Site-Specific Protein Immobilization by Staudinger Ligation

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Microarrays in which proteins are immobilized to a surface enable high-throughput experiments that require only small amounts of analyte.[1] Such protein "chips" can be used to detect protein–ligand, protein–protein, and antibody–antigen interactions. Attaching proteins covalently[2,3] rather than noncovalently[4] produces more robust surfaces. Attaching proteins in a uniform (that is, specific)[2,4] rather than random[3] manner can provide a substantial advantage in assay sensitivity.[5] Here, we report a general method for the covalent, uniform immobilization of peptides and proteins that is both rapid and high-yielding. The method relies on a traceless version of the Staudinger ligation in which an azide and phosphinothioester react to form an amide (Scheme 1).[6] The reaction is known to occur in high yield at room temperature in aqueous or wet organic solvents and is compatible with the unprotected functional groups of proteinogenic amino acids.

As a model protein to immobilize, we chose ribonuclease S′ (RNase S′), which is a truncated form of RNase S. The protease subtilisin prefers to cleave a single peptide bond in RNase A. The product of this cleavage, RNase S, consists of two tightly associated fragments: S-peptide, which derives from residues 1–20 of RNase A, and S-protein, which derives from residues 21–124.[7] Although neither fragment alone has any enzymatic activity, RNase S is as active as intact RNase A. The first 15 residues of S-peptide (S15) bind to S-protein with an affinity similar to that of S-peptide.[8] We reasoned that the immobilization of S15 followed by incubation with S-protein would generate an active RNase S′ tethered to a surface.

The S15 peptide was synthesized with an azido group in either a side chain or its main chain. To install an azido group into a side chain, the ε-amino group of Lys1 was replaced with an azido group to generate (N₃)Lys1 S15. To install an azido group into the main chain, a poly(ethylene glycol) (PEG) fragment with a terminal azido group was attached to the α-amino group of Lys1 to generate N₃-PEG-S15.
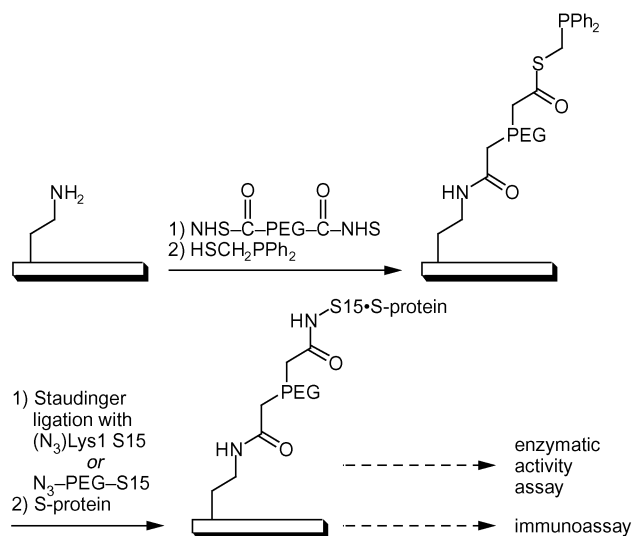

(N₃)Lys1 S15

N₃–PEG–S15
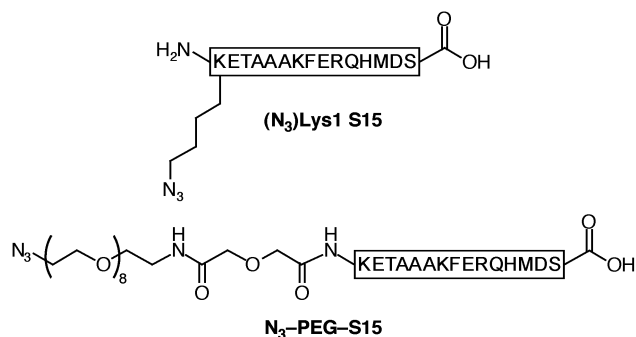
As a surface, we chose glass slides because of their compatibility with standard array and detection equipment. An amine-derivatized


Scheme 1
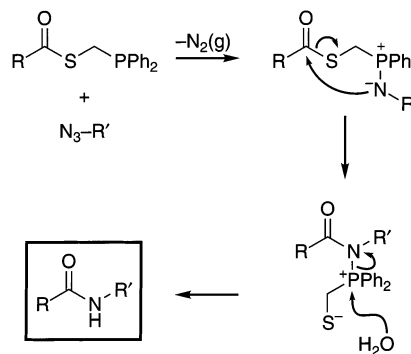
Scheme 2

slide was treated with an excess of PEG having succinimidyl ester termini (NHS-C(O)-PEG-C(O)-NHS) in DMF for 2 h, and diphenylphosphinomethanethiol[5] in DMF for 2 h, resulting in a surface-bound phosphinothioester (Scheme 2).[9] Serial dilutions[10] of (N₃)-Lys1 S15 or N₃-PEG-S15 were spotted on this phosphinothioester-derivatized slide. S-Protein in 0.10 M sodium phosphate buffer (pH 7.2) was spotted on the slide, which was washed thoroughly with buffer.

Two types of assays were used to detect the presence of immobilized RNase S′. First, the ribonucleolytic activity of each spot was determined with a fluorogenic substrate.[11] This assay revealed the amount of active RNase S′ on the surface. After activity assays were performed, the slide was washed thoroughly, and immobilized RNase S′ was visualized by immunostaining.[12] This assay revealed the total amount of RNase S′ on the surface and, hence, the overall yield of the immobilization reaction. Together, the assays indicated that coupling via a side chain (as with (N₃)-Lys1 S15) proceeded in 51% yield[13] and that the enzyme coupled

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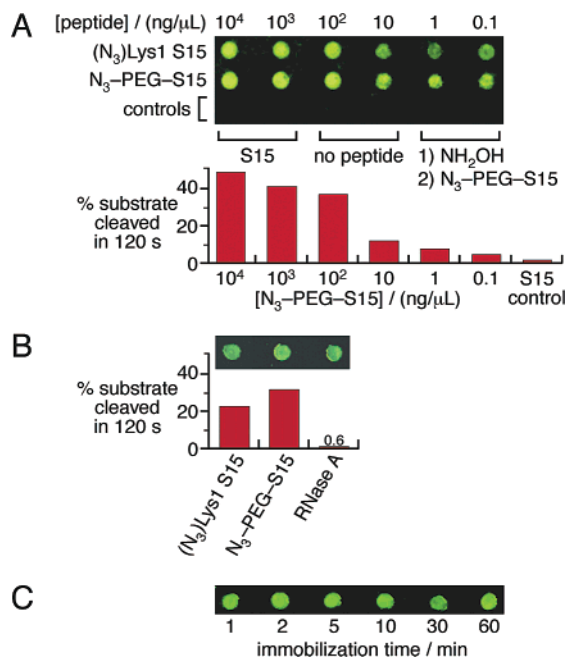
Figure 1. Site-specific protein immobilization by Staudinger ligation. (A) Yield and activity of immobilization by Staudinger ligation. A phosphinothioester-derived slide was spotted for 8 h at room temperature with dilutions[10] of an azido-S15 in DMF/H$_2$O (50:1). The slide was washed thoroughly with DMF, and then with 0.10 M sodium phosphate buffer (pH 7.2). As controls, wild-type S15 (5 μM) was spotted for 8 h, no peptide was spotted, or the slide was treated with NH$_2$OH before spotting for 8 h with N$_3$-PEG-S15. After incubation with S-protein (10 μg/μL) for 8 h, each spot was assayed for ribonucleolytic activity[11] and fluorescence after staining with a primary antibody (to RNase A) and a secondary antibody conjugated to Alexa Fluor 488.[12] (B) Uniform versus random immobilization of protein. A slide displaying NHS-esters was spotted with ribonuclease A (5.0 nM) or phosphinothioester, an azido-S15 (5.0 nM), and S-protein (10 μg/μL). Each spot was assayed as in panel A.[11,12] (C) Timecourse of immobilization by Staudinger ligation. A phosphinothioester-derived slide was spotted with N$_3$-PEG-S15 (5.6 nM) for 1−60 min, and then quenched with NH$_2$OH. After incubation with S-protein (10 μg/μL), each spot was subjected to immunoassay as in panel A.[12]

in this manner had 85% activity.[15] Coupling via the main chain (as with N$_3$-PEG-S15) proceeded in 67% yield,[14] and the enzyme coupled in this manner had 92% activity.[15]

Control experiments indicate that binding occurred only by Staudinger ligation (Figure 1A, row 3). First, S15 without an azido group was spotted on a phosphinothioester-derivatized slide. After incubation of the slide with S-protein, no immobilized peptide was detected with the activity assay or immunoassay. Second, immobilized peptide was necessary for S-protein to bind to the surface, indicating that the phosphinothioester does not react with the functional groups of proteinogenic amino acids. Finally, azido-peptides did not react with a phosphinothioester-derivatized slide that had been treated with NH$_2$OH, which rapidly cleaves thioesters.[16]

Uniform immobilization of a protein analyte yields higher activity than does random immobilization. Amine-derivatized slides were treated with NHS-C(O)-PEG-C(O)-NHS in DMF for 2 h, and then intact RNase A (5.0 nM) in buffer for 8 h. Such immobilization will occur randomly via enzymic amino groups. Coupling proceeded in 45% yield,[17] but the coupled RNase A had only 6% activity (Figure 1B). This low activity is likely due to RNase A being attached through amino groups that are important for function,[18] which is an intrinsic disadvantage of random immobilization.[2−5]

Immobilization by Staudinger ligation is remarkably rapid. A subsaturating concentration of N$_3$-PEG-S15 was spotted for 1−60 min. After addition of S-protein, an immunoassay showed that the

same amount of peptide became attached to the slide in 1 min as in 1 h (Figure 1C) or 8 h (data not shown). Accordingly, the immobilization reaction has $t_{1/2}$ < 1 min.

The Staudinger ligation is the most efficacious method known for the site-specific, covalent immobilization of a protein. No other approach enables more rapid immobilization or a higher yield of active protein (cf.: refs 2−5). Azido-peptides[6] and azido-proteins[19] are readily attainable, and the reactivity of the azido group is orthogonal to that of biomolecules. Accordingly, the Staudinger ligation could be of unsurpassed utility in creating microarrays of functional peptides and proteins.

Supporting Information Available: Procedures and additional data for syntheses and analyses reported herein (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) The PEG layer ($M_r \approx 3400$) prevented the nonspecific attachment of protein to the glass surface.
(10) Peptide concentrations were 5.64 μM−56.4 pM for (N$_3$)Lys1 S15 (M_r 1773.8), and 4.38 μM−43.8 pM for N$_3$-PEG-S15 (M_r 2284.4).
(11) Enzymatic activity assays were performed as described (Kelemen, B. R.; Klink, T. A.; Behlke, M. A.; Eubanks, S. R.; Leland, P. A.; Raines, R. T. *Nucleic Acids Res.* **1999**, *27*, 3696−3701). Briefly, 0.10 M MES−NaOH buffer (pH 6.0) containing NaCl (0.10 M) and 6-carboxyfluorescein-(dA)-rU(dA)$_2$-6-tetramethylrhodamine (6 μM) was added to each spot. At various times, an aliquot was removed from the slide, and its fluorescence was determined.
(12) Immunoassays were performed on each spot by adding rabbit anti-RNase A IgG (2 μg/mL) for 30 min. The slide was then washed with PBS. Each spot was incubated with an Alexa Fluor 488-conjugated secondary antibody (1 μg/mL) for 30 min. The slide was then washed with PBS, and its fluorescence was determined.
(13) Bound peptide, 4.1 fmol/mm^2 (Figure 1A, row 1, column 4); spotted peptide, 8.0 fmol/mm^2.
(14) Bound peptide, 4.2 fmol/mm^2 (Figure 1A, row 2, column 4); spotted peptide, 6.2 fmol/mm^2.
(15) These values are lower limits, as RNase S′ ($K_d = 10^{-8}$ M; ref 7b) could dissociate to some extent during the timecourse of assays.
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