Protein Microarrays

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Site-Selective Protein Immobilization by Staudinger Ligation**

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The site- and chemoselective immobilization of proteins through regions of the macromolecules that are not involved in interactions with other molecules is preferable or even required for the application of protein arrays in proteomics research.[1–4] Recently, expressed protein ligation was employed for this purpose,[5] and Raines and co-workers[6] and Bertozzi and co-workers[7] demonstrated the use of the Staudinger ligation[8,9] for protein immobilization by means of a two-step process in which a peptide is first immobilized followed by noncovalent binding of a protein to the peptide sequence.

Herein, we report the successful site-selective covalent immobilization of proteins by reaction of the azide-modified C terminus of a protein generated by expressed protein

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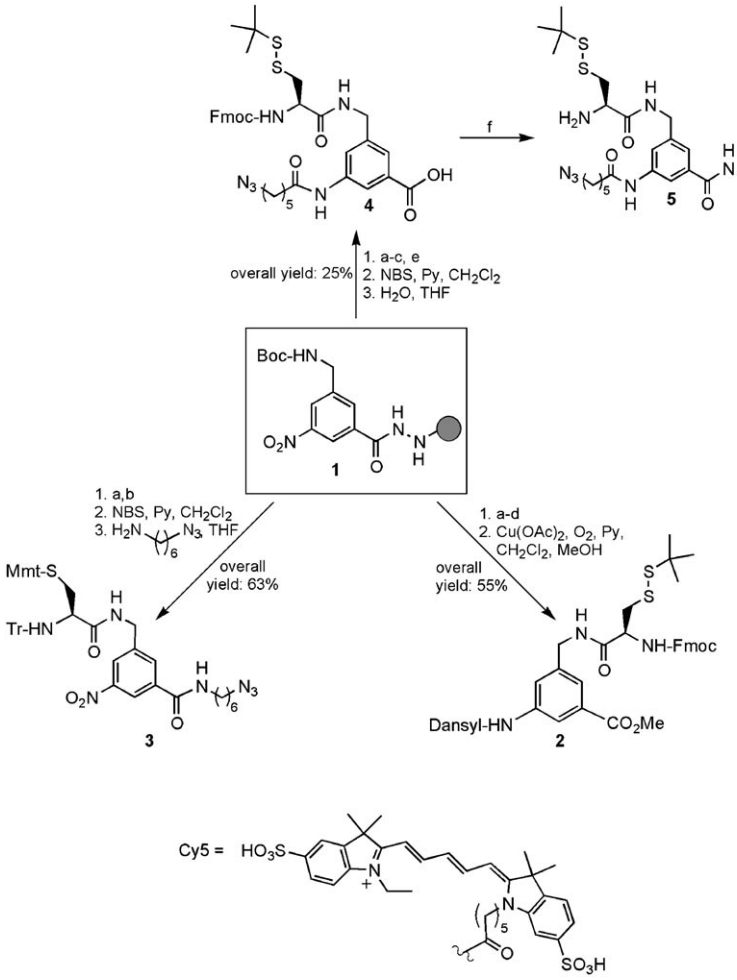
ligation (EPL) in vitro[10] with a phosphane-functionalized glass surface.

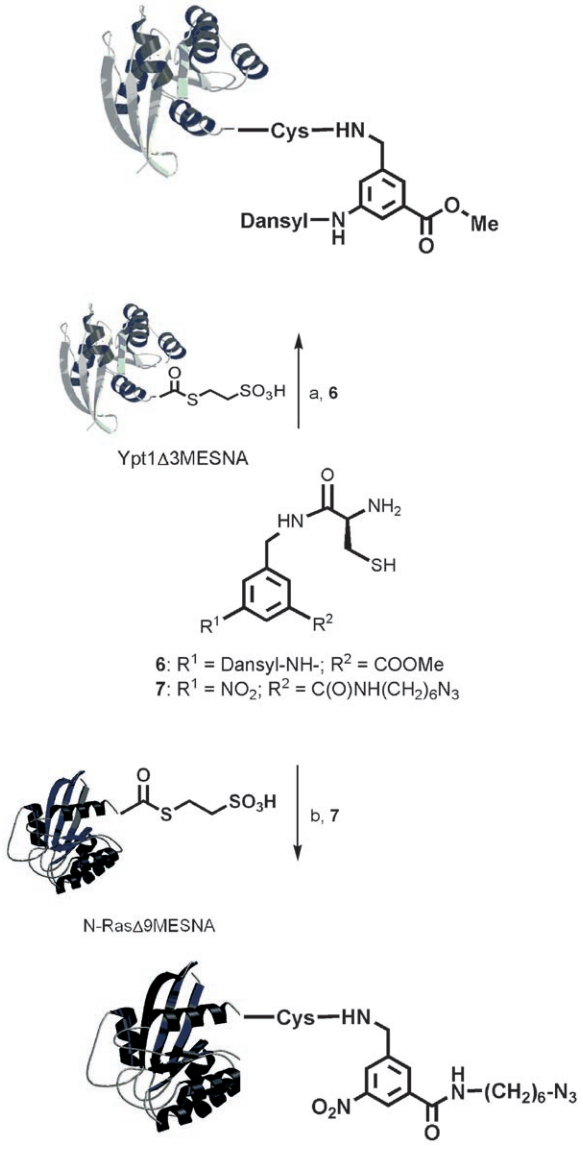
To devise a flexible method we developed a general building block that 1) can be coupled to a protein of interest by EPL, 2) carries an azide group for immobilization to a surface, and 3) embodies a third site that can be equipped with a further functional tag, label, or reporter group, for example, a fluorophore.

To this end, immobilized benzene derivative **1** (Scheme 1) equipped with a triply orthogonal set of functional groups was synthesized. Selective cleavage of the Boc group allowed attachment of a side-chain-protected cysteine derivative for coupling to a thioester group of a protein by EPL (see **2**, **3**, and **4**; Scheme 1). Selective "on-resin" reduction of the nitro group with $SnCl_2$ yielded an amine that can, for instance, be used to introduce a fluorescent label (see **2**; Scheme 1) or an azide-containing amide group (see **4**; Scheme 1). Oxidative activation of the hydrazide linker[11] followed by attack of an appropriate nucleophile on the intermediate acyldiazene gives an ester **2**, the free carboxylic acid **4**, or an amide **3**.

To demonstrate that these building blocks can be coupled successfully to proteins by EPL, the Fmoc group was removed from compound **2**, and the resulting cysteine amide **6** was ligated with the mercaptoethanesulfonic acid (MESNA) thioester of Ypt1Δ3 (Scheme 2), the yeast homologue of the human Rab7 protein that was generated by means of intein technology as described previously.[12] Analysis of the ligation reaction by SDS-PAGE (see the Supporting Information) clearly revealed that the coupling had proceeded successfully. The desired protein was obtained quantitatively.



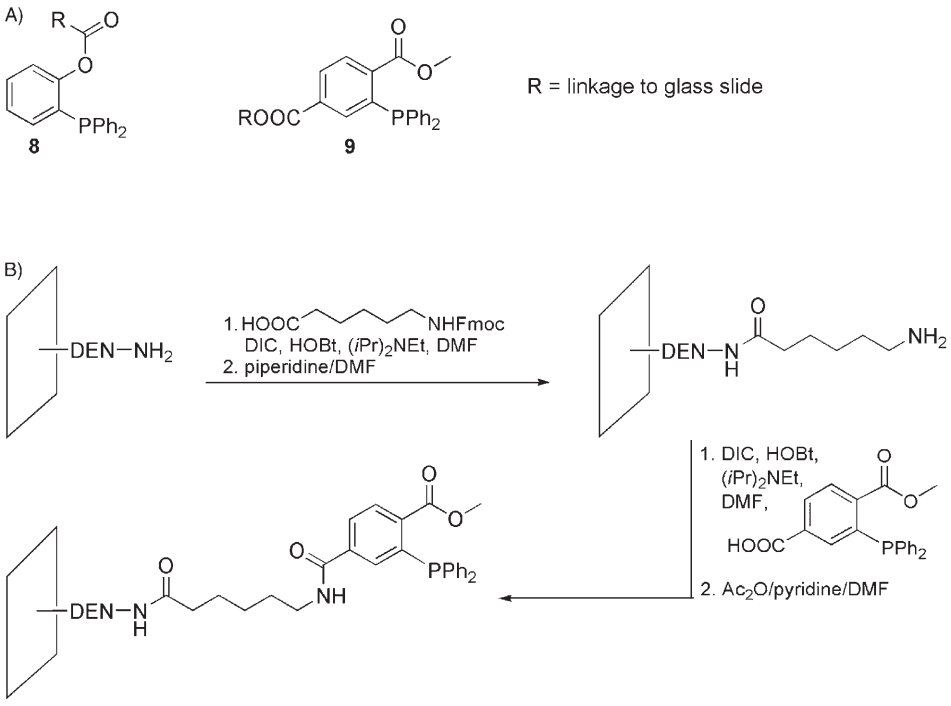
Scheme 1. Synthesis of fluorescently labeled and azide-functionalized building blocks. a) TFA/CH_2Cl_2 (50%), 30 min; b) Fmoc-Cys(*St*Bu)-OH or Tr-Cys(SMmt)-OH (4 equiv), HOBt (4 equiv), DIC, $(iPr)_2NEt$; c) $SnCl_2{\cdot}2H_2O$, DMF, 2 h, room temperature; d) DansylCl (2 equiv), DMAP (0.5 equiv), pyridine (2 equiv), DMF; e) N_3-$(CH_2)_5$-CO_2H (3 equiv), HOBt, DIC, $(iPr)_2NEt$, CH_2Cl_2, 12 h, room temperature; f) Cy5-Et-NH_2 (4 equiv), HOBt (4 equiv), HBTU, $(iPr)_2NEt$, 12 h, room temperature, 45%. Dansyl = 5-(dimethylamino)naphthalene-1-sulfonyl; DIC = 1,3-diisopropylcarbodiimide; DMF = *N*,*N*-dimethylformamide; Fmoc = 9-fluorenylmethyloxycarbonyl; HBTU = *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HOBt = 1-hydroxy-1*H*-benzotriazole; Mmt = (4-methoxyphenyl)-(diphenyl)methyl; TFA = trifluoroacetic acid; Tr = trityl = triphenylmethyl.



Scheme 2. Ligation of building blocks **6** and **7** to Ypt1 thioester and N-Ras thioester. a) Na_2HPO_4/NaH_2PO_4 (50 mM), pH 7.5, $MgCl_2$ (0.1 mM), GDP (2 μM), MESNA (150 mM), 12 h, room temperature. GDP = guanosine-5′-diphosphate; YptΔ3 = Ypt truncated by three amino acids; MESNA = 2-mercaptoethanesulfonic acid.

Encouraged by this finding we aimed at the synthesis of an azide-tagged Ras protein[13] suitable for immobilization on a surface. The hypervariable C-terminal region of the signal-transducing Ras oncoproteins is believed primarily to mediate protein–membrane interactions and to be less important for Ras-effector interactions than other regions of the protein.[13] Therefore, immobilization of Ras through the C terminus is desirable.

Accordingly, we planned to ligate Ras to an azide-carrying building block (Scheme 1) and to immobilize it to a phosphane-functionalized surface by means of the Staudinger ligation technique. To this end, compound **3** was deprotected, and the resulting cysteine derivative **7** was treated with the MESNA thioester of a C-terminally truncated N-RasΔ9 protein (Scheme 2).[14]



Scheme 3. Preparation of phosphane-modified glass slides. A) Phosphanes used for Staudinger ligation; B) functionalization of the glass slides. DEN = fourth-generation polyamidoamine dendrimer.

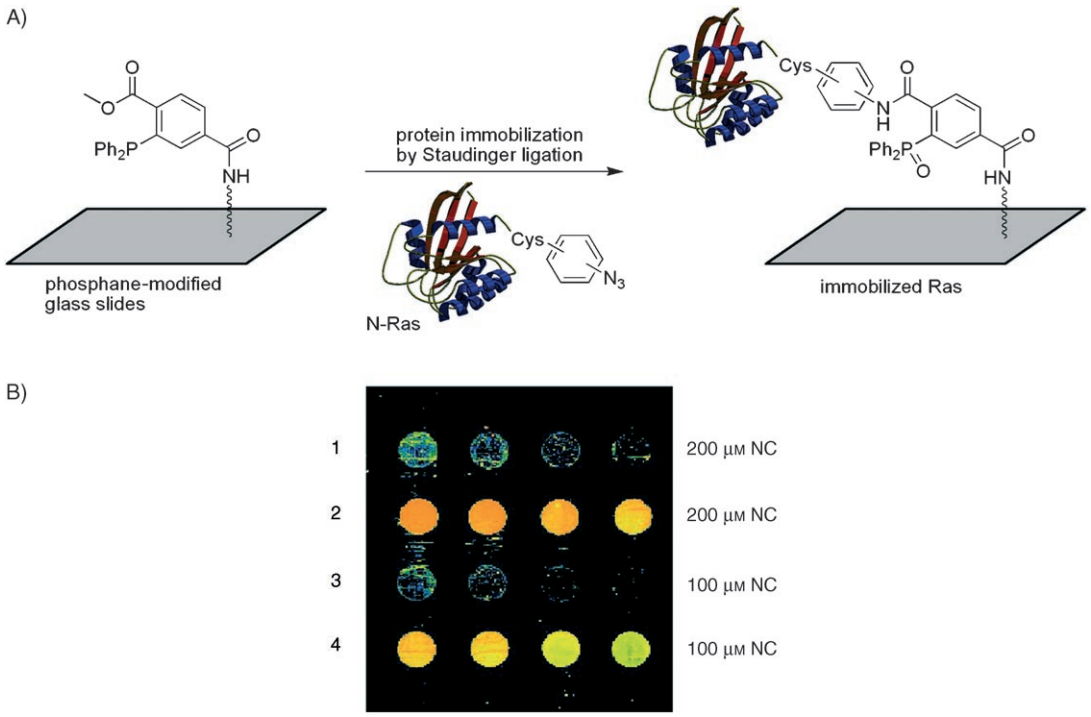
After incubation with a fivefold excess of **7** for 12 h, the semisynthetic protein was obtained pure in 95% yield (see the Supporting Information for details). Notably, the ligation reaction with azide-carrying building block **7** is efficient in the presence of excess thiol, that is, the azide incorporated into **7** is not reduced to the amine under these conditions.

For the immobilization of the azide-functionalized Ras protein, glass slides initially modified with an intermediate layer of generation 4 PAMAM dendrimers were chosen as carriers to allow maximum surface coverage.[9,15] For covalent attachment of the protein by means of Staudinger ligation the surfaces of the slides were decorated with an appropriately functionalized phosphane. Initially it was planned to functionalize the surface with phosphane **8** (Scheme 3). However, Liskamp and co-workers found that 2-diphenylphosphanylphenol ester **8** (Scheme 3A) not only reacts with an azide to give an amide but also undergoes side reactions with the amine groups of lysine side chains,[16] and hence phosphane **9** introduced by Bertozzi and Saxon[8a] was chosen for the immobilization of proteins. This phosphane has been used for in vivo studies in which the Staudinger ligation was applied.[8c] Phosphane groups on surfaces are generally prone to oxidation, but the phosphane used in this case was developed by Bertozzi and co-workers to limit air oxidation.[8d]

For the preparation of the phosphane-functionalized surface an aminocaproic acid linker was introduced to generate distance between the functional groups and the surface, followed by coupling of the phosphane through amide formation (Scheme 3B).

For the immobilization experiments, the azide-modified N-Ras protein (positive control; PC) as well as the unmodified Ras protein as a negative control (NC) (Scheme 4) were dissolved in different buffers at various concentrations and spotted onto the phosphane-modified substrates by using a piezo-driven spotting robot (see the Supporting Information). The slides were washed with buffer at different time intervals and incubated with BSA-containing (BSA = bovine serum albumin) blocking solution to prevent nonspecific binding of protein reagents. The slides were then treated with Cy5-labeled Ras antibody (50 nM, 1 h at room temperature).[17] The fluorescence signals were recorded and quantified after removal of excess reagent. A representative result for the immobilization of azide-modified Ras protein is shown in Scheme 4B. The immobilization of azide-modified N-Ras protein (PC) gave clear fluorescent signals depending on the concentration of N-Ras in the spotting solution. The minimum concentration for acceptable levels of immobilization was found to be approximately 50 μM. An immobilization time of 4 h was sufficient for obtaining clear and reproducible signals. Several buffers are compatible with the immobilization reaction (see the Supporting Information). The immobilization was carried out at pH 7.4–7.6 owing to the pH-dependant stability of the Ras protein. We note, however, that the Staudinger ligation proceeds well over a relatively wide pH range (5–8.5).[18]

These results demonstrate that the immobilization of the azide-modified Ras protein onto the phosphane-modified glass slides was successful. Although the data provide no formal proof of the orientation of the protein on the surface,



Scheme 4. A) Immobilization of the azide-functionalized N-Ras protein on phosphane-functionalized glass slides. B) Binding of Cy5-labeled Ras antibody to immobilized azide-functionalized N-Ras protein (2 and 4) as positive control (PC) and unmodified N-Ras protein (1 and 3) as negative control (NC).

circumstantial evidence suggests that the immobilization occurred regioselectively at the C terminus.

In conclusion, we have developed a new strategy for the site-specific immobilization of proteins onto a glass surface that may be used for the creation of protein microarrays. This approach relies on the Staudinger ligation between azide-modified proteins and a phosphane-functionalized glass surface and employs a central triply orthogonally functionalized building block together with expressed protein ligation for the synthesis of the azide-modified proteins.

The method requires manipulation of expressed proteins before immobilization. Although this extra step may limit the generality of the method, it should still be applicable to the immobilization of numerous proteins.

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