus Site-specific labeling at BCCP/AviTag active site In vivo biotinylation possible with BCCP, separate labeling step not needed AviTag small, 15 amino acids (but requires BirA for biotinylation) (−) Protein purification needed to separate endogenous biotinylated proteins Subject to pros and cons of biotin immobilization (see entry above) Externally introduced BirA required for AviTag labeling
PPTase catalyzed attachment of CoA derivatives	Phosphopantetheinyl section of labeled or immobilized CoA transferred by PPTase enzymes (e.g., Sfp, AcpS) to target protein fused to PCP or small acceptor peptide (e.g., ybbR) (Figure 20).	(+) Site-specific labeling Covalent attachment Tag may be at *C*- or *N*-terminus, or inserted in exposed loop of protein Single-step immobilization, no labeling of protein required after expression Immobilization directly from cell lysate, no purification needed ybbR tag small (11 amino acids), minimal perturbation to target protein (−) Interference from endogenous CoA when attempting conjugation directly from cell lysate Externally introduced PPTase required for labeling Ligand susceptible to hydrolysis, supports must be carefully stored or freshly prepared prior to use

Table 1. Continued

immobilization method	description	advantages (+) and disadvantages (−)
PFTase catalyzed labeling with farnesyl/geranyl pyrophosphate derivatives	Farnesyl/geranyl phosphate bearing bioorthogonal tag attached to target protein fused to CAAX box tag by PFTase catalysis. (Figure 20)	(+) Site-specific labeling Covalent attachment Labeling directly from cell lysate, no purification needed CAAX box tag small (4 amino acids), minimal perturbation to target protein Many possible CAAX box sequences, experimental flexibility (−) CAAX box must be at *C*-terminus of protein Possible that other proteins have CAAX box sequence, may require purification prior to labeling Two-step process, attachment of modified farnesyl/geranyl phosphate, then bioorthogonal immobilization Interference from endogenous ligands when attempting conjugation directly from cell lysate Externally introduced PFTase required for labeling Subject to pros and cons of bioorthogonal ligation used (see entries above)
Transglutaminase catalyzed protein immobilization	Transglutaminase (e.g., MTG, TGase) catalyzes transamidation of Gln amide on one protein to the Lys amine of another protein (Figure 21).	(+) Site-specific labeling Covalent attachment Tag may be at *C*- or *N*-terminus Tags as small as 4 amino acids, minimal perturbation to target protein Wide choice of tags, experimental flexibility MTG does not require cofactors Single-step immobilization, no labeling of protein required after expression MTG commercially available (−) Enzyme has relatively low selectivity, may polymerize protein of interest or result in immobilization at more than one location
Methods Derived from Native Peptide Ligation and Protein Splicing (section 4.4)		
Native peptide ligation (NPL)	Transthioesterification between protein with an *N*-terminal Cys and an immobilized thioester, or protein with a *C*-terminal thioester and an immobilized cysteine, followed by isomerization to give an amide bond (Figure 22).	(+) Site-specific labeling Covalent attachment Single-step immobilization, thioester formed or *N*-terminal Cys residue generated during protein isolation Only requires *N*-terminal Cys residue or *C*-terminal thioester, minimal perturbation of target protein (−) Special methods required to produce proteins with *N*-terminal Cys or *C*-terminal thioester (see below)
Expressed protein ligation (EPL) for *C*-terminal immobilization	Intein at *C*-terminal of target protein undergoes an intramolecular transacylation to give an internal thioester that is transferred to a small molecule thiol or a cysteine attached to a bioorthogonal tag by NPL (Figure 23).	(+) Site-specific labeling Covalent attachment Labeling step included with purification process Intein cleaved prior to immobilization of protein ("traceless" immobilization), minimal perturbation to final immobilized protein (−) Only *C*-terminal attachment (see below for *N*-terminal) Two-step process, NPL to bioorthogonal tag, then immobilization Intein very large (454 amino acids), high metabolic burden during expression and may perturb target protein folding Intein cleavage very slow, tens of hours Subject to pros and cons of bioorthogonal ligation used (see entries above)
Expressed protein ligation (EPL) for *N*-terminal immobilization	The *N*-terminal of target protein fused to intein where a pH change results in self-cleavage of intein, releasing protein with *N*-terminal Cys residue which can be immobilized by NPL (Figures 23 and 24).	(+) Site-specific labeling Covalent attachment Intein cleaved prior to immobilization of protein ("traceless" immobilization), minimal perturbation to final immobilized protein (−) Only *C*-terminal attachment (see above for *N*-terminal) Intein very large (454 amino acids), high metabolic burden during expression and may perturb target protein folding (−) pH change may be detrimental to protein of interest Thioester supports susceptible to hydrolysis, supports must be carefully stored or freshly prepared prior to use
Protein *trans*-splicing	*N*-intein of split intein fused to *C*-terminus of target protein while *C*-intein and *C*-extein are immobilized. The binding of *C*- and *N*-inteins form the active splicing domain which transfers the protein to the immobilized *C*-extein (Figure 25).	(+) Site-specific labeling Covalent attachment All intein components removed after immobilization ("traceless" immobilization), minimal perturbation to final immobilized protein Single-step immobilization, no labeling of protein required after isolation No coupling reagents required, "reagent-free" Relatively small *N*-intein component (123 amino acids)

Table 1. Continued

immobilization method	description	advantages (+) and disadvantages (−)	
		(−) Only *C*-terminal attachment	
		Splicing very slow, tens of hours	
		Nontrivial production of supports with immobilized *C*-intein and -extein	
Non-Enzymatic Biologically Mediated Covalent Immobilization (section 4.5)			
Tyrosine cross-linking	Ni(II)-Catalyzed oxidative cross-linking between Tyr residue adjacent to His_6-tag on protein and Tyr residue adjacent to NTA on support material (Figure 26).	(+) Site-specific labeling	
		Covalent attachment	
		Tag may be at *C*- or *N*-terminus, or inserted in exposed loop of protein	
		Immobilization directly from cell lysate, in situ purification possible	
		Small tag (8 amino acids including His_6-tag)	
		Subject to pros and cons of His_6-tagging (see entry above)	
		(−) Oxidation step may be incompatible with some proteins	
		Possible linkage at other oxidizable residues	
"rpf1368" Tag for 1,3-diketones	Enamine formation between amino moiety of Lys residue in tag and bioorthogonal tags bearing 1,3-diketones (Figure 27).	(+) Site-specific labeling	
		Small tag (21 amino acids)	
		Labeling directly from cell lysate, no purification needed	
		(−) Only *N*-terminal tagging demonstrated	
		Potentially reversible covalent bond	
		Two-step process, attachment of diketone tag then bioorthogonal immobilization	
Insertion of Bioorthogonal Tags at Ribosomal Translation (section 4.6)			
Methionine substitution in methionine auxotrophic *E. coli*	Protein expression by methionine auxotrophic *E. coli* in methionine deficient media incorporate supplemented unnatural amino acids bearing bioorthogonal tagging groups into Met sites of proteins.	(+) Single-step immobilization, no labeling of protein required after isolation	
		Bioorthogonal tag incorporated directly into protein, no additional amino acids needed	
		Relatively straightforward to implement	
		(−) Low incorporation of tag amino acid into protein, mixture of labeled and unlabeled proteins	
		Multiple labeling in proteins with more than one Met residue	} Relatively low selectivity
		Label incorporated into other cellular proteins, purification needed	} Relatively low selectivity
		Subject to pros and cons of bioorthogonal ligation used (see entries above)	
"Amber" codon use for incorporation of unnatural amino acids	Incorporation of unnatural amino acids bearing bioorthogonal tagging groups by employing the "amber" nonsense codon and cognate tRNA to encode for the additional amino acid.	(+) Site-specific labeling	
		Extremely high fidelity incroporation	
		Single-step immobilization, no labeling of protein required after isolation	
		Bioorthogonal tag incorporated directly into protein, no additional amino acids needed	
		(−) Technically complex, need to supply desired acyl tRNA	
		Subject to pros and cons of bioorthogonal ligation used (see entries above)	
Protein Immobilization through DNA and RNA Display (section 4.7)			
	In vitro translation of mRNA-puromycin conjugate generates target protein linked to mRNA *via* the puromycin moiety. The mRNA-puromycin-protein construct is then immobilized on arrays or bead libraries (Figures 28−30).	(+) Site-specific attachment of mRNA to protein (protein−DNA attachment variants also possible, "DNA display")	
		In situ mRNA/DNA attachment at end of translation, no additional labeling step required after translation	
		Very high throughput, *en mass* one-pot translation of multiple mRNA conjugates possible followed by hybridization and immobilization on cDNA array	
		Variations with covalent attachment to array possible	
		(−) Only *C*-terminal attachment	
		Technically complex, combination of multiple techniques	
		Resource demanding	
		Subject to pros and cons of in vitro translation	

biologically mediated methods for site-specific, particularly enzyme-mediated, immobilization of proteins from cell lysates should enable the future fabrication of more highly sensitive protein microarrays and biosensors as wells as finding new applications in nanotechnology and single-molecule enzymology.

Many of the immobilization strategies presented here are extensions of methods of site-specific protein labeling;[169] it is therefore expected that future strategies for the covalent immobilization of proteins will continue to exploit these new bioorthogonal reactions and methods of site-specific protein modification. In this respect, there have been a number of recent reports detailing new protein sequences that can be selectively tagged that were not discussed here.[170−173] These include the sortase transpeptidase-mediated ligation,[174,175] the formylglycine generating enzyme (FGE) approach,[176,177] and the use of lipoic acid and biotin ligases for protein post-translational modification with lipoic acid[178] and biotin analogues.[179,180] No doubt the application of these methodologies for protein immobilization will be a subject of future research. Finally, many proteins within the cell exist and function as part of larger biological assemblies. Therefore, other future challenges in this field will include the functional immobilization of multimeric protein complexes, membrane-bound proteins, and other higher-ordered structures.[181−183]

These are active areas of research, and new strategies to tackle the issues related to these proteins will undoubtedly be developed in future.

6. List of Abbreviations

ACP	acyl carrier protein
AGT	O^6-alkylguanine transferase
AFM	atomic force microscopy
AP	alkaline phosphatase
β-Gal	β-galactosidase
BCCP	biotin carboxyl carrier protein
BirA	biotin ligase/synthetase (*E. coli*)
BSA	bovine serum albumin
BSE	bovine spongiform encephalopathy
CBD	chitin binding domain
cDNA	complementary DNA
CJD	Creutzfeldt−Jakob disease
CoA	coenzyme A
DHFR	dihydrofolate reductase
EDCI	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA	ethylenediaminetetraacetic acid
eGFP	enhanced green fluorescent protein
FN3	Fibronectin III
GFP	green fluorescent protein
GST	glutathione *S*-transferase
IMAC	immobilized metal affinity chromatography
Luc	Luciferase
MESNA	2-mercaptoethanesulfonic acid
MBP	maltose binding protein
MTG	microbial transglutaminase
NHS	*N*-hydroxysuccinimide
NPL	native peptide ligation
NTA	nitrilotriacetic acid
ORF	open reading frame
PAR	polyacrylic resin
PCP	peptidyl carrier protein
PEG	polyethylene glycol
PFTase	protein farnesyltransferase
PPTase	phosphopantetheine transferases
mRNA	messenger RNA
SAM	self-assembled monolayer
SARS	severe acute respiratory syndrome
scFv	single-chain fragment of the variable region of an antibody
SPR	surface plasmon resonance
TCypD	Cyclophilin D
TGase	transglutaminase 2 (human)
Trx	Thioredoxin
TOR	target of rapamycin (protein)
V_{HH}	single-domain antibody fragment

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