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Bioorthogonal Chemistry for Site-Specific Labeling and Surface Immobilization of Proteins

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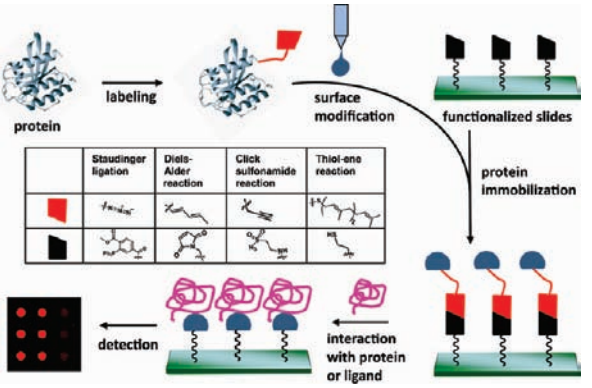

Understanding protein structure and function is essential for uncovering the secrets of biology, but it remains extremely challenging because of the high complexity of protein networks and their wiring. The daunting task of elucidating these interconnections requires the concerted application of methods emerging from different disciplines. Chemical biology integrates chemistry, biology, and pharmacology and has provided novel techniques and approaches to the investigation of biological processes. Among these, site-specific protein labeling with functional groups such as fluorophors, spin probes, and affinity tags has greatly facilitated both in vitro and in vivo studies of protein structure and function.

Bioorthogonal chemical reactions, which enable chemo- and regioselective attachment of small-molecule probes to proteins, are particularly attractive and relevant for site-specific protein labeling. The introduction of powerful labeling techniques also has inspired the development of novel strategies for surface immobilization of proteins to create protein biochips for in vitro characterization of biochemical activities or interactions between proteins. Because this process requires the efficient immobilization of proteins on surfaces while maintaining structure and activity, tailored methods for protein immobilization based on bioorthogonal chemical reactions are in high demand.

In this Account, we summarize recent developments and applications of site-specific protein labeling and surface immobilization of proteins, with a special focus on our contributions to these fields. We begin with the Staudinger ligation, which involves the formation of a stable amide bond after the reaction of a preinstalled azide with a triaryl phosphine reagent. We then examine the Diels–Alder reaction, which requires the protein of interest to be functionalized with a diene, enabling conjugation to a variety of dienophiles under physiological conditions. In the oxime ligation, an oxyamine is condensed with either an aldehyde or a ketone to form an oxime; we successfully pursued the inverse of the standard technique by attaching the oxyamine, rather than the aldehyde, to the protein. The click sulfonamide reaction, which involves the Cu(I)-catalyzed reaction of sulfonylazides with terminal alkynes, is then discussed. Finally, we consider in detail the photochemical thiol–ene reaction, in which a thiol adds to an ene group after free radical initiation.

Each of these methods has been successfully developed as a bioorthogonal transformation for oriented protein immobilization on chips and for site-specific protein labeling under physiological conditions. Despite the tremendous progress in developing such transformations over the past decade, however, the demand for new bioorthogonal methods with improved kinetics and selectivities remains high.

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Introduction

The characterization of function and interaction in networks of proteins is crucial for an understanding of biological processes. However, this task is extremely challenging due to the high complexity and diversity of proteins after their maturation, folding, and posttranslational modification in cells. Although numerous established biological tools have been successfully developed to meet this goal, a need for alternative novel techniques persists.[1]

An increasingly important field of chemical biology is the development of new strategies and methods for modification of proteins via attachment of additional functional groups, such as fluorophores (either proteins or small molecules), spin probes, photoactivatable groups, or affinity tags. Protein labeling techniques together with biophysical and biochemical methods have facilitated the study of protein structural dynamics, interaction networks, and enzymatic activity.[2,3] In addition, protein labeling with fluorophores in combination with modern microscopy techniques has enabled tracking of protein localization in cells, imaging of protein interactions with other cellular components, as well as the visualization of protein expression, trafficking, and degradation in response to regulatory signals.[4]

Site-specific protein labeling has distinct advantages over random protein labeling. Established methods employ the native functionalities of protein residues for chemical modification. For example, the thiol group of cysteine can selectively be alkylated with maleimides or α-halo acids, the ε-amine group of lysine can react with *N*-hydroxysuccinimide (NHS) esters or isocyanates, and an *N*-terminal cysteine can be targeted with aldehydes.[5] However, proteins usually contain several residues with similar reactivity complicating site-specific labeling.

To meet this challenge, a variety of new strategies for site-specific protein labeling has been developed. Notable examples are the application of novel chemoselective reactions such as particular metal-mediated transformations to modify native protein residues,[6] the fusion to genetically encoded functional tags (such as the green fluorescent protein and its variants),[7] the incorporation of unique amino acids into sequences by means of genetic methods such as amber codon suppression mutagenesis,[8] the use of enzyme-catalyzed protein modifications,[9] and the application of bioorthogonal chemical reactions for specific attachment of small molecule probes.[10] Bioorthogonal chemical reactions proceed under physiological conditions and are compatible with both the native functional groups of biomolecules and with biological media. Relevant examples of bioorthogonal chemical reactions include the Staudinger ligation,[11] the Cu(I)-catalyzed or strain promoted [3+2] azide−alkyne cycloaddition,[12,13] and native chemical ligation.[14]

In addition to applications in biochemistry and cell biology, chemically modified proteins can also be used for surface immobilization to create protein biochips. Protein biochips provide an effective and efficient platform mainly for in vitro characterization of biochemical activities or interactions of proteins in a high-throughput manner, only requiring small quantities of samples.[15] The feature that distinguishes biochips from microtiter plate solution phase assays is the attachment of proteins to a planar surface.[16] By immobilizing proteins on solid surfaces, thousands of samples can be evaluated on a single chip without tedious separation or purification. However, since proteins are generally delicate and sensitive, protein biochips require milder conditions and more tailored immobilization methods than DNA microarrays, in order to preserve the native conformation, integrity, and biological function of the immobilized proteins.[17] Thus, reactions required for protein immobilization should proceed chemoselectively and preferably under aqueous conditions, to avoid any potential denaturing cosolvent and additives, such as catalysts, that may be harmful for proteins. In this respect, protein immobilization strategies have strongly benefited from significant advances in the development of new site-specific protein labeling methods. Additionally, since protein immobilization proceeds in a two-phase system, extra factors that affect this heterogeneous process, such as chemical or physiological properties of the surface, have to be considered.[17]

Similarly to labeling, protein immobilization can be achieved via either random or site-specific attachment. Both covalent and noncovalent chemical strategies have been used to generate protein biochips with random immobilization. In the covalent nonspecific immobilization, native functionalities of proteins, such as the side-chain amino group of lysine, or the thiol group of cysteine, directly react with functionalized surfaces containing corresponding reactive functionalities such as NHS esters, aldehydes, epoxides, or maleimides. Alternatively, proteins can be nonspecifically and noncovalently linked to surfaces such as nitrocellulose membranes and glass slides modified with nitrocellulose or poly(L-lysine) through hydrophobic, polar, or ionic interactions.[18] Although these straightforward methods do not require extensive protein modification, random protein

immobilization suffers from the risk of blocking the proteins active sites thereby decreasing activity, which may result in false negative signals and heterogeneous data. Furthermore, random protein orientation may impact protein interactions and function.[19]

In the light of these facts, site-specific protein immobilization has recently attracted substantial attention in the development of protein biochips. This approach can generate homogeneous protein layers as well as guarantee accessibility to the binding site after oriented immobilization of proteins via a site distant to the active center.[18] Site-specific protein conjugation strategies can also be divided into covalent and noncovalent binding. Two common noncovalent protein immobilization strategies are the immobilization of proteins fused to a polyhistidine (His_6) affinity tag on nickel-coated chips[20] and the conjugation of biotinylated proteins with either avidin- or streptavidin-modified slides.[21] Nevertheless, in terms of linkage stability and protein activity, covalent site-specific protein immobilization methods are particularly advantageous since they avoid undesired protein dissociation. Generally, these methods include two key steps, that is, the site-specific protein modification with non-native chemical functionalities or tags, followed by the conjugation of the activated proteins with appropriately modified chip surfaces through bioorthogonal chemical reactions or enzyme-mediated transformations.

In this Account we primarily discuss our contributions to the repertoire of methods for site-specific protein modification and protein immobilization on surfaces.

The Staudinger Ligation for Protein Labeling and Immobilization

For selective covalent protein immobilization, the incorporation of biocompatible non-native functional groups or tags into proteins using, for instance, amber codon suppression mutagenesis,[8] native chemical ligation,[14] metabolic engineering,[22] and enzymatic attachment[9] is a viable strategy. However, bioorthogonal chemical reactions that can be applied for protein immobilization have to be robust, high yielding, fast, and chemoselective at low protein concentration under reaction conditions compatible with biological systems. Only few methods meet these demands.

The Staudinger reaction is a classical method for the reduction of an azide to an amine through the formation of an aza-ylide intermediate by treatment with a phosphine.[23] In 2000, Bertozzi and Saxon developed a triaryl phosphine reagent containing an electrophilic carbonyl group that can trap the aza-ylide intermediate and finally form a stable amide bond in aqueous media.[11] Since this modified Staudinger reaction generates a stable linkage of two molecules, it is termed as Staudinger ligation. The exquisite bioorthogonality of azides and triarylphosphines, which are absent in biological systems, as well as the high ligation yield under physiological conditions have enabled the use of the Staudinger ligation for in vitro and in vivo labeling of biomolecules.[11,24] Raines et al. demonstrated the suitability of the Staudinger ligation for protein immobilization by means of a two-step process in which a peptide bearing an azide group was first immobilized on a phosphinothioester derivatized surface via Staudinger ligation followed by noncovalent binding of a protein to the peptide sequence.[25]

We envisaged that the combination of expressed protein ligation (EPL) with Staudinger ligation could serve as useful platform for site-specific covalent immobilization of proteins on biochips.[26] EPL was first developed by Muir et al. for protein semisynthesis.[27] It generates a recombinant protein thioester by thiolysis of an intein fusion protein which reacts with a synthetic peptide bearing an *N*-terminal cysteine to yield a native amide bond (native chemical ligation; NCL[14]). Thus, by means of EPL, proteins can be modified at the C- or N-terminus and directly immobilized on appropriate functionalized slides.[28,29] Lin et al.[30] demonstrated that C-terminal alkyne-modified maltose binding protein (MBP) generated by means of EPL could be successfully immobilized on azide-functionalized slides through Cu(I)-catalyzed 1,2,3-triazole formation. By analogy, a protein thioester could be ligated with a peptide containing an N-terminal cysteine and an azide group, and the resulting protein was subsequently immobilized on a phosphine-functionalized glass surface by Staudinger ligation. To prove the concept of combining EPL and Staudinger ligation for protein immobilization, the strategy was first applied for the generation of a Ras protein microarray.[26] Ras proteins regulate numerous cellular programs and are involved in the establishment of cancer.[31] The hypervariable C-terminal region of Ras proteins is essential for mediating protein–membrane interactions but has less influence on Ras–effector interactions,[31] which makes Ras proteins tolerant to C-terminal modification by EPL. Accordingly, the thioester of a C-terminally truncated N-RasΔ9 protein generated by thiolysis of an intein fusion protein was ligated with a compound embodying a cysteine and an azide moiety (Figure 1A). The azide-functionalized Ras protein (positive control) as well as an unmodified Ras protein (negative control) were spotted on a phosphine-coated slide that can specifically conjugate with azide-functionalized Ras by

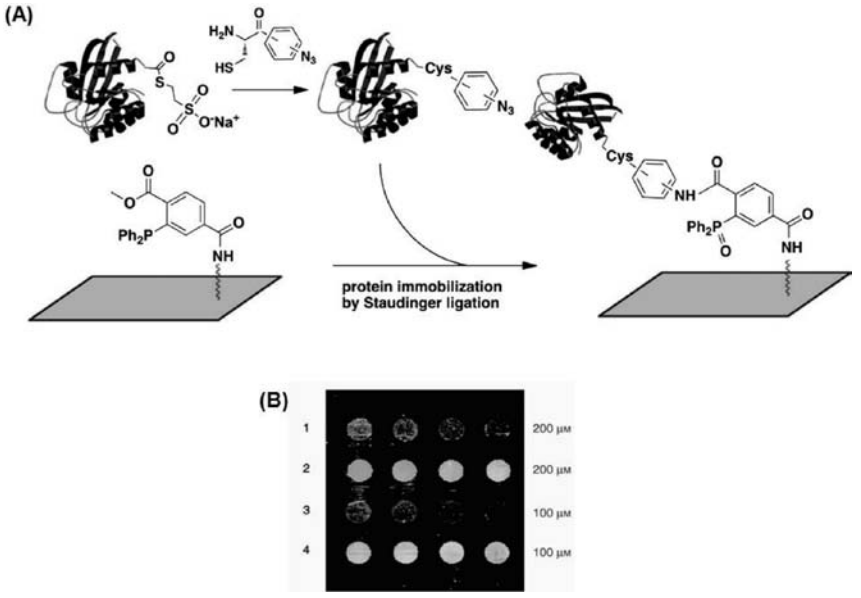

FIGURE 1. (A) Immobilization of an azide-functionalized N-Ras protein on a phosphine-functionalized glass slide. (B) Binding of Cy5-labeled Ras antibody to immobilized azide-functionalized N-Ras protein (2 and 4) as positive control and unmodified N-Ras protein (1 and 3) as negative control. The figure was reproduced from ref 26. Copyright 2006 Wiley-VCH Verlag GmbH & Co.

Staudinger ligation (Figure 1A). Sequentially, the slide was treated with a fluorescent Cy5-labeled Ras antibody that specifically recognizes a helix located in the active site region of the protein, followed by fluorescence read-out and quantification. The efficiency of protein immobilization by Staudinger ligation was demonstrated by fluorescence scanning (Figure 1B). The results indicate that the immobilization of azide-modified Ras and most likely other proteins on phosphine-modified glass slides is specific and efficient, and that the immobilized proteins do retain their three-dimensional structure.

Raines et al. reported a similar strategy to immobilize azide-modified bovine pancreatic ribonuclease (azido-RNase) onto a phosphinothioester-displaying self-assembled monolayer on a gold surface by Staudinger ligation.[32] A bifunctional reagent bearing an α-hydrazino acetamido and an azido group instead of thiol reagents was employed to cleave the transient C-terminal thioester of the target protein fused to an intein, thereby directly labeling the protein with an azide group at the C-terminus in a single step.

We investigated the use of the Staudinger ligation for protein labeling making use of the possibility to introduce additional functional groups into proteins by means of an enzymatic reaction.[33] Many proteins in cells are posttranslationally modified with a farnesyl group at the C-terminal cysteine. This farnesyl moiety is introduced by a farnesyltransferase (FTase) that specifically recognizes a C-terminal "CAAX-box" tetrapeptide sequence (where C is cysteine, A is an aliphatic amino acid, X is one of a variety of amino acids) of the protein substrate. This modification can also be performed in vitro by incubation of the protein substrate with FTase and farnesyl pyrophosphate (Fpp) as the farnesyl donor.[34]

With the aim of using this enzymatic reaction for site-specific protein modification, several azide- and diene-functionalized analogues of Fpp were synthesized.[33] Attachment of the modified Fpps was successfully achieved by incubation of the protein substrates, containing the required CAAX-box, with FTase, which indicates the high substrate tolerance of this particular enzyme.[37] Using this strategy, an azide- and a diene-modified cyan fluorescent protein (CFP) were generated (Figure 2). Their applicability was shown by further derivatization of the modified protein by Staudinger ligation with a phosphine or Diels–Alder reaction with 6-maleimidohexanoic acid.[33]

The Diels–Alder Reaction for Protein Labeling and Immobilization

The high biocompatibility and efficiency of the Diels–Alder reaction have allowed the successful surface immobilization of oligonucleotides and ligation of peptides.[35,36] Since this reaction proceeds with high selectivity and efficiency in aqueous solution,[37] we decided to investigate its application for site-specific protein labeling.[38] Accordingly, the protein of interest needed to be functionalized with a diene for further conjugation to different dienophiles under physiological conditions.

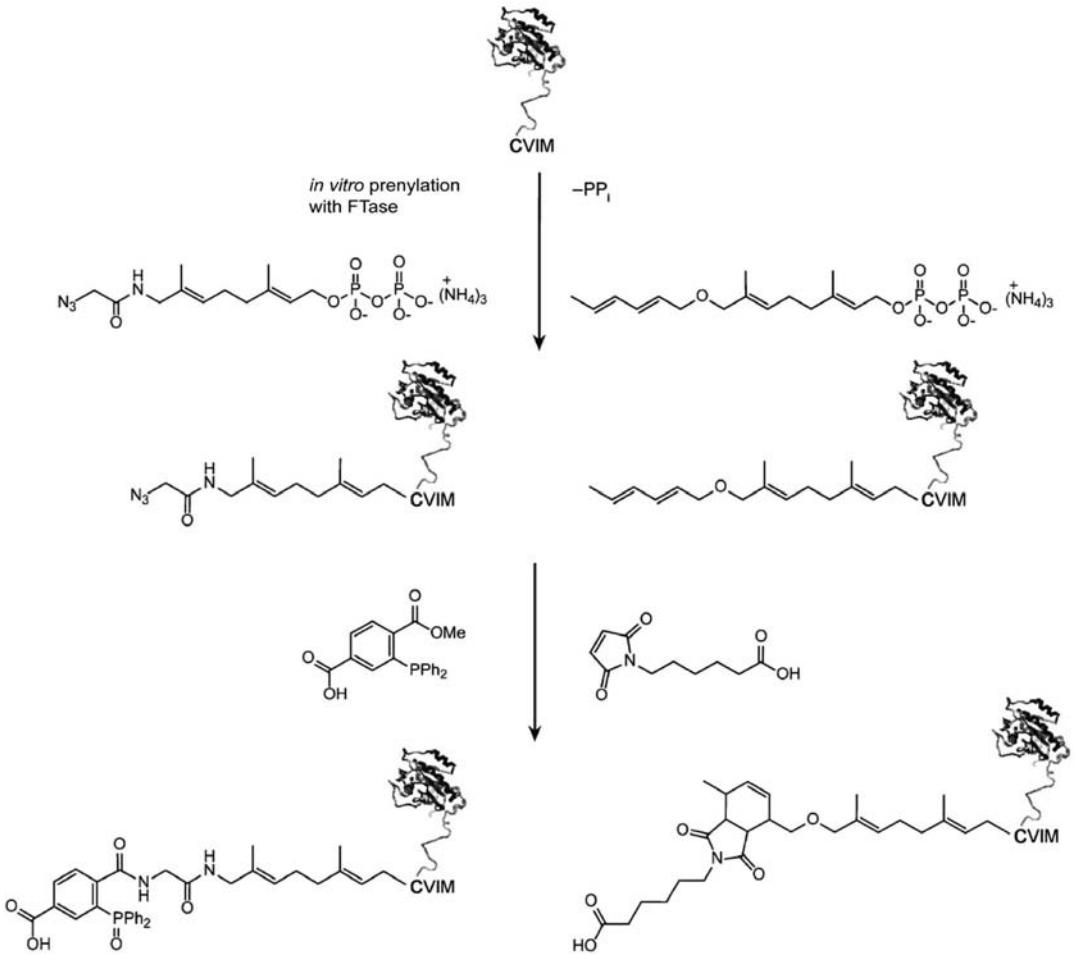

FIGURE 2. Overview of in vitro prenylation of CFP-CAAX followed by subsequent Staudinger ligation with phosphine and Diels–Alder cycloaddition with 6-maleimidohexanoic acid. The figure was reproduced from ref 33. Copyright 2007 Wiley-VCH Verlag GmbH & Co.

The strategy was initially applied for the labeling of streptavidin as a model system.[38] A diene group was first attached to streptavidin by means of complex formation with a biotinylated hexadienyl-peptide ester or by covalent attachment of a NHS-ester bearing a hexadiene group to the side chains of lysine residues. The diene-functionalized streptavidin was treated with maleimide-derivatized peptides or fluorophores in aqueous solution. Under slightly acidic conditions the Diels–Alder reaction proceeds chemoselectively at room temperature, thus proving the suitability of the approach for protein labeling.[38] The Diels–Alder reaction was applied in a combination with EPL for site-specific labeling of Rab7 with fluorophores, a protein that plays a key role in regulating vesicular transport.[39] Rab proteins labeled with different probes are versatile tools for biochemical and biophysical investigations. A recombinant truncated Rab7 thioester was prepared by thiolysis of an intein fusion protein and then ligated with a peptide containing a C-terminal hexadienyl ester under mild conditions to yield a Rab7 protein hexadienyl ester. This Rab7 protein reacted with maleimide-functionalized fluorophores (Figure 3A). Structural integrity and activity of the labeled Rab7 proteins were confirmed in fluorescence-based assays monitoring their interaction with the accessory Rab escort protein 1 (REP-1), which facilitates prenylation of Rab and recognizes Rab proteins with correct tertiary structure.[40]

The Diels–Alder reaction has further been applied for the immobilization of diene-modified proteins on glass slides.[38] Initially, streptavidin was modified with a cyclopentadiene derivative and the resulting diene-functionalized streptavidin (positive control) as well as unmodified streptavidin (negative control) were spotted on a maleimide-modified microarray slide (Figure 3B). The slide was subsequently treated with fluorophore-labeled Cy5-biotin, which tightly binds to streptavidin, and this interaction was then detected and quantified by fluorescence scanning. As shown in Figure 3C, the immobilization of diene-modified streptavidin

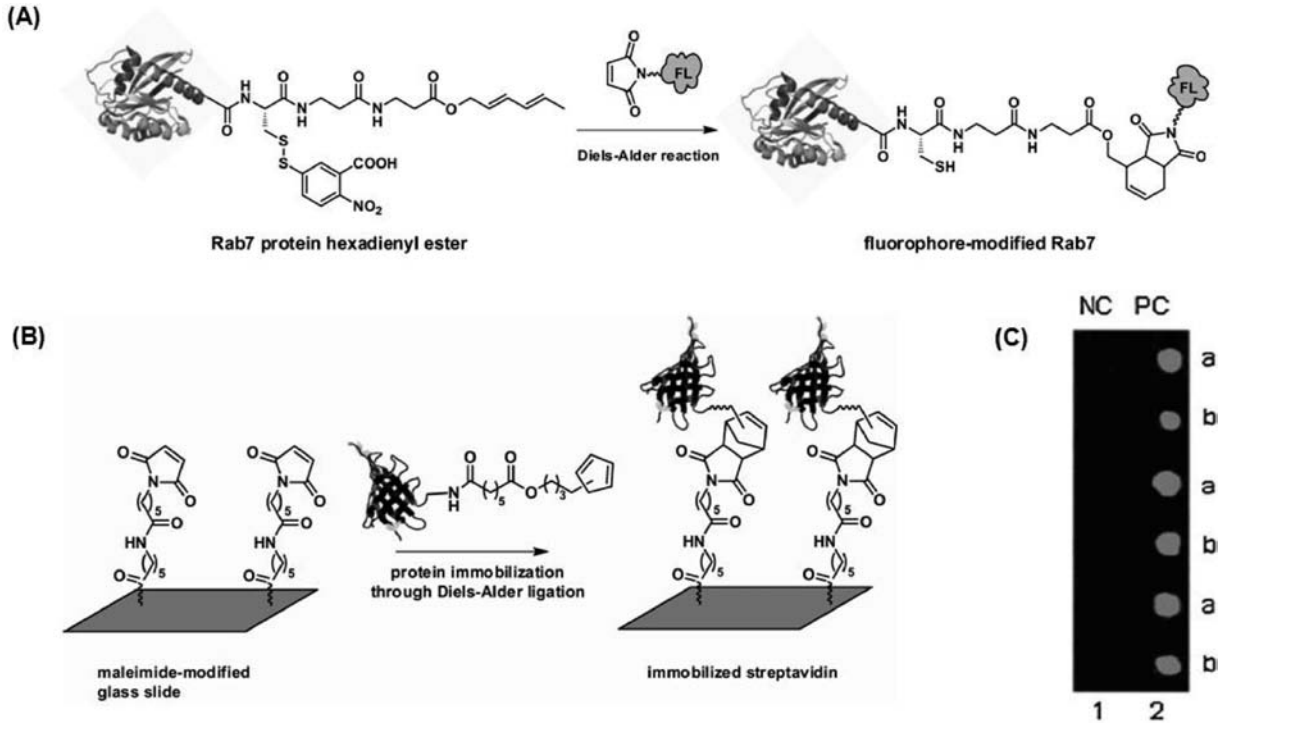

FIGURE 3. (A) Site-specific modification of the Rab7 protein with fluorophores by means of Diels–Alder reaction. FL: fluorophore. (B) Immobilization of diene-functionalized streptavidin on maleimide-functionalized glass slides. (C) Binding of Cy5-labeled biotin to immobilized diene-functionalized streptavidin (2) as a positive control (PC) and unmodified streptavidin (1) as a negative control (NC). The figure was reproduced from ref 38. Copyright 2006 Wiley-VCH Verlag GmbH & Co.

gave intense fluorescence signals, whereas no signal was observable for the negative control.

Oxime Ligation for Site-Specific Labeling of Proteins

The term "oxime ligation" refers to the condensation of an oxyamine and an aldehyde or ketone to form an oxime linkage. The oxyamine and aldehyde/ketone functionalities are bioorthogonal, and their conjugation proceeds fast and in high yield under physiological conditions. Therefore, the oxime ligation has been used in surface immobilization and labeling of proteins.[41,42] However, the described methods based on oxime ligation mainly employ proteins equipped with an aldehyde, and the difficulty of introducing such a functionality into proteins has limited its application. In order to expand the application of the oxime formation for protein labeling, it was planned to incorporate an oxyamine group into the C-terminus of a protein by EPL, which could in turn be conjugated with aldehyde/ketone-modified probes.[43]

A truncated Rab7Δ2-thioester generated by thiolysis of an intein fusion protein was efficiently ligated with a readily accessible bis(oxyamine), to yield Rab7Δ2-ONH_2 with a stable hydroxamic acid bond and another oxyamine group available for further reaction with aldehyde/ketone-containing probes.[43] This Rab7Δ2-ONH_2 protein was modified further with ketone-functionalized fluorophores, such as coumarin, via oxime ligation (Figure 4). The bioactivity of the fluorophore-modified Rab7 protein was confirmed by detection of complex formation with REP-1 using size exclusion chromatography. To further demonstrate applicability of the method, a site-specific dansyl-labeled Rab7Δ7 protein was generated using the same strategy. In this case, its interaction with REP-1 was investigated using a fluorescence-based assay, yielding a K_d value of 2.6 nM, which is close to the K_d value (1.0 nM) determined previously with mant-GDP loaded Rab7.[43]

Dual-color protein labeling enables the study of dynamic protein structure changes by means of fluorescence resonance energy transfer (FRET). Therefore, methods to obtain doubly labeled proteins are of high interest. The oxime ligation was applied to synthesize a doubly fluorophore-labeled Ypt7Δ3 protein, the yeast analogue of the human Rab7 protein (mCherry-Ypt7Δ3-fluorescein) from recombinant mCherry-Ypt7Δ2 thioester. The emission spectra of mCherry-Ypt7D3-fluorescein showed a clear FRET effect between mCherry and fluorescein thus proving the applicability of the method.[43]

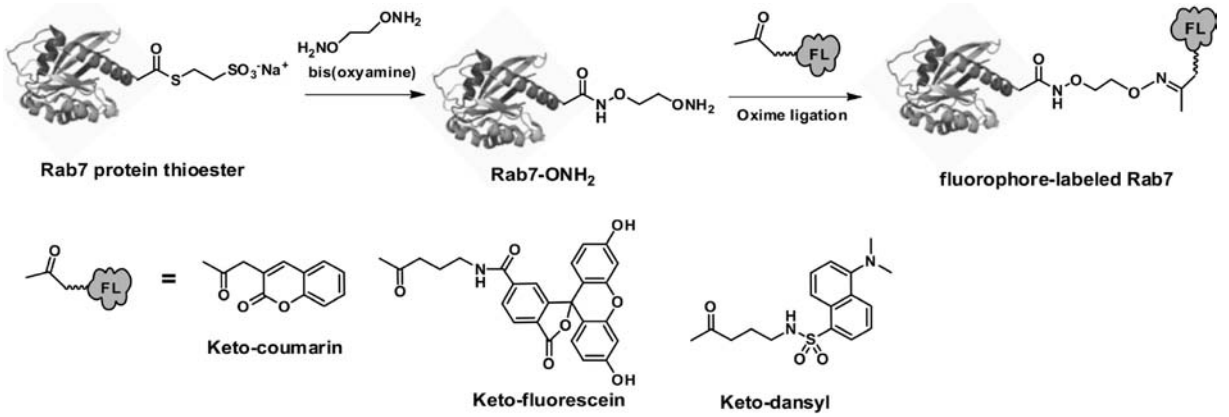

FIGURE 4. Fluorescent labeling of Rab7 protein at the C-terminus by oxyamine-ketone ligation. The figure was reproduced from ref 43. Copyright 2010 Wiley-VCH Verlag GmbH & Co.

The "Click Sulfonamide" Reaction for Surface Immobilization of Proteins

Bioorthogonal sulfonylazides and terminal alkynes can react under the catalysis of Cu(I) to form stable *N*-acylsulfonamides in aqueous solution as reported by Chang et al. and Fokin et al.[44,45] This "click sulfonamide reaction" (CSR) is related to the Cu(I)-catalyzed [3+2] azide−alkyne cycloaddition and characterized by high chemoselectivity and robustness under mild conditions. Therefore, we applied CSR for site-specific surface immobilization of proteins.[46]

Initially, small biomolecules, such as biotin, α-D-mannose, and phosphopeptides, were modified by introducing an alkyne moiety and then ligated with a benzyloxycarbonyl-protected tauryl sulfonylazide in aqueous solution in the presence of different cosolvents, Cu(I)-catalyst, and additives.[46] The reaction reached 90−95% conversion within 2−4 h without undesired side reactions, thus indicating the potential of CSR in surface immobilization. To demonstrate the applicability of CSR for site-specific modification of full-length proteins, a fluorescent mCherry-Ypt7 protein was equipped with an alkyne moiety at its C-terminus by means of EPL. The alkyne-modified mCherry-Ypt7 and the corresponding nonmodified protein were spotted onto sulfonylazide-functionalized slides (Figure 5A). mCherry-Ypt7 embodying an alkyne group gave clear fluorescence signals at the minimum concentration of 2 mM, which indicated successful protein immobilization.

Protein immobilization by means of CSR was also applied for the surface immobilization of the Ras-binding domain (RBD) of cRaf1.[46] The interaction between the cRaf kinase and the Ras protein plays a prominent role in the Ras-MAP kinase signaling pathway.[31] The RBD can specifically recognize the Ras protein in its active form (GTP loaded) as compared to the inactive form (GDP loaded). Thus, the RBD of cRaf1 was modified with an alkyne at the *C*-terminus by EPL and then immobilized on sulfonylazide-functionalized microarray slides that subsequently were treated with either GppNHp- (a nonhydrolyzable GTP analogue) or GDP-loaded Ras protein. The slides were incubated with a solution of Cy5-labeled Ras-specific antibody followed by fluorescence detection (Figure 5B). Only the spots treated with GppNHp-loaded Ras protein yielded a clear fluorescence signal in a concentration-dependent manner, which confirmed that CSR immobilization yielded a fully functional protein (Figure 5C).

The Photochemical Thiol−Ene Reaction for Protein Immobilization

The term "thiol−ene" denotes the addition of a thiol to an ene group via a free radical mechanism upon initiation either by a chemical radical initiator or by light.[47] The thiol−ene reaction occurs upon irradiation of a thiol and an alkene with UV light at wavelengths of 365−405 nm (close to visible) to form a stable thioether bond. It has been used successfully in carbohydrate and peptide chemistry as well as in polymer and materials synthesis.[48] Due to its specificity for olefins and robustness in aqueous buffer, the thiol−ene reaction can be considered bioorthogonal. The reaction proceeds almost quantitatively in aqueous solutions and does not require the presence of catalysts or additives. Therefore, the photochemically induced coupling of thiols and olefins was investigated for its potential applicability in surface patterning and immobilization of biomolecules on functionalized slides.

In initial experiments, this reaction was used for photochemical attachment of a biotin equipped with an olefin

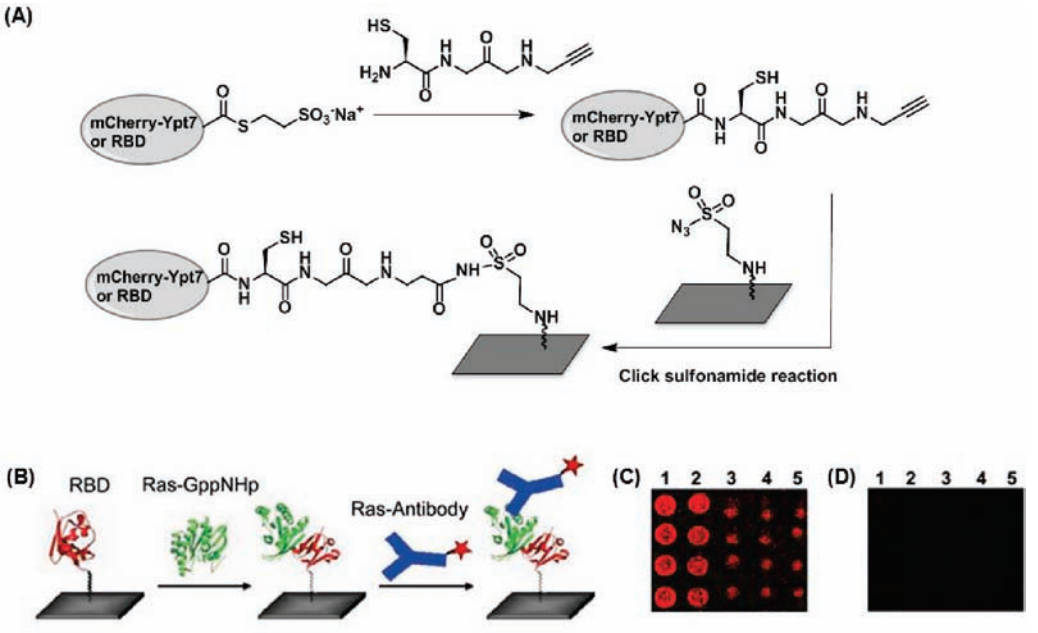

FIGURE 5. (A) Site-specific immobilization of alkyne-modified proteins on sulfonylazide-functionalized glass slides. (B) Schematic for detection of Ras binding to immobilized Raf-RBD. (C) A RBD-immobilized slide was treated with activated Ras:GppNHp (at five concentrations, (1) 50 μM, (2) 25 μM, (3) 12.5 μM, (4) 6.2 μM, (5) 3.1 μM) followed by Cy5-labeled Ras antibody and then scanned for fluorescence. (D) RBD slide incubated with Ras:GDP followed by Cy5-labeled Ras antibody. The figure was reproduced from ref 46. Copyright 2008 The Royal Society of Chemistry.

derivative to a thiol-functionalized surface that was subsequently treated with Cy5-labeled streptavidin (SAv) to produce a fluorescent SAv-patterned surface (Figure 6).[49] In order to demonstrate its applicability, biotinylated calf-intestine alkaline phosphatase (AlkPh) was anchored to a SAv-patterned surface through biotin-SAv binding. The enzymatic activity of the AlkPh linked to the SAv-patterned surface was retained, as was proven by fluorescence assays using the AlkPh substrates, Vector Red, and Attophos. When treated with Vector Red, the slide showed distinctive fluorescent patterns due to the precipitation of a fluorescent dye produced upon dephosphorylation of Vector Red. The rate constant for the dephosphorylation reaction between AlkPh and its fluorescent substrate Attophos was in accordance with the constant measured using free AlkPh in solution.

These SAv-patterned slides could also be used for the study of protein–protein interactions. In these experiments, the RBD of cRaf1 and GppNHp loaded Ras proteins were chosen as model pairs. After immobilization of biotinylated Ras proteins (either GppNHp- or GDP-loaded) on the SAv-patterned surface and subsequent treatment of the slides with fluorescent YFP-RBD protein, clear YFP fluorescent patterns were recorded for Ras:GppNHp. In contrast, no YFP fluorescent patterns were observed for Ras:GDP, proving the functionality of the surface-immobilized proteins.

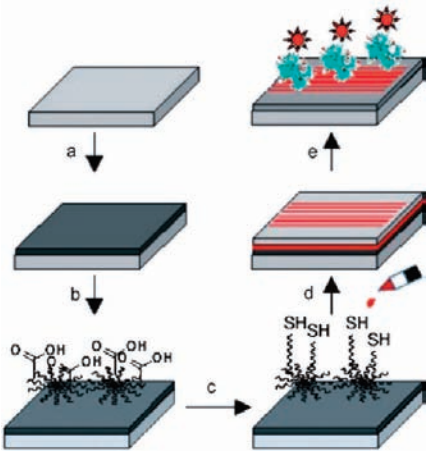

FIGURE 6. Schematic illustration of the surface-patterning technology using the thiol–ene reaction. (a) activation (plasma-enhanced chemical vapor deposition, silanization); (b) optimization (aminocaproic acid linker, dendrimers); (c) functionalization with thiols; (d) drop casting of biotin equipped with olefin and immediate coverage with a photomask to prevent drying of the liquid film prior to irradiation (10 min, 365–405 nm); (e) removal of the mask. Patterns of biotin are visualized by incubating the surface with SAv-Cy5. The figure was reproduced from ref 49. Copyright 2008 Wiley-VCH Verlag GmbH & Co.

In a further step, the photochemical thiol–ene reaction was applied for direct and covalent immobilization of olefin-functionalized biomolecules. In a proof-of-principle experiment, phosphopeptides carrying an olefin moiety at the N-terminus were incubated with thiol-functionalized slides

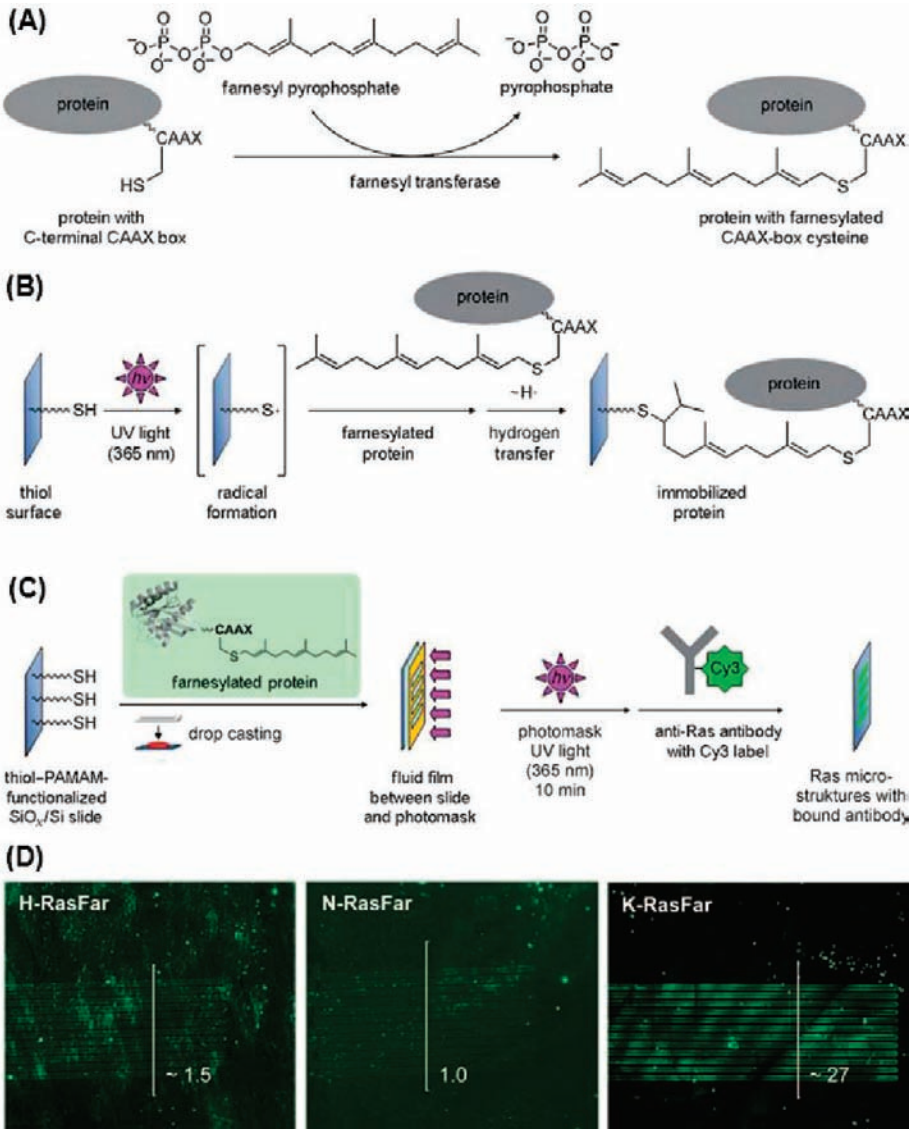

FIGURE 7. Oriented immobilization of farnesylated Ras proteins by the photochemical thiol−ene reaction to generate Ras microstructures. (A) Farnesylation of proteins by farnesyltransferase (FTase). (B) Proposed mechanism for the thiol−ene photoimmobilization of farnesylated proteins. (C) Schematic depiction of the thiol−ene photomicrostructuring of farnesylated Ras isoforms. (D) Fluorescence images obtained after the photomicrostructuring of farnesylated Ras isoforms. Average relative fluorescence intensities obtained from cross sections of the microstructure lines (white lines) are indicated. The figure was reproduced from ref 50. Copyright 2010 Wiley-VCH Verlag GmbH & Co.

and exposed to light at wavelengths of 365−405 nm for 10 min. The successful immobilization was confirmed by clear fluorescent patterns on the slides after treatment with a Cy5-labeled anti-phospho-tyrosine antibody (pY antibody).[49]

Direct application of the photochemical thiol−ene reaction for immobilization of full-length proteins requires incorporating an olefin group into the protein. The farnesyl group, a known lipid posttranslational modification at the C-terminus of proteins, contains three olefins that could be directly used for the thiol−ene reaction (Figure 7A). Therefore, we employed the farnesyl transferase (FTase) catalyzed farnesylation of proteins embodying a CAAX-box tag for direct immobilization of proteins on thiol-functionalized chips (Figure 7B).[50] FTase-catalyzed protein modifications by synthetic alkyne- or azide-functionalized farnesyl analogues together with the Huisgen [3+2] cycloaddition or Staudinger ligation have alternatively been used for protein immobilization.[51,52]

Recombinant Ras isoforms (including H-Ras, N-Ras, and K-Ras)[31] bearing a CAAX box were separately farnesylated in vitro by FTase. The resulting farnesylated proteins were immobilized on thiol-modified slides upon exposure to UV light at a wavelength of 365 nm for 10 min. Incubation with a fluorescent Cy3-labeled antibody against Ras isoforms

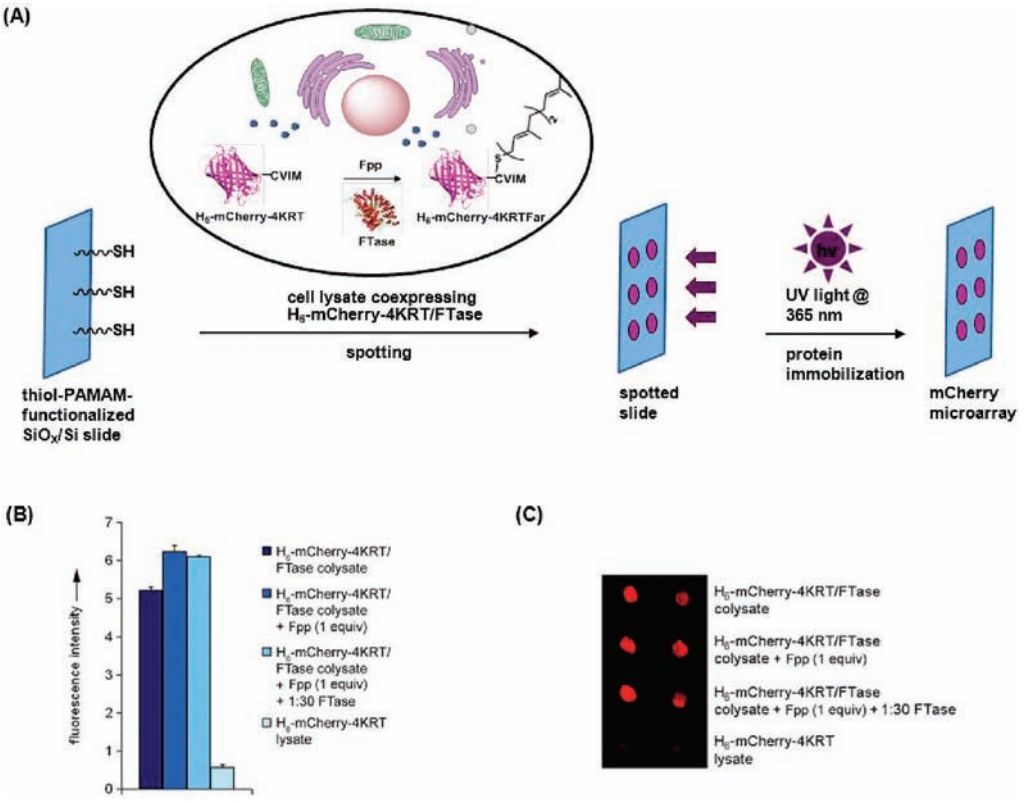

FIGURE 8. Immobilization of farnesylated proteins directly from cell lysate. (A) Generation of a mCherry protein microarray by the thiol−ene reaction using cell lysate coexpressing H_6-mCherry-4KRT and FTase. (B) Fluorescence intensity observed for H_6-mCherry-4KRT (78 μM)/FTase coexpression lysate and H_6-mCherry-4KRT (75 μM) lysate on a microarray. Additional Fpp and a combination of Fpp and FTase were separately added to two colysate samples. (C) Fluorescence image of a subsection of the colysate microarray described in (B). The figure was reproduced from ref 50. Copyright 2010 Wiley-VCH Verlag GmbH & Co.

clearly produced Ras positive microstructures (Figure 7C), which confirmed that the Ras proteins were successfully immobilized and correctly folded. However, the immobilization efficiency for farnesylated K-Ras was substantially higher than for farnesylated H-Ras or N-Ras (Figure 7D). The main difference between K-Ras and H-Ras/N-Ras is the presence of a unique polybasic stretch at the C-terminus of K-Ras. It was hypothesized that this polybasic stretch containing eight lysines could play an important role in improving the efficiency of protein immobilization, probably due to ionic interactions between the positively charged K-Ras C-terminus (abbreviated as KRT) and carboxylic acid groups of PAMAM dendrimers present on the surface of the slides. Control experiments using polybasic stretches of different lengths and different numbers of lysines confirmed this assembly and showed that a tetralysine-based KRT-like C-terminus (4KRT) was sufficient to enhance protein immobilization.

Therefore, as a general approach for protein immobilization, a tetralysine-based KRT-like stretch was genetically attached to the C-terminus of the target proteins. After enzymatic attachment of the corresponding farnesyl group, proteins would be ready for surface immobilization via photoinduced thiol−ene reaction. Indeed, the fluorescent protein H_6-mCherry-4KRT (H_6 indicates a hexa-histidine tag), containing the polybasic stretch as well as the CAAX box required for the enzymatic reaction, was generated, and after purification the protein was farnesylated in vitro through incubation with FTase and Fpp. The resulting protein could then be successfully immobilized on microarray slides. Moreover, a recombinant Rab6A protein variant with a hexalysine-based KRT-like C-terminus (6KRT) was also farnesylated in vitro and the generated Rab6A-6KRTFar could be photoimmobilized on thiol-functionalized slides. The attached protein was efficiently recognized by the minimal Rab6A-binding domain of bicaudal D2 fused to an enhanced green fluorescent protein (eGFP-bicaudalD2).

A technique that allows for the immobilization of proteins directly from expression lysates without additional isolation, purification, or chemical derivatization would be of major interest. We envisioned that this goal could be reached by in

vivo farnesylation of recombinant KRT-tagged proteins through coexpression of FTase in *E. coli* cells, and direct use of cell lysates for thiol−ene photoimmobilization of target proteins without further purification.[50] To prove this strategy, *E. coli* cell lysates coexpressing H_6-mCherry-4KRT and FTase with or without additional Fpp were directly applied for immobilization on thiol-modified microarray slides through exposure to UV light for 20 min (Figure 8A). Fluorescence scanning of the slides with cell lysate containing only H_6-mCherry-4KRT as control showed that farnesylation of H_6-mCherry-4KRT by coexpressed FTase employing endogenetic Fpp is sufficient to allow for specific protein immobilization of cell lysates via photochemical thiol−ene reaction (Figure 8B and C). The strategy was further successfully used to generate a Ypt1 protein microarray, indicating its general applicability.

The results of these investigations demonstrate that the photochemical thiol−ene reaction is a powerful tool for both patterning and immobilization of proteins to generate microstructures and microarrays on functionalized slides.

Site-specific immobilization of proteins on surfaces is a powerful technique for characterization of protein interactions. The corresponding immobilization methods need to preserve protein three-dimensional structure and activity. Consequently, tailored methods for protein immobilization are in high demand. The bioorthogonal ligation methods summarized here make use of different functionalities and reactions and fulfill these requirements. The choice of the particular method to be employed may be dictated by the properties of the proteins to be immobilized. For example, the method based on the "click sulfonamide" reaction should be avoided if the protein to be immobilized is sensitive to copper, as this metal is required for the immobilization. Similarly, the photochemical thiol−ene reaction is not a suitable choice if UV sensitive proteins have to be immobilized. For labile proteins, methods characterized by short reactions times may be preferable to minimize protein denaturation. To this end, the photochemical thiol−ene reaction (30 min), the Staudinger ligation (4 h), or the "click sulfonamide" reaction (4 h) may be more suitable than, for example, the Diels−Alder cycloaddition that requires longer reaction time (8 h).

Each method described has advantages and disadvantages that need to be taken into account. We point out that the photochemical thiol−ene reaction in conjunction with in vivo enzymatic attachment of a farnesyl moiety to proteins by coexpressed FTase can be applied widely. It allows immobilization of proteins directly from expression lysates without additional manipulation and thereby facilitates the efficient generation of protein microarrays.

Summary and Perspectives

The bioorthogonal reactions described in this Account and related transformations (see above) have proven to be powerful tools. Thus, the Staudinger ligation, the Diels−Alder cycloaddition, the oxime ligation, the click sulfonamide reaction, and the photochemical thiol−ene reaction can successfully be used for site-specific protein immobilization on biochips or for protein labeling under physiological conditions. By means of these strategies the orientation of immobilized proteins can be controlled while maintaining protein structure and activity, which largely facilitates the use of protein chips for biological research. Expanding the repertoire of bioorthogonal reactions has provided novel opportunities for site-specific labeling of proteins and the production of protein-biochips. However, despite the success in developing such transformations in the past decade, the demand for new bioorthogonal methods with improved kinetics and selectivities remains high.

BIOGRAPHICAL INFORMATION

Yong-Xiang Chen was born in 1980 in Hunan, China. She received her Ph.D. in organic chemistry from Tsinghua University under the guidance of Prof. Dr. Y.M. Li in 2007, after which she is working with Prof. Dr. H. Waldmann at the MPI of Molecular Physiology in Dortmund as a postdoctoral fellow, supported by an Alexander von Humboldt fellowship. Her research interests include synthesis and biochemical studies of lipoproteins, and protein microarray technology.

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Herbert Waldmann was born on June 11, 1957 in Neuwied. He received his Ph.D. in 1985 from the University of Mainz under the guidance of Prof. H. Kunz in organic chemistry after which he completed a postdoctoral appointment with Prof. Dr. G. Whitesides at Harvard University. He was appointed as Professor of Organic Chemistry at the University of Bonn (1991), full Professor of Organic Chemistry at the University of Karlsruhe (1993), and Director at the MPI of Molecular Physiology Dortmund, and Professor of Organic Chemistry at the University of Dortmund (1999). His research interests lie in chemical biology research

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FOOTNOTES

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