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Activity-Based Profiling

Triple Bioorthogonal Ligation Strategy for Simultaneous Labeling of Multiple Enzymatic Activities**

*Lianne I. Willems, Nan Li, Bogdan I. Florea, Mark Ruben, Gijsbert A. van der Marel, and Herman S. Overkleeft**

Bioorthogonal chemistry plays an important role in chemical biology research by creating the means to carry out selective chemical transformations in complex biological samples. A ligation reaction classifies as being bioorthogonal when it can be performed in a biological sample in a chemoselective manner without any interference with the biological system. Bioorthogonal reactions have been used in cell-surface labeling of glycoproteins and studies of biological processes that involve post-translational modifications.[1] Another area of research that has benefited from bioorthogonal chemistry is two-step activity-based protein profiling (ABPP),[2] where it enables the temporal separation of a reporter group and a chemical probe that is directed to the active site of an enzyme (such a chemical probe is also called activity-based probe, ABP). Two-step ABPP strategies are of particular interest when the presence of a tag interferes with selectivity, affinity, cell-permeability, or bioavailability of the probe. A further advantage of tandem labeling strategies is the option to use different reporter groups depending on the type of experiment and the desired method of analysis while using a single ABP.

Several bioorthogonal ligation strategies have been described,[3] and continuing efforts are being made to develop ligations that are more selective and efficient than existing methods. At the same time the high complexity of biological processes often requires the study of multiple targets simultaneously, thereby creating a need for ligation reactions that are orthogonal with respect to each other and can thus be used concurrently in a single experiment. Over the past decade, a number of tandem ligation strategies has been described for use in bioconjugation.[4] The first report of a tandem bioorthogonal ligation in complex biological samples involved a Staudinger–Bertozzi ligation and Diels–Alder cycloaddition procedure, which utilizes mutually orthogonal reagents but suffers from the need to mask free thiol groups prior to the ligation step to avoid nonspecific labeling.[5] More recently, it was reported that a copper-free azide–cyclooctyne cycloaddition can be used concurrently with an inverse-electron-demand Diels–Alder reaction between tetrazine and *trans*-cyclooctene for the simultaneous labeling of two different receptors on cell surfaces, provided that the proper reagents are carefully selected so that cross-reactivity is minimized.[6] Herein, we describe a triple ligation strategy employing the tetrazine ligation, Staudinger–Bertozzi ligation, and copper(I)-catalyzed Huisgen [2+3] cycloaddition ("click" reaction)[7] for the selective and simultaneous labeling of three different enzymatic activities in a single experiment (Scheme 1a).

Several examples of two-step ABPP strategies using click chemistry[8] and Staudinger–Bertozzi ligation[9] have been described previously. The tetrazine ligation, however, has thus far not been used for this purpose. Therefore we set out to develop a two-step ABPP strategy in which an ABP is functionalized with norbornene as a ligation handle that can react with a tetrazine reagent conjugated to a reporter group to enable detection and analysis of labeled proteins. As a model system for our studies we selected the 20S proteasome, containing three catalytically active subunits (β1, β2, and β5) that can be targeted by either broad-spectrum or subunit-specific ABPs. We designed two proteasome ABPs that are functionalized with norbornene as a ligation handle: ABP **1** is derived from the pan-reactive proteasome inhibitor epoxomicin,[10] and ABP **2** has a different scaffold based on a β5-subunit-selective proteasome inhibitor[11] (Scheme 1b). Furthermore, we chose to create a panel of three tetrazine reagents functionalized with different tags, being Bodipy-TMR (**3a**), BodipyFL (**3b**), and biotin (**3c**). Other reagents used herein for two-step labeling of the proteasome by click chemistry and Staudinger ligation are shown in Scheme 1c. The synthesis of all reagents and competition experiments confirming the ability of the ABPs to target all proteolytically active proteasome β subunits (**1**, **4**, **5**) or only the β5 subunit (**2**) in cell extracts and/or in living cells can be found in the Supporting Information.

The applicability of the tetrazine ligation for two-step labeling of endogenous proteasome activity was tested by exposing human embryonic kidney (HEK) cell lysates to norbornene-functionalized ABP **1** in a concentration that results in complete proteasome binding followed by ligation with one of the tetrazine reagents **3a–c** for one hour at 37 °C. Analysis of labeled proteins by SDS-PAGE using either fluorescent readout or detection by streptavidin Western blotting (Figure 1a and Figure S2 in the Supporting Information) showed that ligation with all three tetrazine reagents results in labeling of the three catalytically active proteasome β subunits in a concentration-dependent manner.[12] In this

[*] L. I. Willems, N. Li, Dr. B. I. Florea, M. Ruben, Prof. G. A. van der Marel, Prof. H. S. Overkleeft
Leiden Institute of Chemistry and Netherlands Proteomics Centre
Gorlaeus Laboratories
Einsteinweg 55, 2333 CC Leiden (The Netherlands)
E-mail: h.s.overkleeft@chem.leidenuniv.nl

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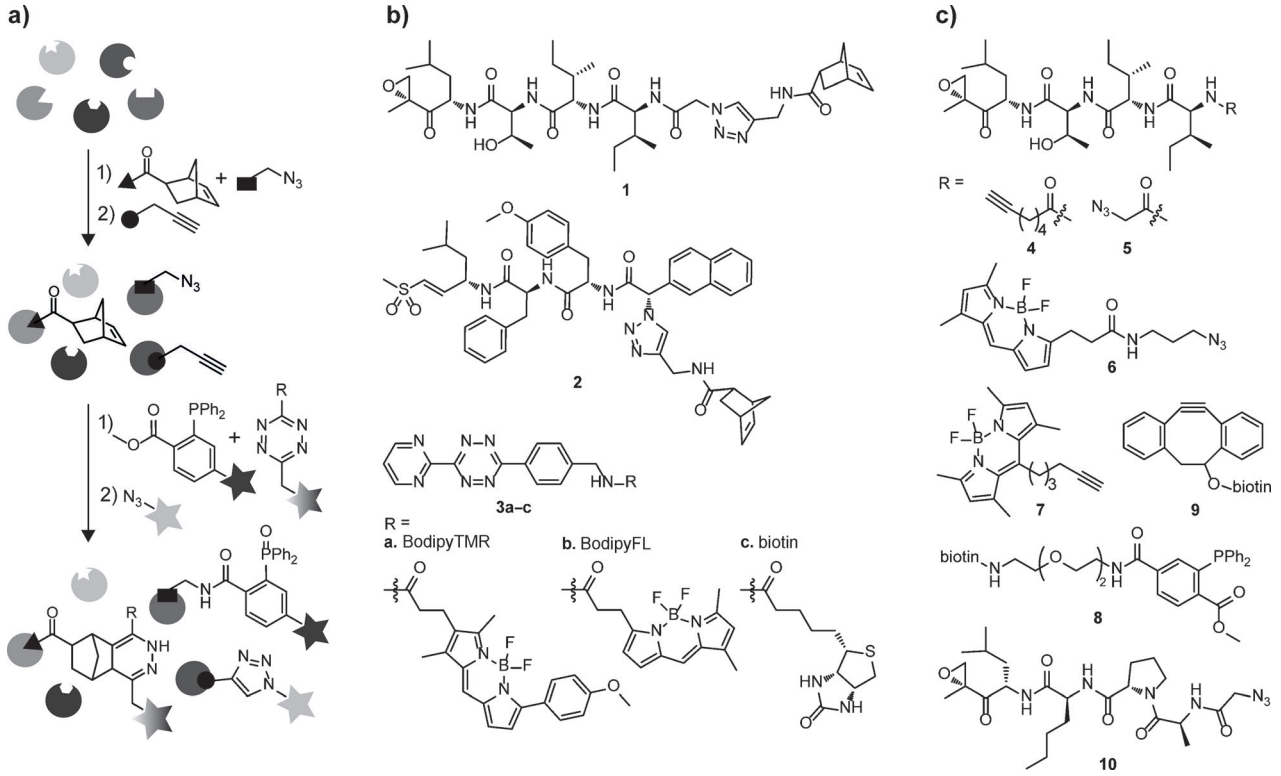

Scheme 1. a) Schematic overview of the triple ligation strategy for simultaneous labeling of multiple enzymatic activities involving tetrazine–norbornene cycloaddition, Staudinger–Bertozzi ligation, and copper(I)-catalyzed click reaction. The triangle, rectangle, and circle depict different enzyme-reactive probes and the stars correspond to different reporter groups (fluorescent or biotin). b) Structures of norbornene-functionalized proteasome ABPs **1** and **2** and tetrazines with a fluorescent Bodipy tag (**3a**, **3b**) or biotin (**3c**). c) Structures of alkyne (**4**)- and azide (**5**)-functionalized proteasome ABPs, azide (**6**)- or alkyne (**7**)-derivatized fluorescent click reagents, biotin-phosphine **8** for Staudinger–Bertozzi ligation, biotinylated dibenzocyclooctyne (DIBO) **9** for strain-promoted click reaction and azide-functionalized ABP **10**, selective for the β1 subunit of the proteasome.

experimental setup the minimal tetrazine concentration that was needed to detect specific labeling was approximately 5 μM (Figure S3 in the Supporting Information). The high selectivity and specificity of the ligation reaction is apparent from the low degree of background labeling in the absence of the ABP **1** and from the absence of proteasome labeling when enzymatic activity is inhibited by an excess of epoxomicin or heat inactivation prior to the labeling procedure (Figure S6 in the Supporting Information). Optimization of the reaction time and temperature revealed that, at 50 μM of tetrazine, labeling can be achieved within 30 min and appears to be complete within 1–2 h (Figure S4 in the Supporting Information). The reaction can also be performed at room temperature, but at higher temperature (50 °C) or a prolonged reaction time (overnight) nonspecific labeling is increased. We also tested the compatibility of the ligation reaction with different pH values (between 4 and 8) and found no marked effects on reaction efficiency or selectivity.

Next, we examined the efficacy of the tetrazine ligation inside living cells. HEK cell cultures were treated with ABP **1**, washed, and subsequently exposed to tetrazines **3a**, **3b**, or **3c** for one hour at 37 °C. After cell lysis the proteins were analyzed by SDS-PAGE (Figure 1b). In situ tetrazine ligation with **3a** and **3b** resulted in efficient labeling of the active proteasome β subunits, with labeling visible at concentrations as low as 1 μM, thus demonstrating that the tetrazine probes are cell-permeable and that the ligation reaction can be performed successfully inside living cells. Furthermore, the ligation proceeded very selectively with even less nonspecific labeling than in cell lysates (see also Figure S2 in the Supporting Information). Ligation with tetrazine **3c** was less efficient, presumably owing to a reduced cell permeability caused by the biotin moiety.

In a next set of experiments, we set out to compare the efficiency and selectivity of the tetrazine ligation in cell extracts with three other commonly used ligation methods (Figure 2 and Figure S5 in the Supporting Information). The tetrazine ligation procedure showed similar results to Staudinger–Bertozzi ligation using azide-functionalized ABP **5**[14] and biotin-phosphine **8**[15] and to both copper(I)-catalyzed click procedures. A remarkable difference can be seen between the click reaction using azide-functionalized ABP **5** and alkyne-Bodipy **7**,[14] which appears to proceed somewhat more efficiently but also gives more background fluorescence, and ligation using alkyne-ABP **4** and azido-Bodipy **6**, which shows much higher selectivity. In the case of strain-promoted click reaction with DIBO-biotin **9**[16] a very high amount of background labeling was present; thus specific signals were not detectable. This result confirms our previous finding that

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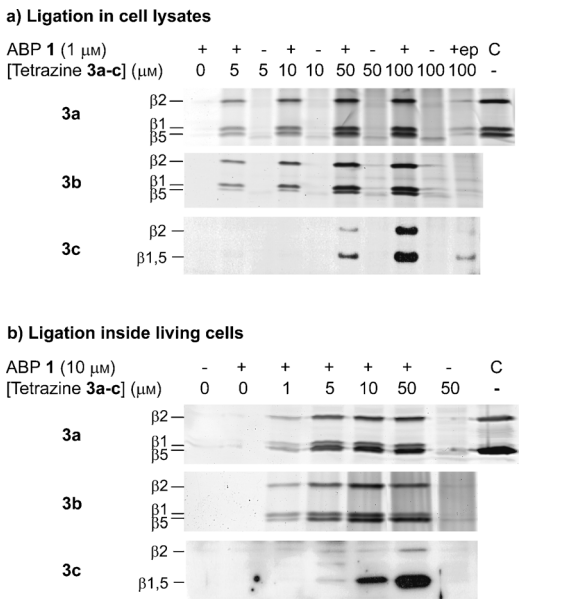

Figure 1. Two-step profiling of proteasome activity by tetrazine ligation in cell extracts and inside living cells. a) HEK cell extracts were exposed to ABP **1** (1 μM) for one hour at 37°C and then reacted with the indicated concentrations of tetrazine reagents **3a–c** for one hour at 37°C. In control experiments tetrazine ligation was performed in the absence of ABP **1** or after competition by an excess of epoxomicin ("+ep"). Alternatively, extracts were labeled with fluorescent ABP MV151[13] (1 μM) as a positive control ("C"). b) HEK cells were exposed to ABP **1** (10 μM) for three hours at 37°C, washed, and then reacted with the indicated concentrations of tetrazines **3a–c** for one hour, after which the cells were washed and lysed. Proteins were analyzed by SDS-PAGE and detected by in-gel fluorescent readout (**3a**, **3b**) or streptavidin Western blotting (**3c**). Annotation of the proteasome subunits is based on reported labeling profiles.[13]

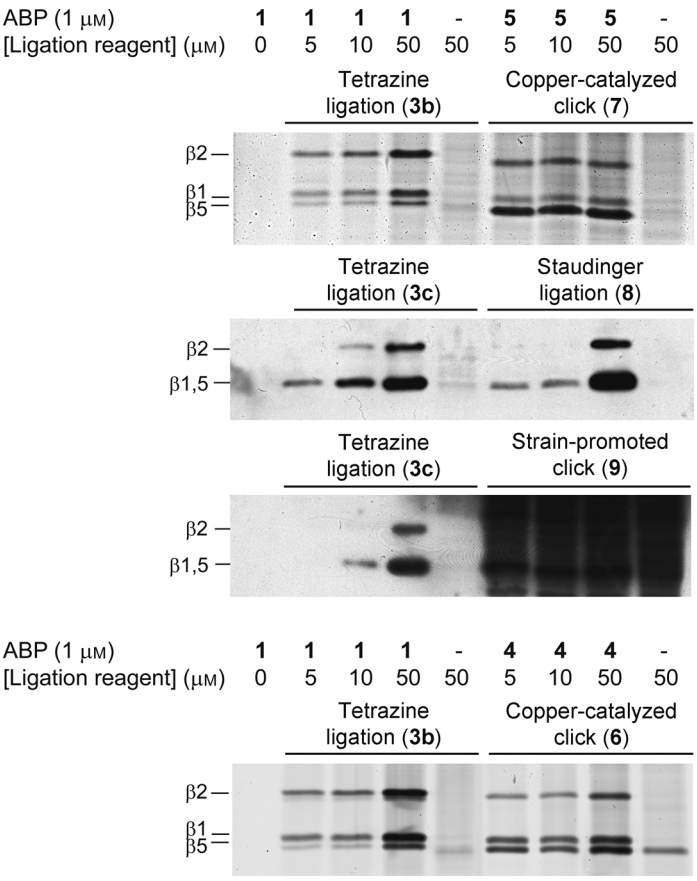

Figure 2. Two-step profiling of proteasome activity by different ligation strategies. HEK cell extracts were exposed for one hour at 37°C to proteasome ABP **1** (1 μM) and then reacted with the indicated concentrations of tetrazine **3b** or **3c** for one hour at 37°C. Alternatively, ABP **4** (1 μM) was used for ligation to Bodipy-azide **6** or ABP **5** (1 μM) for ligation to Bodipy-alkyne **7**, biotin-phosphine **8**, and DIBO-biotin **9**. Copper-catalyzed click reactions with **6** and **7** were performed in the presence of $CuSO_4$ (1 mM), TCEP (1 mM), and TBTA (100 μM). Proteins were analyzed by SDS-PAGE using detection by in-gel fluorescent readout (**3b**, **6**, **7**) or streptavidin Western blotting (**3c**, **8**, **9**). TCEP = tris(2-carboxyethyl)phosphine, TBTA = tris(benzyltriazolylmethyl)amine.

strain-promoted click chemistry is poorly compatible with cell extracts.[17]

To investigate the orthogonality between tetrazine ligation, Staudinger–Bertozzi ligation, and copper(I)-catalyzed click reaction, we tested for potential cross-reactivity between norbornene-, azide-, and alkyne-tagged proteasome ABPs (**1**, **4**, and **5**) and azide-, alkyne-, phosphine-, and tetrazine-functionalized reagents (**3b**, **6**, **7**, and **8**) in a gel-based assay (Figure S6 in the Supporting Information). In this assay no cross-reactivity could be detected, opening up the way to perform all three ligation reactions simultaneously. However, we found that tetrazine reagents are not compatible with click conditions and give a very high degree of background fluorescence owing to the presence of copper sulfate. As a result, the tetrazine ligation and copper(I)-catalyzed click reaction cannot be performed at the same time. Nevertheless we designed a triple ligation experiment in which HEK cell extracts were first treated with β5-selective norbornene-functionalized ABP **2** and β1-selective azide-functionalized ABP **10**[18] and subsequently with alkyne-derivatized probe **4**, which then labels only the available proteasome β2 subunits. Next the lysates were exposed to tetrazine-BodipyTMR **3a** together with biotin-phosphine **8** for one hour, excess reagents was removed by buffer exchange, and click ligation was performed for one hour with azido-BodipyFL **6**, after which the proteins were analyzed by SDS-PAGE (Figure 3 and Figure S7 in the Supporting Information). Using this tandem labeling strategy we were able to selectively label the three catalytic activities of the proteasome by the three different ligation reactions in a single sample.

In conclusion, we have developed a two-step ABPP strategy based on the tetrazine ligation and have shown that this method can be used to label endogenous proteasome activity with high selectivity in cell extracts and inside living cells. We have demonstrated that the reagents used for the tetrazine ligation are orthogonal to those used in Staudinger–Bertozzi ligation and copper(I)-catalyzed click reactions and have used these three ligation reactions for the simultaneous labeling of multiple enzymatic activities in a single experiment. Although tetrazines are not directly compatible with copper-catalyzed click chemistry, this problem can easily be overcome by a simple washing step between the two ligation reactions. The triple ligation strategy will not only be useful

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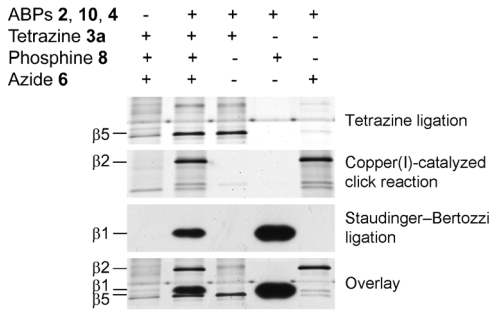

Figure 3. Triple ligation strategy using tetrazine ligation, Staudinger–Bertozzi ligation, and copper(I)-catalyzed click reaction in a single sample. HEK cell extracts were exposed to β5-selective ABP **2** (1 μM) and β1-selective azide-functionalized ABP **10** (5 μM) for one hour at 37°C and then to ABP **4** (1 μM) for one hour at 37°C. Next the lysates were reacted with tetrazine **3a** (25 μM) and phosphine **8** (100 μM) for one hour at 37°C, followed by buffer exchange to click buffer (50 mM Tris pH 7.5, 1 mM CuSO4, 1 mM TCEP, 100 μM TBTA) and reaction with azide **6** (25 μM) for one hour at 37°C. Proteins were then analyzed by SDS-PAGE and detected by in-gel fluorescent readout (Cy2 (**6**) and Cy3 (**3a**) settings) and streptavidin Western blotting (**8**).

for application in ABPP but also for the study of other biological processes such as post-translational modifications, allowing the selective monitoring of multiple targets at the same time. Moreover, the possibility to perform both tetrazine and Staudinger–Bertozzi ligation inside living cells[19,9] and in living animals[20] should also enable tandem labeling procedures in vivo.

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