Protein Modification

DOI: 10.1002/anie.200600756

Site-Specific Protein Modification through Cu^{I}-Catalyzed 1,2,3-Triazole Formation and Its Implementation in Protein Microarray Fabrication**

*Po-Chiao Lin, Shau-Hua Ueng, Mei-Chun Tseng, Jia-Ling Ko, Kuo-Ting Huang, Sheng-Chieh Yu, Avijit Kumar Adak, Yu-Ju Chen, and Chun-Cheng Lin**

Site-specific modification of proteins is an important yet challenging task in biochemical and biophysical research. In particular, during the fabrication of protein microarrays, the activity of the protein may be significantly influenced by its orientation on the solid surface.[1] Although many reports have detailed the noncovalent immobilization of proteins with orientational control (for example, biotin–streptavidin and His tag–Ni interactions), there are few such methods for the site-specific covalent immobilization of proteins.[2] However, the most commonly used chemistries for protein modification, such as amide-bond formation and reductive amination, involve random covalent-bond formation,[3] which inevitably has a negative impact on protein activity and, hence, the sensitivity of activity-based assays. Thus, given the presence of multiple reactive amino acids in proteins under physiological conditions, there is a considerable need for highly selective orthogonal reactions.[4] To address this issue, we have combined the intein fusion protein expression system with Cu^{I}-catalyzed 1,2,3-triazole formation to specifically modify a target protein at its C terminus. Furthermore, the use of a diazido linker yielded a covalent link between monomers of a homodimeric protein. We also applied this method to the fabrication of a protein microarray by site-

[*] P.-C. Lin, Prof. Y.-J. Chen, Prof. C.-C. Lin
Department of Chemistry
National Tsing Hua University
Hsinchu (Taiwan)
Fax: (+886) 3-571-1082
E-mail: cclin66@mx.nthu.edu.tw
and
Chemical Biology and Molecular Biophysics
Taiwan International Graduate Program
Academia Sinica, Taipei (Taiwan)

P.-C. Lin, Dr. S.-H. Ueng, M.-C. Tseng, J.-L. Ko, K.-T. Huang, S.-C. Yu, Dr. A. K. Adak, Prof. Y.-J. Chen, Prof. C.-C. Lin
Institute of Chemistry and Genomic Research Center
Academia Sinica, Taipei (Taiwan)

J.-L. Ko
Department of Chemistry
National Taiwan Normal University
Taipei (Taiwan)

[**] We acknowledge financial support from the National Tsing Hua University (Academia Sinica Research Project on Nano Science and Technology) and the National Science Council, Taiwan.

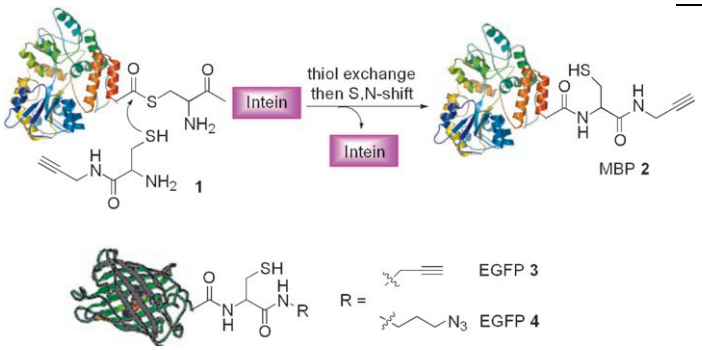
Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

specific covalent-bond formation, which resulted in the retention of the protein activity.

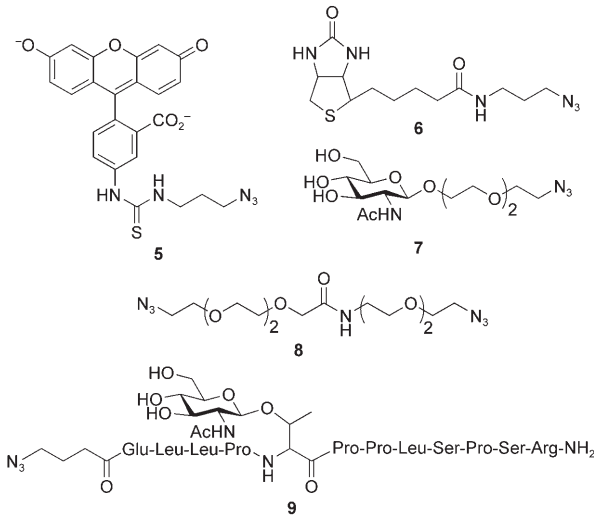
Recent studies have shown that site-specific modification of proteins can be achieved either by incorporating non-natural amino acids containing orthogonally reactive groups[5] or by the technique of expressed protein ligation (EPL)[6] to include terminal cysteine-containing molecules at the C termini. Although EPL has been used to attach a wide variety of molecules to target proteins[6–11] and to fabricate protein microarrays,[10–12] it has limited practical application for diverse modifications because of the instability of thioesters in proteins. As a result of the rapid expansion of array-based methods with diverse applications, there is an urgent need to develop methodologies to establish core template proteins that may be readily modified in a variety of ways; moreover, the systems must be amenable to long-term storage.

To develop an efficient and practical ligation method, Sharpless et al. and Meldal et al. exploited the effective formation of 1,2,3-triazoles between azides and terminal acetylenes in the presence of a Cu^I catalyst.[13] This azide–alkyne cycloaddition offers good reproducibility and a high degree of specificity and biocompatibility with water, which makes it potentially appropriate for a variety of in vitro and in vivo applications.[14–18] The Cu^I-catalyzed azide–alkyne cycloaddition[13] is highly predictable, very fast, and resistant to side reactions. Herein, we describe the use of EPL and the Cu^I-catalyzed formation of 1,2,3-triazole to achieve C-terminal protein modification and to fabricate a protein microarray by site-specific covalent-bond formation.

To demonstrate the concept, maltose binding protein (MBP) was chosen as the target protein because it has a good expression yield as an intein fusion protein in *Escherichia coli*.[7] MBP was expressed using the commercially available IMPACT (intein-mediated purification with affinity chitin-binding tag) vector system. The fusion protein was purified with chitin beads and treated with cysteine alkyne **1**, followed by a spontaneous S–N acyl shift to give core-alkynated MBP **2** (Scheme 1). The purified MBP **2** had a mass of 43217 Da, as determined by electrospray mass spectrometry. By a similar method, we prepared core-modified enhanced green fluorescent proteins (EGFPs) **3** and **4**, which contain alkyne and azide functional groups at their respective C termini. Initially, we used core-alkynated MBP **2** as the core template protein, with the C-terminal alkyne available for ligation with various azido molecules. To demonstrate the general applicability of the approach, **2** was conjugated with several target azide-containing molecules, such as fluorescein isothiocyanate (FITC; **5**), biotin (**6**), *N*-acetylglucosamine (**7**), diazide linker (**8**), and a glycopeptide (**9**; Scheme 2).



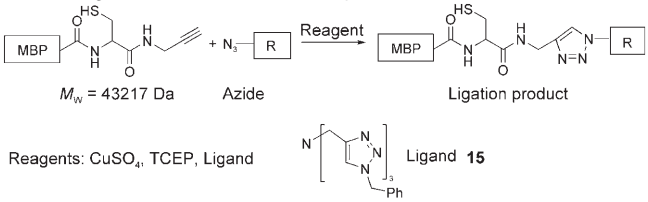
Scheme 1. Preparation of recombinant alkynated MBP **2**, EGFP **3**, and EGFP **4**.



Scheme 2. Azido molecules for ligation with alkynated MBP **2**.

Conjugation of **2** with azido molecules was achieved by 1,2,3-triazole formation catalyzed by Cu^I, which was produced from the reaction of $CuSO_4$ (1 mM) with tris(carboxyethyl)-phosphine (TCEP; 2 mM) in phosphate-buffered saline (PBS; pH 8.0, 0.1M; Table 1). As unligated and ligated **2** have similar masses (difference of <1 kDa) and hydrophobicity, common protein separation methods, such as SDS-PAGE and size-exclusion HPLC, could not be used to distinguish them. Thus, electrospray mass spectrometry was used to monitor both unligated and ligated **2** during the reaction and to evaluate the efficiency of cycloaddition.

Table 1: Ligation of various azido compounds with MBP **2**.



Azide	Ligation product	M_{obs} [Da]	M_{calcd} [Da]
5	**10**	43704	43704
6	**11**	43539	43544
7	**12**	43596	43594
8	**13**	43619	43606
9	**14**	44838	44836

In an attempt to maintain the native protein structure, the initial ligation between alkynated MBP **2** and **5** was performed overnight at 0 °C. However, these conditions resulted in a low product yield. To increase the reaction rate and yield, tri(triazolyl)amine ligand **15**

(2 mM)[15] was added and the reaction temperature was increased to 25 °C. As depicted in Figure 1 a, the electrospray mass spectrum revealed the time-dependent reactant/product ratio during the reaction. The *m*/*z* value of 43 704 clearly

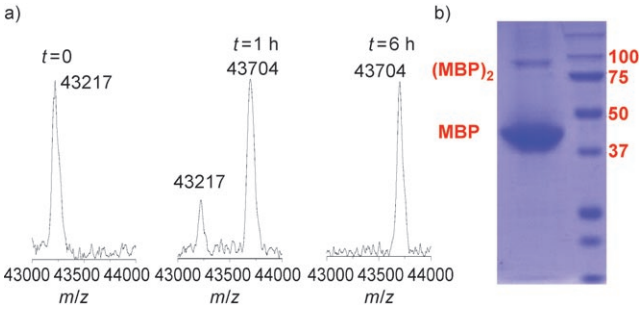


Figure 1. a) Ligation of alkynated MBP **2** and FITC (**5**) with reaction times of 0, 1, and 6 h. b) SDS-PAGE of homoligation of alkynated MBP 2 and **13**.

showed that ligation was complete in 6 hours, thus forming FITC-modified MBP **10** and indicating the efficiency of C-terminal modification. To demonstrate the application and versatility of the site-specific modification method, we tested some common reagents in bioevaluation, such as fluorescent probes and affinity tags. The observed masses of the modified protein products under different ligation reactions are shown in Table 1. The consistency between the calculated and observed masses of ligated proteins unambiguously shows that the correct ligation products were obtained. The observed yields clearly demonstrate that alkyne–azide cycloaddition presents a very efficient method for protein conjugation. In general, all ligations were complete within 12 h.

In addition to ligation with small molecules, the linker-conjugated MBP **13** was designed to demonstrate the applicability of the method to protein–protein conjugation. Alkynated MBP **2** was ligated with excess diazido linker **8** to give MBP **13**, which was again conjugated with MBP **2** to yield a covalently linked MBP homodimer (see Figure 1 b). SDS-PAGE of the ligation product showed a new band migrating at approximately 86 kDa, which is in good agreement with the expected mass of a covalent homodimer of MBP **2** (monomer 43 kDa). The ligation reaction was inefficient, however, probably because of the short length of the polyethylene glycol linker, which resulted in steric hindrance during close contact with the second MBP molecule. This putative steric effect was more significant with shorter linkers (data not shown). Further optimization of linker length, thereby minimizing steric hindrance, could improve the effective yield of the protein modification.

To demonstrate the utility of 1,2,3-triazole formation during the fabrication of a protein microarray, the alkyne-modified EGFP **3** and azide-modified EGFP **4** were used as fluorescent proteins that were covalently and site-specifically attached to glass slides by triazole formation, respectively. To prepare the alkynated and azidated glass slides, a slide coated with amino groups was incubated with glutaric acid bis-*N*-hydroxysuccinimide ester (10 mM) and then treated with 3-azidopropanylamine (10 mM) or monopropargylamine (10 mM) followed by capping with ethanolamine (Figure 2). The alkynated EGFP **3** (2.7 μg mL^{-1}) and azidated EGFP **4** (2.4 μg mL^{-1}) were spotted on the azide- and alkyne-functionalized glass slides, respectively, and unmodified EGFP

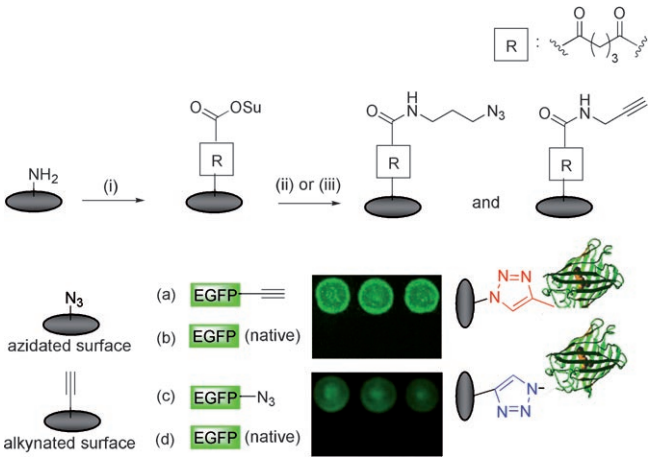


Figure 2. Fabrication of an EGFP microarray by 1,2,3-triazole formation for a) alkynated EGFP **3** and b) native EGFP with azido slide; c) azido-EGFP **4** and d) native EGFP with alkynated slide. Reaction conditions: i) immersed in a solution of glutaric acid bis-*N*-hydroxysuccinimide ester (10 mM) in DMF at room temperature for 24 h; ii) a solution of 3-azidopropanylamine (10 mM) in DMF, and iii) a solution of monopropargylamine (10 mM) in DMF incubated with activated slide in the presence of *N*,*N*-diisopropylethylamine (100 mM) at room temperature for 24 h.

was used as control for both functionalized glass slides. The ligation reaction was incubated for 12 h at room temperature in the presence of $CuSO_4$ (2 mM) and TCEP (4 mM) in PBS buffer (pH 8.0), and the protein-modified slide was then extensively washed with PBST (PBS + 0.04 % Tween 20). Positive EGFP fluorescence, as measured at 530 nm, was evident for both ligations on the glass slides, whereas the negative control gave no signal (Figure 2). These results indicate that chemoselective attachment was achieved by 1,2,3-triazole formation and that the protein tertiary structure was maintained.

However, the azide-modified slide presented a much better surface for efficient protein ligation relative to the alkyne-modified surface (Figure 2 a versus 2 c). This effect may be because Cu^I coordinates with alkynes in solution more rapidly and with higher affinity than with the azide, thereby enhancing the rate of 1,2,3-triazole formation with the surface azido group.[19] A similar preference for the surface functional group has also been reported when labeling azido-modified cowpea mosaic virus with an alkynated dye molecule through triazole formation.[15b] Significantly, in the current study a protein concentration of only 1 μM was needed to achieve well-detectable levels of immobilization, which demonstrates that Cu^I-catalyzed 1,2,3-triazole formation is a very efficient reaction in surface chemistry.[12]

To address the importance of orientation in protein immobilization, alkynated MBP **2** was covalently attached to a glass slide in a site-specific manner by triazole formation, as described in the fabrication of the EGFP microarray.

Biotinylated maltose **16** was then used as the probe to interact with **2** on the glass surface, followed by fluorescence visualization with streptavidin-Cy3. As shown in Figure 3a, the relative intensity of the signal measured at 570 nm indicated that chemoselective attachment was achieved by 1,2,3-triazole formation.

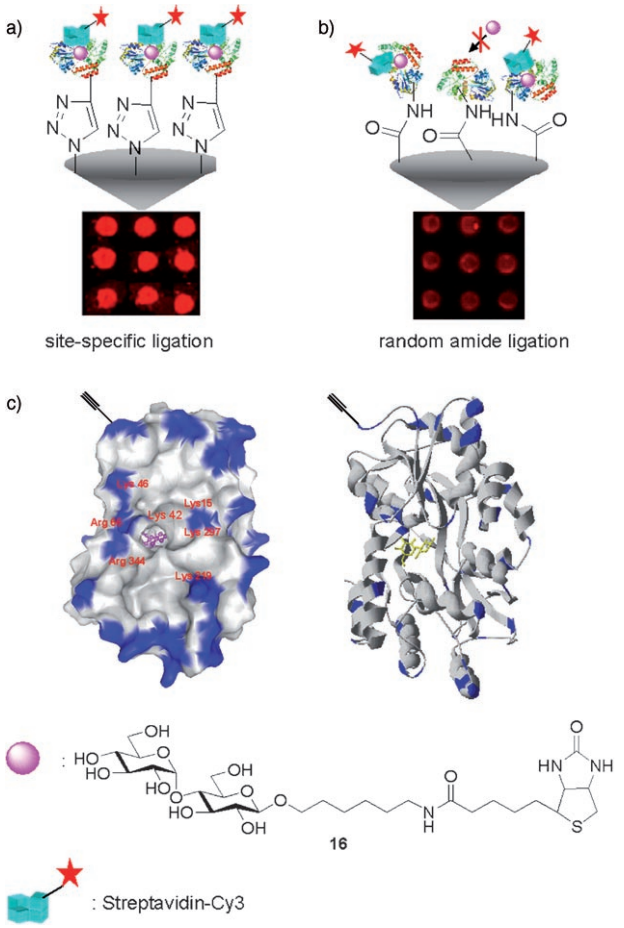


Figure 3. Fabrication of a protein microarray by a) site-specific covalent-bond (1,2,3-triazole) formation or b) random amide-bond formation. c) X-ray structure of MBP, with blue moieties indicating Lys and Arg residues.

To compare the effect of site-specific or random conjugation on maltose binding activity, MBP **2** was immobilized on *N*-hydroxysuccinimide (OSu)-activated slides by random amide-bond formation (Figure 3b). The site-specific immobilization technique yielded higher protein binding activity (Figure 3a versus 3b). The X-ray crystal structure of MBP[20] reveals that seven of 41 lysine and arginine residues on the protein surface are located near the maltose binding site (Figure 3c). These amino-containing residues may react with reagents involved in protein immobilization, which could, upon covalent attachment to a solid surface, cause a partial or complete blockade of the maltose binding site. By contrast, the C terminus of MBP is far from the binding site, and thus immobilization at this site would have minimal impact on the native conformation of the maltose binding site. Our data demonstrate that the site-specific fabrication of a protein array preserves greater protein activity relative to random protein attachment methods, thereby significantly improving assay sensitivity.

In conclusion, by combining the intein fusion protein expression system with Cu^{I}-catalyzed 1,2,3-triazole formation, the target protein was efficiently modified at its C terminus. This method allows the immobilized core template protein to be modified by diverse small molecules in a site-specific manner. In addition, site-specific protein–protein conjugation was achieved by using a diazido linker, a method that should be applicable to the preparation of protein heterodimers. Moreover, we demonstrated the importance of site-specific immobilization of a protein in an array-based assay; when protein molecules are oriented in an optimal manner, the native conformation is more likely to be preserved, thereby retaining greater activity. Given the flexibility and ease of this site-specific modification/immobilization technique, the procedure reported herein constitutes an efficient and reproducible method for the fabrication of protein microarrays or other solid-phase assays.

Received: February 27, 2006
Published online: May 31, 2006

Keywords: copper · dimerization · ligation · nitrogen heterocycles · protein modifications

[1] T. Cha, A. Guo, X.-Y. Zhu, *Proteomics* **2005**, *5*, 416–419.
[2] a) K.-Y. Tomizaki, K. Usui, H. Mihara, *ChemBioChem* **2005**, *6*, 782–799; b) A. D. De Araújo, J. M. Palomo, J. Cramer, M. Köhn, H. Schröder, R. Wacker, C. Niemeyer, K. Alexandrov, H. Waldmann, *Angew. Chem.* **2006**, *118*, 302–307; *Angew. Chem. Int. Ed.* **2006**, *45*, 296–301.
[3] G. T. Hermanson, *Bioconjugate Techniques*, Academic Press, San Diego, **1996**.
[4] For a review, see: G. A. Lemieux, C. R. Bertozzi, *Trends Biotechnol.* **1998**, *16*, 506–513.
[5] L. Wang, P. G. Schultz, *Angew. Chem.* **2004**, *116*, 34–66; *Angew. Chem. Int. Ed.* **2005**, *44*, 34–66.
[6] T. W. Muir, *Annu. Rev. Biochem.* **2003**, *72*, 249–289.
[7] T. J. Tolbert, C.-H. Wong, *J. Am. Chem. Soc.* **2000**, *122*, 5421–5428.
[8] J. A. Pezza, K. N. Allen, D. R. Tolan, *Chem. Commun.* **2004**, 2412–2413.
[9] T. Durek, K. Alexandrov, R. S. Goody, A. Hildebrand, I. Heinemann, H. Waldmann, *J. Am. Chem. Soc.* **2004**, *126*, 16368–16378.
[10] M. Lovrinovic, R. Seidel, R. Wacker, H. Schroeder, O. Seitz, M. Engelhard, R. S. Goody, C. M. Niemeyer, *Chem. Commun.* **2003**, 822–823.
[11] M.-L. Lesaicherre, R. Y. P. Lue, G. Y. J. Chen, Q. Zhu, S. Q. Yao, *J. Am. Chem. Soc.* **2002**, *124*, 8768–8769.
[12] J. A. Camarero, Y. Kwon, M. A. Coleman, *J. Am. Chem. Soc.* **2004**, *126*, 14730–14731.
[13] a) V. V. Rostovtsev, L. G. Green, V. V. Fokin, K. B. Sharpless, *Angew. Chem.* **2002**, *114*, 2708–2721; *Angew. Chem. Int. Ed.* **2002**, *41*,2596–2599; b) C. W. Tornøe, C. Christensen, M. Meldal, *J. Org. Chem.* **2002**, *67*, 3057–3064; c) for a review, see: V. D. Bock, H. Hiemstra, J. H. Van Maarseveen, *Eur. J. Org. Chem.* **2006**, 51–68.
[14] a) H. C. Kolb, K. B. Sharpless, *Drug Discovery Today* **2003**, *8*, 1128–1137; b) A. Krasinski, Z. Radic, R. Manetsch, J. Raushel, P. Taylor, K. B. Sharpless, H. C. Kolb, *J. Am. Chem. Soc.* **2005**, *127*, 6686–6692.

[15] a) T. R. Chan, R. Hilgraf, K. B. Sharpless, V. V. Fokin, *Org. Lett.* **2004**, *6*, 2853 – 2855; b) Q. Wang, T. R. Chan, R. Hilgraf, V. V. Fokin, K. B. Sharpless, M. G. Finn, *J. Am. Chem. Soc.* **2003**, *125*, 3192 – 3193.
[16] A. E. Speers, B. F. Cravatt, *Chem. Biol.* **2004**, *11*, 535 – 546.
[17] a) J. P. Collman, N. K. Devaraj, C. E. D. Chidsey, *Langmuir* **2004**, *20*, 1051 – 1053; b) N. K. Devaraj, G. P. Miller, W. Ebina, B. Kakaradov, J. P. Collman, E. T. Kool, C. E. D. Chidsey, *J. Am. Chem. Soc.* **2005**, *127*, 8600 – 8601; c) J. K. Lee, Y. S. Chi, I. S. Choi, *Langmuir* **2004**, *20*, 3844 – 3847.
[18] M. C. Bryan, F. Fazio, H.-K. Lee, C.-Y. Huang, A. Chang, M. D. Best, D. A. Calarese, O. Blixt, J. C. Paulson, D. Burton, I. A. Wilson, C.-H. Wong, *J. Am. Chem. Soc.* **2004**, *126*, 8640 – 8641.
[19] S. Punna, J. Kuzelka, Q. Wang, M. G. Finn, *Angew. Chem.* **2005**, *117*, 2255 – 2260; *Angew. Chem. Int. Ed.* **2005**, *44*, 2215 – 2220.
[20] N. V. Adikesavan, S. S. Mahmood, S. Stanley, Z. Xu, N. Wu, M. Thibonnier, M. Shoham, *Acta Crystallogr. F* **2005**, *61*, 341.

