Synthetic Methods

DOI: 10.1002/anie.200804514

Dual Labeling of Biomolecules by Using Click Chemistry: A Sequential Approach**

Péter Kele, Gábor Mezö, Daniela Achatz, and Otto S. Wolfbeis**

Dedicated to Professor Ferenc Sebestyén on the occasion of his 70th birthday

Imaging biomolecules by means of fluorescent tags is an important tool for the study of complex biological processes both in vitro and in vivo. The introduction of these reporter tags rely on their selective and efficient reaction, under physiological conditions, with the available functional groups on the biomolecule of interest. Bioorthogonal chemical reporters which are "non-native, non-perturbing chemical handles that can be modified in living systems through highly selective reactions with exogenously delivered probes" have drawn much attention lately.[1a] Among bioorthogonal tagging reactions the Staudinger ligation[1] and the click reaction, involving the copper(I)-catalyzed azide alkyne cycloaddition (CuAAC),[2] are the most valuable. These two methods are superior to other labeling techniques because of the inertness of the chemical reporters and the exogenously delivered probes, and the selective and efficient reaction between the reporter and the probe. The extreme rareness of azide and alkyne functions in biological systems additionally increases the importance of tagging by the means of a copper catalyzed azide/alkyne cycloaddition (CuAAC; a click reaction). This reaction has been shown to be quite versatile in terms of biological applications.[3] CuAAC also finds widespread applications in the high throughput screening of libraries.[4] Recently, Bertozzi et al. have proposed a copper-free version of tagging by using click reactions of strained cycloalkynes.[5] Boons et al. recently reported the synthesis of novel and potent dibenzocyclooctynols which react with azides at very high rate.[6] The efficiency of these strained cyclooctynes in click reactions was found to be comparable to that of CuAAC, which paves the way to bioorthogonal chemical reporters that can be easily labeled with more than one fluorescent tag. This option is particularly useful when these reporters are to be transformed into resonance energy transfer (RET) or fluorescence resonance energy transfer (FRET) systems, by using the appropriate fluorescent tags.[7] The introduction of multiple labels onto biomolecules still remains a challenging task. During the preparation of this manuscript, Carell et al. published modular labeling of DNA sequences with multiple click labels using a combination of alkyne protecting groups.[8]

To tag peptide sequences post-synthetically with multiple labels by using click chemistry, we first tried to differentiate the terminal alkyne groups by using protecting groups. Our efforts to introduce protected alkyne groups to appropriately modified model peptide sequences failed, probably as a result of the lability of the alkyne protecting groups. We also tried to introduce protected alkyne groups to aryl-halide modified peptides by using the Sonogashira reaction.[9] However, this route was found to not be efficient as very low overall yields were observed, so we then focused on the cyclooctyne moiety known[5] to react with azides in an uncatalyzed click reaction, which is in contrast to the behavior of terminal alkynes.

Herein we demonstrate the feasibility of sequential tagging using click labels on bioorthogonal model systems in solution by sequentially exploiting copper-free and copper-mediated click chemistry. We believe that this is a very versatile method that has numerous applications, especially because the reactions proceed readily under conditions similar to physiological conditions (i.e., room temperature, aqueous solution, pH 7).

The introduction of multiple labels onto a biomolecule was first tested for simple model systems. Several cyclooctyne derivative candidates are known,[5c] but for the sake of facile and concise synthesis we chose compound **A**[5b] (Figure 1) to elucidate the conditions for copper-free labeling using fluorescent azides. Amongst the azido labels considered, compound **2** (Figure 2) had the salient feature that only its click products are fluorescent, thus eliminating background fluorescence of unreacted starting material.[10] To introduce a novel long wavelength azido label that emits in the red region of the visible spectrum, we have prepared compound **3** (see the Supporting Information for experimental details).[11] Its design was derived from the Stokes dye family developed by Czerney et al.[12]. This family possesses remarkable photostability, high quantum yields, and large Stokes shifts.

[*] Dr. P. Kele
Institute of Chemistry, Eötvös Loránd University
1117 Pázmány Péter sétány 1a, Budapest (Hungary)
Fax: (+36) 1-372-2909
E-mail: kelep@elte.hu
D. Achatz, Prof. Dr. O. S. Wolfbeis
Institute of Analytical Chemistry, Chemo- and Biosensors, University of Regensburg
93040 Regensburg (Germany)
Fax: (+49) 941-943-4065
E-mail: otto.wolfbeis@chemie.uni-regensburg.de
Prof. Dr. G. Mezö
Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Eötvös L. University (Hungary)

[**] P.K. thanks the Humboldt Foundation for a Humboldt fellowship (3.3-UNG/1126507). Financial support from GVOP-3.2.1.-2004-04-0005/3.0, T 049814 (OTKA) and H07-B-74291 (NKTH-OTKA) are greatly acknowledged. The authors wish to thank Dr. K. Hegyi and Dr. Z. Novák for helpful discussions.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200804514.

CyOCOOMe **A**

CyOCO-Ala-PrGly-NH_2 **Pep1**

CyOCO-Gly-Pro-Leu-Gly-Val-Arg-PrGly-NH_2 **Pep2**

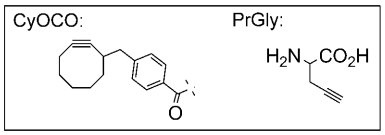

Figure 1. Model systems.

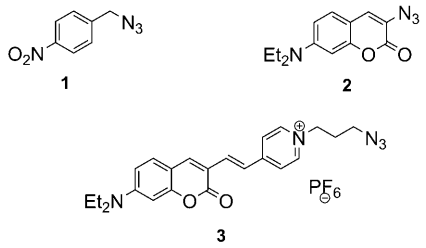

Figure 2. Fluorophore-containing azides for click chemistry. **1** is a nonfluorescent azide designed for use as a dark quencher, whereas **2** is a nonfluorescent azide that becomes fluorescent after undergoing a click reaction. **3** is strongly fluorescent both as the azide and its click product.

Compound **A** readily reacted with azides, in the absence of Cu^I and in aqueous media, to give both regioisomers of the triazole products, which is in accordance with earlier observations.[5] Next we incorporated the cyclooctyne and the terminal alkyne moieties into model compounds. **Pep1** and **Pep2** were prepared on Rink-amide MBHA (MBHA = 4-methylbenzhydrylamine) resin by using standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. In a first attempt, the cyclooctyne moiety was attached to resin-bound peptide chains. However, the crude and purified peptides had mass values that were different from the calculated mass values. The CyOCO-Ala-DL-PrGly-NH_2 showed 426.2 Da instead of 408.0, probably the result of the presence of one firmly bound water molecule. The CyOCO-Gly-Pro-Leu-Gly-Val-Arg-DL-PrGly-NH_2 had a mass of 1056.3 Da which is higher by 140 Da than expected. By using the ESI MS/MS method we found that in both cases the cyclooctyne derivatives were modified by the addition of the scavengers, applied during the cleavage of the peptides from the resin (details not shown here), to the triple bond.

The synthetic strategy was therefore partially modified such that the peptides were built up on the resin and then the cyclooctyne derivative was attached in solution. This synthetic route was successful and the expected compounds were identified by mass spectrometry (m/z 408.0 and 916.4, respectively). Although the application of the racemic forms of PrGly and CyOCO− may lead to four diastereomers, HPLC analysis revealed only two peaks for each of the peptides (see the Supporting Information). At this point there were no attempts to separate the isomers. This synthetic approach also revealed that the cyclooctyne moiety is sensitive to the acidic conditions of peptide cleavage from the Rink-amide MBHA resin. Other resins requiring milder cleavage conditions are potentially more adequate for the incorporation of the CyOCO− moiety onto the solid-phase. Alternatively, direct labeling of support-bound sequences may also be possible prior to cleavage from the resin.

Dual labeling of the peptides was carried out in acetonitrile/water (1:1). The solution containing the peptide was treated with an equimolar quantity of the first label. The solutions were stirred at room temperature for 12 hours, and then the crude products were analyzed by using HPLC methods (Table 1). The monolabeled peptide was then treated with an equimolar quantity of the second label in the presence of $CuSO_4$, ascorbic acid, and triethylamine, and then stirred at room temperature for 12 hours. HPLC analyses indicated that both coupling steps proceeded with good to excellent yields (Table 1). MS analyses of the crude or purified products were in complete agreement with the expected data (see the Supporting Information). For **Pep1** the second labeling proceeded with lower yields compared to **Pep2**, potentially because of the increased steric hindrance present on the smaller peptide.

Table 1: Dual labeling of model compounds.[a]

Entry	Model compound	Label 1	Yield **1** [%][b,c]	Label 2	Yield **2** [%][b,c]
1	**Pep1**	**2**	96	**1**	71
2	**Pep1**	**2**	98	**3**	73
3	**Pep1**	**3**	76 (66)	–	–
4	**Pep2**	**2**	>99 (82)	**1**	>99 (65)
5	**Pep2**	**2**	98 (89)	**3**	95 (60)
6	**Pep2**	**3**	78 (68)	–	–
7	**Pep2**	**2**	98	**2**	96

[a] Click reactions in solution. [b] Determined by integration of the HPLC traces of the crude products at 220, 410 and 500 nm after 12 h. [c] Numbers in parentheses indicate yields of isolated products.

The sequence of **Pep2** represents a synthetic substrate for the enzyme matrix metalloproteinase-2 (MMP-2) whose elevated activity is a diagnostic indicator for tissue tumors. The renown substrate sequence[13] of Gly-Pro-Leu-Gly-Val-Arg-Gly-Lys-Cys-NH_2 was slightly altered for our convenience to give **Pep2**. Enzymatic scission occurs between Gly^4-Val^5. To demonstrate the importance of double labeling we used **Pep2** to construct a FRET system by using an appropriate combination of labels and our sequential labeling technique.

The resulting substrate has not yet been optimized with respect to cleavage by MMP-2, but despite this and the fact that it is a mixture of diastereomers and regioisomers, the dually labeled substrate **2**–**Pep2**–**3** is readily cleaved by the enzyme as can be seen from the decrease in the efficiency of FRET (Figure 3). The large Stokes shift of label **3** certainly is an additional advantage in terms of signal separation; in fact, the fluorescence at 620 nm could be easily measured even without the use of optical filters.

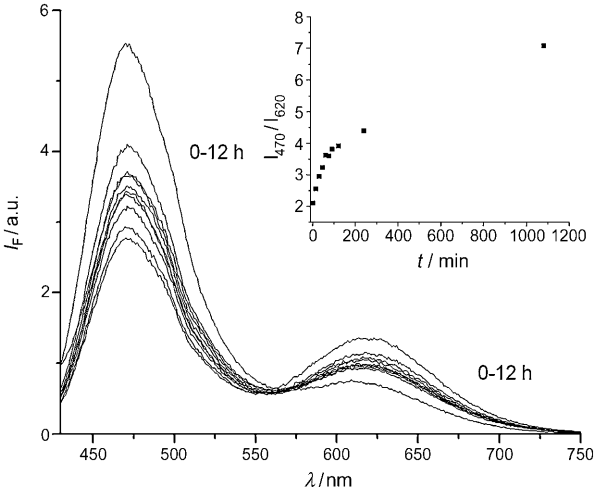

Figure 3. Spectral changes of **2**–**Pep2**–**3** (1 μM) in the presence of activated MMP-2 (4 nM) in TCNB buffer at 37 °C; inset: changes in the ratio of the monitored wavelengths. TCNB = tris/calcium/sodium buffer.

To provide additional examples of sequential labeling by using copper-free and copper-mediated 1,3-dipolar cycloadditions, we attempted to label bovine serum albumin (BSA) with our FRET pair. BSA was treated first with a CyOCO-modified maleimide linker (see the Supporting Information) to create a copper-free anchor through the free sulfhydryl group of BSA. This step was then followed by treatment with pentynoic acid succinimide ester[14] to provide numerous sites on the protein for the copper-mediated click reactions. The modified BSA was then separated from excess reagents by gel filtration and then label **3** was introduced. After removal of the unreacted dyes the monolabeled BSA was reacted with label **2** in the presence of Cu^I (Figure 4). As expected, the intensity of both emission bands increased as the second click-reaction proceeded. Changes of the second band at 620 nm also indicated the presence of FRET (see the Supporting Information).

A third piece of evidence for the versatility of the sequential labeling is presented by using silica nanoparticles doped with **2**.[11] **Pep1** was linked to the doped particles bearing azido functions by using the copper-free click chemistry. The **Pep1**-functionalized fluorescent particles were then separated from unreacted peptides. Subsequent labeling by using a copper-mediated click-reaction with **3** yielded dually labeled nanoparticles (Figure 5, see details in the Supporting Information). This method can be used to efficiently functionalize doped nanoparticles by using click chemistry with biologically active, labeled motifs.

We presume that the method for sequential labeling presented herein can be extended to various systems including small synthetic protein oligomers, bioorthogonalized proteins, organic messengers, polysaccharides, or nucleic acids and nanoparticles. Its efficiency allows the whole labeling process to take place in the same solution by administering the labels sequentially to the appropriate acceptors.

In summary, we have shown for the first time that the applications of copper-free and copper-mediated click reactions (both are highly selective and efficient) represents a viable tool to sequentially labeling biological targets with two labels without protecting the functional groups. Given the mild experimental conditions, the method is likely to work in vivo, provided that the alkyne- or azide-modified building blocks are taken up by cells through metabolic pathways. Subsequent sequential labeling with fluorescent tags bearing the counterpart functionality enables one to introduce multiple labels into these modified biological targets. An extension of the method towards labeling even more complex systems, as well as labeling doped silica nanoparticles with an MMP-2 substrate is in progress and will be reported in due course.

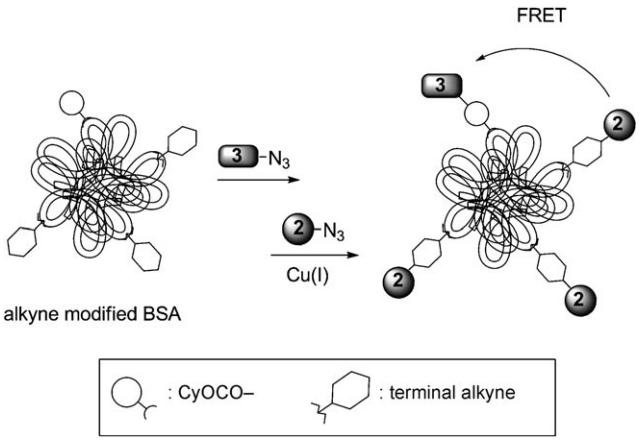

Figure 4. Schematic representation of dual labeling of alkyne modified BSA.

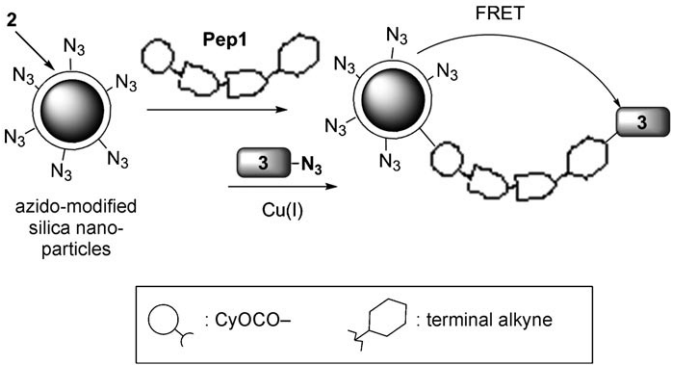

Figure 5. Loading biomolecules onto azido-functionalized silica nanoparticles and subsequent labeling.

Received: September 13, 2008
Published online: December 9, 2008

Keywords: click chemistry · copper · FRET · peptides

[1] a) J. A. Prescher, C. R. Bertozzi, *Nat. Chem. Biol.* **2005**, *1*, 13; b) P. V. Chang, J. A. Prescher, M. J. Hangauer, C. R. Bertozzi, *J. Am. Chem. Soc.* **2007**, *129*, 8400; c) K. L. Kiick, E. Saxon, D. A. Tirrell, C. R. Bertozzi, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 19.
[2] a) C. W. Tørnoe, C. Christensen, M. Meldal, *J. Org. Chem.* **2002**, *67*, 3057; b) V. V. Rostovtsev, L. G. Green, V. V. Fokin, K. B.

Sharpless, *Angew. Chem.* **2002**, *114*, 2708; *Angew. Chem. Int. Ed.* **2002**, *41*, 2596.
[3] a) O. S. Wolfbeis, *Angew. Chem.* **2007**, *119*, 3038; *Angew. Chem. Int. Ed.* **2007**, *46*, 2980; b) A. Dondoni, *Chem. Asian J.* **2007**, *2*, 700.
[4] M. Hintersteiner, M. Auer, *Ann. N. Y. Acad. Sci.* **2008**, *1130*, 1.
[5] a) N. J. Agard, J. A. Prescher, C. R. Bertozzi, *J. Am. Chem. Soc.* **2004**, *126*, 15046; b) N. J. Agard, J. M. Baskin, J. A. Prescher, A. Lo, C. R. Bertozzi, *ACS Chem. Biol.* **2006**, *1*, 644; c) J. M. Baskin, J. A. Prescher, S. T. Laughlin, N. J. Agard, P. V. Chang, I. A. Miller, A. Lo, J. A. Codelli, C. R. Bertozzi, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 16793; d) S. T. Laughlin, J. M. Baskin, S. L. Amacher, C. R. Bertozzi, *Science* **2008**, *320*, 664; e) E. M. Sletten, C. R. Bertozzi, *Org. Lett.* **2008**, *10*, 3097; f) J.-F. Lutz, *Angew. Chem.* **2007**, *119*, 1036; *Angew. Chem. Int. Ed.* **2007**, *46*, 1018.
[6] X. Ning, J. Guo, M. A. Wolfert, G.-J. Boons, *Angew. Chem.* **2008**, *120*, 2285; *Angew. Chem. Int. Ed.* **2008**, *47*, 2253.
[7] B. Valeur, *Molecular Fluorescence*, Wiley-VCH, Weinheim, **2002**.
[8] a) P. M. E. Gramlich, S. Warncke, J. Gierlich, T. Carell, *Angew. Chem.* **2008**, *120*, 3491; *Angew. Chem. Int. Ed.* **2008**, *47*, 3442; b) G. A. Burley, J. Gierlich, M. R. Mofid, S. T. H. Nir, Y. Eichen, T. Carell, *J. Am. Chem. Soc.* **2006**, *128*, 1398.
[9] K. Sonogashira, Y. Tohda, N. Hagihara, *Tetrahedron Lett.* **1975**, *16*, 4467.
[10] K. Sivakumar, F. Xie, B. M. Cash, S. Long, H. N. Barnhill, Q. Wang, *Org. Lett.* **2004**, *6*, 4603.
[11] H. Mader, X. Li, S. Saleh, M. Link, P. Kele, O. S. Wolfbeis, *Ann. N. Y. Acad. Sci.* **2008**, *1130*, 218.
[12] P. Czerney, M. Wenzel, B. Schweder, F. Lehmann, US20040260093, **2004**.
[13] C. Bremer, C. H. Tung, R. Weissleder, *Nat. Med.* **2001**, *7*, 743.
[14] X-M. Liu, H.-T. Lee, R. A. Reinhardt, L. A. Marky, D. Wang, *J. Controlled Release* **2007**, *122*, 54.

