Development of trifunctional probes for glycoproteomic analysis†

Charng-Sheng Tsai,[a] Po-Yu Liu,[ab] Hsin-Yung Yen,[ab] Tsui-Ling Hsu[a] and Chi-Huey Wong*[ac]

Received 11th March 2010, Accepted 16th April 2010
First published as an Advance Article on the web 13th May 2010
DOI: 10.1039/c0cc00345j

A new trifunctional probe, assembled using a cleavable linker, is useful for efficient enrichment and detection of alkynyl sugar-tagged biomolecules.

Glycosylation is an important co-/post-translational modification that has profound effects on protein structure and function.[1] However, the complex and non-templated nature of glycans has been a barrier for studying many of their basic features, especially on a proteome-wide scale. In order to understand the role of glycans in numerous physiological and pathological processes, metabolic oligosaccharide engineering (MOE) has been employed to insert sugar analogues appended with bioorthogonal alkynyl or azido groups in place of the native sugars *via* glycan synthesis pathways in cells.[2] Following further labeling with proper probes *via* Cu(I)-catalyzed Azide–Alkyne [3 + 2] Cycloaddition (CuAAC),[3] the tagged glycoproteins can be visualized and profiled on a proteome-scale. Click-activated fluorogenic probes have widely emerged as indispensable tools for labeling biomolecules,[2c,d,4] as these probes give lower background signals that are commonly observed in many fluorochromes.[5] Fluorogenic probes bearing azido or alkynyl groups can form a 1,2,3-triazole ring under CuAAC to trigger fluorescence.[2c,d,5b,c] Our previous studies have demonstrated that peracetylated alkynyl sugar derivatives of fucose (Fucyne) and *N*-acetylmannosamine (ManNAcyne) can be incorporated into fucosylated and sialylated proteins, respectively, where they are selectively labeled by fluorogenic azido-1,8-naphthalimide or coumarin probes using CuAAC.[2c,d] These click-activated probes can be used for visualizing and quantitatively studying cellular glycoconjugates.

In this paper, we report the synthesis of trifunctional probes,[6] which combines the features of labeling alkynyl group-tagged biomolecules, generating fluorescence upon CuAAC, and enabling affinity enrichment of labeled biomolecules, for testing their applications in glycoproteomic analysis. These trifunctional probes consist of three major components: an azido group and click-activated fluorescent and biotin moieties.

Here we examine the applications of two trifunctional probes with different linkers for their abilities in both target detection *via* 3-azido-7-aminocoumarin and enrichment *via* biotin, when reacting with alkynyl sugars (Fig. 1). Probe **I** carries an azido group for both conjugation and fluorescent triggering. When the 1,2,3-triazole ring was formed by CuAAC, it activated the fluorescent property of 3-azido-7-aminocoumarin to provide a high fluorescent signal for rapid and sensitive target detection, while the biotin group in probe **I** offered the advantage of glycoprotein enrichment. In order to increase the elution yield of labeled targets that bind to streptavidin matrices, we further introduced a cleavable linker (cystamine) between 3-azido-7-aminocoumarin and biotin in probe **II**. Thus after binding to streptavidin matrices, the labeled targets can be recovered by treatment with reducing agents such as dithiothreitol (DTT) or *tris*-(2-carboxyethyl) phosphine (TCEP)[7] to break the disulfide bond and give fluorescent labeled biomolecules.

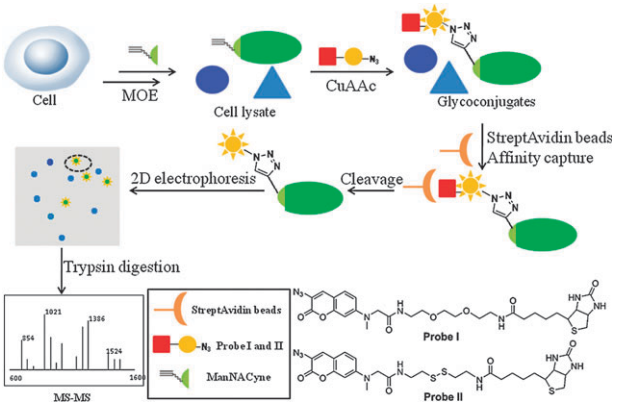


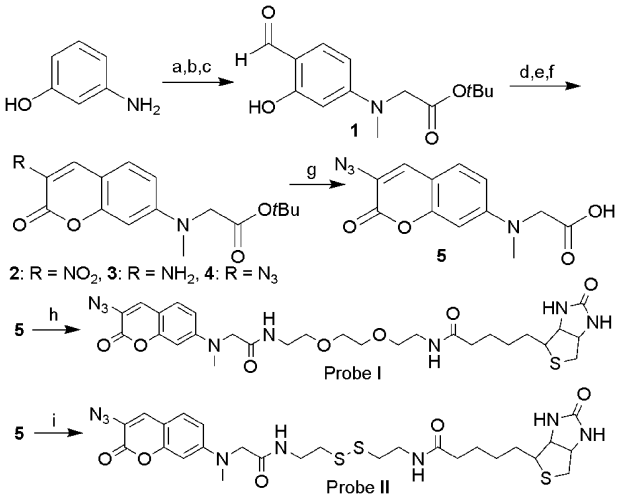
Fig. 1 Application of trifunctional probes in glycoproteomics and the structure of trifunctional probes **I** and **II**.

As shown in Scheme 1, the synthesis of probes **I** and **II** started with two alkylations and formylation of 3-aminophenol to give **1**, which was further condensed with ethyl nitroacetate under the Knoevenagel condensation reaction[8] to yield **2**. The nitro group was subsequently reduced in presence of Lindlar catalyst to give **3**. The azido group was introduced to the C-3 position of **3** using *tert*-butyl nitrite (*t*-BuONO) and azidotrimethylsilane ($TMSN_3$) to yield **4**,[9] followed by treatment with trimethylsilyl triflate (TMSOTf) and 2,6-lutidine to give **5**. Probes **I** and **II** were constructed by amide bond formation between **5** and the TFA salt of the dioxaoctanediamine derivative of biotin or the TFA salt of the cystamine derivative of biotin in the presence of EDCI.

The reactivity and click-activated fluorogenic property of probes **I** and **II** were tested on ManNAcyne. As expected, the ligation reaction produced a significant increase in fluorescence intensity (Fig. 2). The wavelength of maximum emission

[a] *Genomics Research Center, Academia Sinica, 128 Academia Road Section 2, Nankang, Taipei 115, Taiwan. E-mail: chwong@gate.sinica.edu.tw; Fax: +886-2-2785-3852; Tel: +886-2-2789-9400*
[b] *Institute of Biochemical Science, National Taiwan University, 1 Roosevelt Road Section 4, Taipei 106, Taiwan*
[c] *Department of Chemistry, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA. E-mail: wong@scripps.edu*

† Electronic supplementary information (ESI) available: Synthetic procedures for probes **I** and **II**, 2D-gel and table of identified proteins. See DOI: 10.1039/c0cc00345j



Scheme 1 Synthesis of probes **I** and **II**. *Reagents and conditions*: (a) CH_3I, K_2CO_3, DMF, 100 °C, 60%; (b) *tert*-butyl bromoacetate, 2,6-lutidine, DMF, rt, 60%; (c) $POCl_3$, DMF, rt, 95%; (d) ethyl nitroacetate, piperidine, AcOH, *n*-butanol, reflux, 96%; (e) Lindlar catalyst, H_2, MeOH, rt; (f) *t*BuONO, $TMSN_3$, CH_3CN, rt, 65% in two steps; (g) TMSOTf, 2,6-lutidine, CH_2Cl_2, rt; (h) the TFA salt of dioxaoctanediamine derivative of biotin, EDCI, HOBT, TEA, DMF, rt, 55% in two steps; (i) the TFA salt of cystamine derivative of biotin, EDCI, HOBT, TEA, DMF, rt, 58% in two steps.

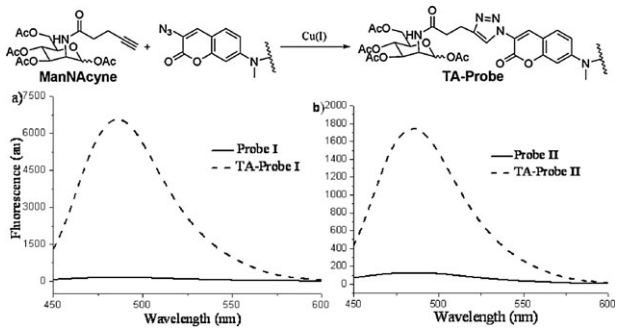


Fig. 2 The fluorescence spectra of probe **I** and probe **II** and their click products TA-probe **I** and TA-probe **II**.

for both probes **I** and **II** was 495 nm when excited at 430 nm, whereas the parent compounds of probes **I** and **II** gave very low fluorescence under this setting (Fig. 2a and b). TA-probe **I** and TA-probe **II** (reaction products with triazole rings) displayed 46-fold and 18-fold fluorescence enhancement compared to probe **I** and probe **II**, respectively. The spectral properties of probes **I** and **II**, and TA-probes **I** and **II** were similar to those of the corresponding 3-azido-7-diethylaminocoumarin and their CuAAC adducts (the compounds without biotin moiety), respectively.[5b]

We next examined whether these probes could be applied to analyze sialylated glycoproteomes. After feeding with ManNAcyne or control ManNAc, prostate cancer PC3 cells were lysed for harvesting their protein extracts. The soluble fractions of lysates were next labeled with probe **I** or probe **II** and then separated by gel electrophoresis. The fluorescence imaging of protein gels showed specific signals in the lysates derived from PC3 cells fed with ManNAcyne (Fig. 3, lanes 6 and 8) but not ManNAc (Fig. 3, lanes 5 and 7). The signal

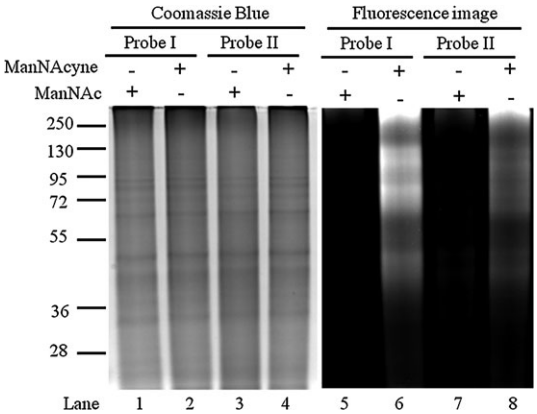


Fig. 3 Detection of ManNAcyne-labeled glycoproteins in PC3 cell extracts. Lanes 1–4: Coomassie blue stain for visualizing total proteins of each sample. Lanes 5–8: protein gel analyzed by fluorescence imaging (Ex: 419 nm; Em: 490 ± 25 nm).

intensity generated from probe **I** was stronger than that from probe **II**, which was similar to their corresponding fluorescent moieties alone.

We also tested whether these trifunctional probes were able to enrich sialylated proteins. Probe-labeled ManNAcyne-treated cell extracts were incubated with streptavidin beads. After removing unbound proteins, probe **I**- and probe **II**-labeled proteins were eluted with 1 mM biotin and 50 mM TCEP, respectively. Our results showed that the elution yield of probe **I**-labeled lysate was less than 1% and the elution yield of probe **II**-labeled lysate was more than 90%. We observed specific enrichment and efficient elution of sialylated proteins labeled by probe **II** (Supporting Information Fig. S1†). In summary, probe **II** is more suitable for the enrichment of sialylated proteins.

We further analyzed the enriched glycoproteins by 2D electrophoresis. Labeled glycoproteins from the cells fed with ManNAcyne showed specific fluorescent signals compared to the cells fed with control ManNAc (Fig. 4). The results showed detectable protein spots in the ManNAcyne-, but not the ManNAc-treated sample, indicating that the enrichment by probe **II** is specific. Comparing the signals given by probe **II** and protein stain, the spots can be classified as either high or low sialic acid/protein ratios. According to this, we picked up five spots (two and three with high or lower sialic acid/protein ratio, respectively) for protein identification by LC-MS/MS (Supporting Information Table S1†). Among these five spots, two were predicted to have potential glycosylation sites (spots 2 and 4) and three have been proved to be glycoproteins (spots 1, 3, and 5),[10] validating that probe **II** enabled the enrichment of glycoproteins appended with sugar analog(s). Here, the results demonstrated that our trifunctional probing system is useful for glycoproteomic study.

In conclusion, we have developed and synthesized two trifunctional probes. Notably, probe **II** is useful for the efficient enrichment and detection of alkynyl sugar-tagged biomolecules (*e.g.*, sialylated proteins). Therefore, this trifunctional probe can be a powerful tool for use in profiling and comparing low-abundant glyco-biomolecules at different physiological/pathological stages for identification of glycan-related biomarkers and targets, for example, in cancer cells.

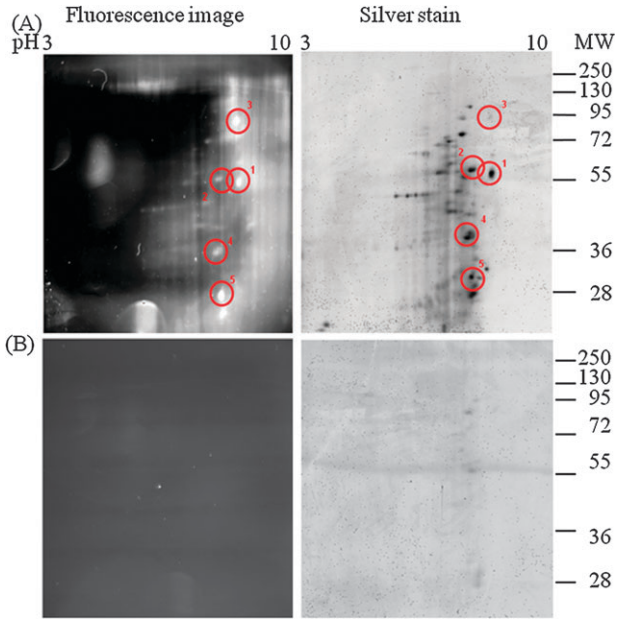


Fig. 4 After reaction of probe **II** with PC3 cell lysate was resolved in 2D electrophoresis (A) fluorescence image (left) and silver stain (right): enriched PC3 cell lysate treated with ManNAcyne then clicked with probe **II**. (B) Fluorescence image (left) and silver stain (right): enriched PC3 cell lysate clicked with probe **II**, PC3 cell (Ex: 419 nm; Em: 490 ± 25 nm).

We thank Dr Chung-Yi Wu for critical comments on this manuscript, and Chien-Hung Chen for technical support on MS analysis.

Notes and references

1 A. Varki, R. Cummings, J. D. Esko, H. Freeze, G. W. Hart and J. Marth, *Essentials of Glycobiology*, Cold Spring Harbor Press, Cold Spring Harbor, NY, 1999.

2 (*a*) J. A. Prescher and C. R. Bertozzi, *Cell*, 2006, **126**, 851; (*b*) S. T. Laughlin, N. J. Agard, J. M. Baskin, I. S. Carrico, P. V. Chang, A. S. Ganguli, M. J. Hangauer, A. Lo, J. A. Prescher, C. R. Bertozzi and F. Minoru, *Methods in Enzymology*, Academic Press, New York, 2006, vol. 415, pp. 230–250; (*c*) M. Sawa, T.-L. Hsu, T. Itoh, M. Sugiyama, S. R. Hanson, P. K. Vogt and C.-H. Wong, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 12371–12376; (*d*) T.-L. Hsu, S. R. Hanson, K. Kishikawa, S.-K. Wang, M. Sawa and C.-H. Wong, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 2614–2619; (*e*) S. R. Hanson, T.-L. Hsu, E. Weerapans, K. Kishikawa, G. M. Simon, B. F. Cravatt and C.-H. Wong, *J. Am. Chem. Soc.*, 2007, **129**, 7266–7267; (*f*) P. V. Chang, X. Chen, C. Smyrniotis, A. Xenakis, T. Hu, C. R. Bertozzi and P. Wu, *Angew. Chem., Int. Ed.*, 2009, **48**, 4030–4033; (*g*) P. V. Chang, J. A. Prescher, E. M. Sletten, J. M. Baskin, I. A. Miller, N. J. Agard, A. Lo and C. R. Bertozzi, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 1821–1826; (*h*) M. A. Breidenbach, J. E. G. Gallagher, D. S. King, B. P. Smart, P. Wu and C. R. Bertozzi, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 3988–3993.

3 (*a*) V. V. Rostovtsev, L. G. Green, V. V. Fokin and K. B. Sharpless, *Angew. Chem., Int. Ed.*, 2002, **41**, 2596–2599; (*b*) H. C. Kolb and K. B. Sharpless, *Drug Discovery Today*, 2003, **8**, 1128–1137.

4 (*a*) K. E. Beatty, F. Xie, Q. Wang and D. A. Tirrell, *J. Am. Chem. Soc.*, 2005, **127**, 14150–14151; (*b*) K. E. Beatty, J. C. Liu, F. Xie, D. C. Dieterich, E. M. Schuman, Q. Wang and D. A. Tirrell, *Angew. Chem., Int. Ed.*, 2006, **45**, 7364–7367; (*c*) H. E. Murrey and L. C. Hsieh-Wilson, *Chem. Rev.*, 2008, **108**, 1708–1731; (*d*) P. M. Clark, J. F. Dweck, D. E. Mason, C. R. Hart, S. B. Buck, E. C. Peters, B. J. Agnew and L. C. Hsieh-Wilson, *J. Am. Chem. Soc.*, 2008, **130**, 11576–11577; (*e*) Z. Wang, K. Park, F. Comer, L. C. Hsieh-Wilson, C. D. Saudek and G. W. Hart, *Diabetes*, 2008, **58**, 309–317; (*f*) A. B. Neef and C. Schultz, *Angew. Chem., Int. Ed.*, 2009, **48**, 1498–1500.

5 (*a*) G. A. Lemieux, C. L. De Graffenried and C. R. Bertozzi, *J. Am. Chem. Soc.*, 2003, **125**, 4708–4709; (*b*) K. Sivakumar, F. Xie, B. M. Cash, S. Long, H. N. Barnhill and Q. Wang, *Org. Lett.*, 2004, **6**, 4603–4606; (*c*) F. Xie, K. Sivakumar, Q. Zeng, M. A. Bruckman, B. Hodges and Q. Wang, *Tetrahedron*, 2008, **64**, 2906–2914.

6 (*a*) S. C. Alley, F. T. Ishmael, A. D. Jones and S. J. Benkovic, *J. Am. Chem. Soc.*, 2000, **122**, 6126–6127; (*b*) G. C. Adam, E. J. Sorensen and B. F. Cravatt, *Mol. Cell. Proteomics*, 2002, **1**, 828–835; (*c*) M.-R. Lee, D.-W. Jung, D. Williams and I. Shin, *Org. Lett.*, 2005, **7**, 5477–5480; (*d*) W.-W. Qiu, J. Xu, D.-Z. Liu, J.-Y. Li, Y. Ye, X.-Z. Zhu, J. Li and F.-J. Nan, *Bioorg. Med. Chem. Lett.*, 2006, **16**, 3306–3309; (*e*) G. Guizzunti, T. P. Brady, V. Malhotra and E. A. Theodorakis, *Bioorg. Med. Chem. Lett.*, 2007, **17**, 320–325.

7 J. A. Burns, J. C. Butler, J. Moran and G. M. Whitesides, *J. Org. Chem.*, 1991, **56**, 2648–2650.

8 G. Brufola, F. Fringuelli, O. Piermatti and F. Pizzo, *Heterocycles*, 1996, **43**, 1257.

9 K. Barral, A. D. Moorhouse and J. E. Moses, *Org. Lett.*, 2007, **9**, 1809–1811.

10 (*a*) R. Chen, X. Jiang, D. Sun, G. Han, F. Wang, M. Ye, L. Wang and H. Zou, *J. Proteome Res.*, 2009, **8**, 651–661; (*b*) J. Hedou, B. Bastide, A. Page, J. C. Michalski and W. Morelle, *Proteomics*, 2009, **9**, 2139–2148; (*c*) M. E. Grace and G. A. Grabowski, *Biochem. Biophys. Res. Commun.*, 1990, **168**, 771–777.