JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

pubs.acs.org/JACS

Live-Cell Labeling of Specific Protein Glycoforms by Proximity-Enhanced Bioorthogonal Ligation

Peter V. Robinson,[‡] Gabriela de Almeida-Escobedo,[‡] Amber E. de Groot, Julia L. McKechnie, and Carolyn R. Bertozzi*

Departments of Chemistry and Molecular and Cell Biology and the Howard Hughes Medical Institute, University of California, Berkeley, California 94720, United States

Supporting Information

ABSTRACT: Reagents for detecting post-translational modifications in the context of their protein scaffold are powerful tools, but are challenging to develop for glycosylated epitopes. We describe a strategy for detecting protein-specific glycosylation through the use of cyclooctyne-aptamer conjugates. These molecules selectively ligate to azidosugar-labeled glycans exclusively on a target protein on live cells. We characterized aptamer conjugates against two different cell surface glycoproteins and show that these reagents are amenable to detecting protein sialoforms by mass spectrometry, Western blotting, and flow cytometry. Given the abundance of aptamers that bind cell surface targets, we expect this technology will be a useful platform for investigating the roles of protein-specific glycosylation in various cellular contexts.

Glycosylation of cell surface proteins serves many biological functions such as the generation of ligands for cell−cell interactions as well as reporting on the internal metabolic status of the cell.[1] While it is appreciated that global changes in glycosylation drive biological phenomena,[2,3] the importance of particular glycoprotein actors is emerging as a prominent theme in cell biology.[4−7] Therefore, developing tools to detect specific protein glycoforms is a valuable yet challenging endeavor.

The study of glycosylated proteins is facilitated by the use of affinity reagents such as antibodies or lectins that bind their pendant glycan structures. These reagents are indispensable for many gold-standard experimental platforms such as enrichment for mass spectrometry, Western blot, fluorescence microscopy, ELISAs, and proximity ligation.[8] However, these reagents recognize only the isolated glycan epitopes while remaining agnostic to the protein scaffold upon which the modification resides.

One solution is the generation of glycospecific antibodies through the immunization of animals with synthetic peptides bearing the desired glycan modification.[9] Unfortunately, this strategy is labor-intensive and low-throughput, often requiring extensive purification and screening to generate high-affinity reagents.[10] For many complex glycan targets, this may not be feasible due to the difficulty of obtaining homogeneously glycosylated antigen. Additionally, many glycans and glycopeptides tend to be T-independent antigens and elicit relatively low affinity IgM subtype antibodies instead of the preferred high affinity IgG subtypes.[11] While several glycospecific antibodies are known, most only detect small glycan epitopes, such as the monomeric Tn antigen on MUC1 peptides[12] and the intracellular O-GlcNAc modification.[13] Therefore, the development of alternative labeling protocols for specific protein glycoforms remains an important goal in the field of glycobiology.

Our group has previously developed a two-step process for labeling glycans by appropriating normal glycan biosynthesis with azide-bearing substrates that covalently react with cyclooctynes conjugated to a reporter group.[14] While this strategy is valuable for monitoring global glycosylation,[15,16] it does not afford protein selectivity. We and others were recently able to combine metabolic labeling with FRET microscopy to image specific protein glycoforms.[17−19] However, these methods do not address the need for reagents that form a physical connection to the glycoprotein to enable enrichment.

We surmised that an affinity reagent capable of detecting a specific protein glycoform might be designed by accelerating the reaction rate of cyclooctyne probes with azide-bearing glycans on a specific target through enhancement of the local concentration surrounding that protein (Figure 1). We took inspiration from previous work using nucleic acid aptamers to deliver reactive functional groups to singular protein targets amidst complex biological systems. For example, Brody et al. incorporated photo-cross-linkable bases into aptamers that covalently attach to their protein target upon exposure to UV light.[20] The proximity induced by aptamer binding is thought to increase the local concentration of the UV reactive groups to accelerate the rate of photo-cross-linking only with nearby proteins. We predicted that analogous selectivity could be achieved with cyclooctyne-azide chemistry to label specific glycoforms on the surfaces of live cells.

To test this hypothesis, we identified two aptamers: sgc8c[21] and TD05.1,[22] which target protein tyrosine kinase-7 (PTK7) and the mu heavy chain of the B-cell receptor (mIgM), respectively. As test substrates, we used Jurkat cells, which bear PTK7 but lack mIgM, and Ramos cells, which bear mIgM but lack PTK7 (Figure S1). PTK7 is a co-receptor in the Wnt signaling pathway,[23] while mIgM is a major component of the B-cell receptor. Both single-stranded DNA aptamers were purchased with a biotin group at the 5′ end for easy detection by Western blot and an amine at the opposing terminus for further functionalization. We then synthesized a panel of cyclooctyne-aptamer conjugates where we varied the site of attachment and linker length of the cyclooctyne attachment to

Received: April 24, 2015
Published: August 4, 2015

DOI: 10.1021/jacs.5b04279
J. Am. Chem. Soc. 2015, 137, 10452−10455

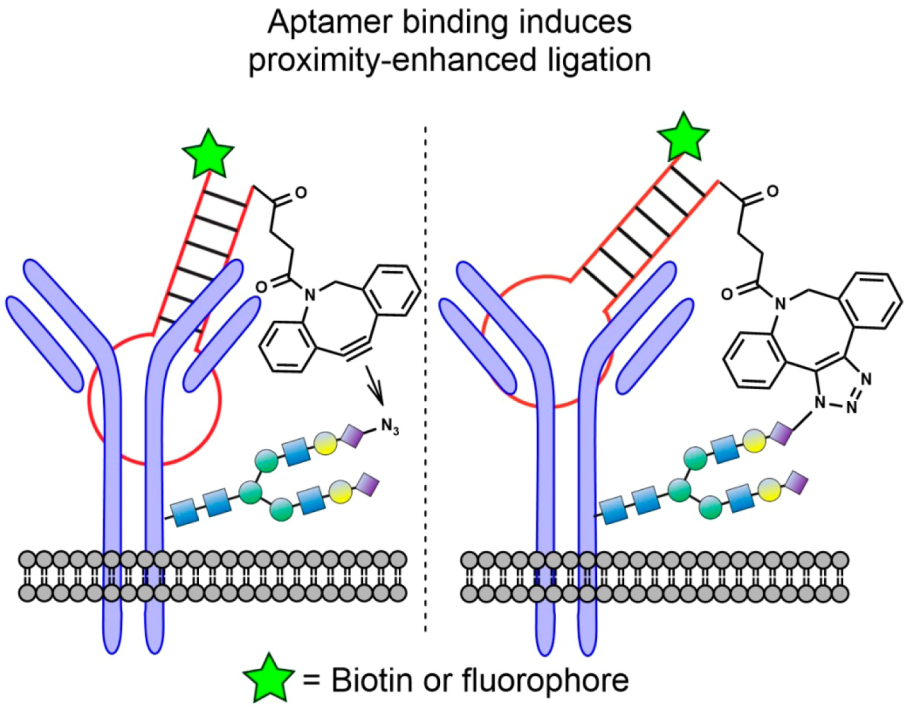

Figure 1. A chemical strategy for the selective labeling of protein glycoforms on live cell surfaces. Cells are incubated with Ac_4ManNAz, which when metabolized introduces SiaNAz into surface glycoproteins. Cells are then treated with a functionalized aptamer bearing a cyclooctyne and a reporter group on the 5′ and 3′ termini, respectively. Aptamer binding positions the cyclooctyne to ligate preferentially to SiaNAz residues in proximity on the same glycoprotein.

determine the optimal configuration (Table S3). We appended a cyclooctyne to either terminus by reacting the corresponding terminal amino group with commercially available DBCO-PEG_4-NHS ester (Scheme S1). We reasoned that the 1−2 nm distance provided by the PEG_4 linker would allow for enough flexibility to reach a large portion of the protein surface while still restricting access to neighboring glycoproteins.

Next, we cultured either Jurkat or Ramos cells with peracetylated *N*-azidoacetylmannosamine (Ac_4ManNAz) to generate cell surface glycoproteins bearing *N*-azidoacetylneuraminic acid (SiaNAz) and then incubated the cells with low micromolar concentrations of a cyclooctyne-aptamer conjugate. The cells were washed in PBS and then lysed. The lysates were resolved by SDS-PAGE and then analyzed for biotinylated proteins by Western blotting with streptavidin-HRP. Irrelevant bands corresponding to endogenously biotinylated carboxylases[24] are indicated in the Western blots.

For Jurkat cells labeled with the cyclooctyne-sgc8c aptamer conjugate, a 170-kDa band was observed, corresponding to the reported molecular weight of PTK7, with some off-target labeling (Figure 2A,B). Western blot analysis of Ramos cells labeled with the TD05.1 cyclooctyne-aptamer conjugate exhibited two bands with molecular weights that correspond to the heavy (60 kDa) and light chains (30 kDa) of mIgM (Figure 2C,D). In the case of the light chain IgM, laddering is observed, likely due to the addition of multiple aptamer-conjugates to this low molecular weight protein. At higher conjugate concentrations, additional bands began to appear, suggesting selectivity is achieved only at low concentrations, where the proximity effect of the cyclooctyne-aptamer conjugate is allowed to dictate the kinetics of the reaction. While many factors likely contribute to the specificity of any specific aptamer-conjugate, we observed the greatest selectivity at low micromolar concentrations of the probe.

An endogenously biotinylated carboxylase of similar molecular weight to PTK7 was potentially masking the banding pattern. To confirm the specific labeling of PTK7, we treated Jurkat cells with an sgc8c-cyclooctyne aptamer conjugate, a mismatched TD05.1-cyclooctyne aptamer conjugate, and a sgc8c-biotin conjugate lacking a cyclooctyne. Detergent lysates of these labeled cells were enriched for biotinylated proteins with Neutravidin resin. Bound proteins were stripped by boiling in SDS and then analyzed by an anti-PTK7 Western blot. Satisfyingly, a single 170 kDa band appeared only in the lane with the correctly matched aptamer-cyclooctyne conjugate (Figure S6).

In order to confirm that labeling was covalent and not simply the result of a strong noncovalent interaction between the aptamer and its antigen, we treated cells with aptamers lacking a cyclooctyne. As an additional negative control, we labeled Jurkat cells with the TD05.1 cyclooctyne-aptamer conjugate and Ramos cells with the sgc8c cyclooctyne-aptamer conjugate. These aptamers target proteins that are absent from the respective cell surfaces. Both cases led to minimal labeling, suggesting that the aptamer is protein selective and primarily reacts through the cyclooctyne (Figure 2B,D). Furthermore, sialidase-treated samples produced no detectable labeling with the cyclooctyne-aptamer conjugate (Figure 3A,C). Specific glycoprotein labeling required the cells to be Ac_4ManNAz-treated and were absent in cells treated only with DMSO (Figure 3B,D).

To further improve the labeling efficiency, we sought to structrually optimize our aptamer-cyclooctyne conjugate probes by varying the length of the linker between the cyclooctyne and the aptamer. *A priori*, it was difficult for us to reason whether or not a long or short linker would provide optimal labeling of protein sialoforms. A long linker might provide greater access to sialic acids far from the epitope binding site of the aptamer, but a shorter linker might increase the reactivity rate by constraining

DOI: 10.1021/jacs.5b04279
J. Am. Chem. Soc. 2015, 137, 10452−10455

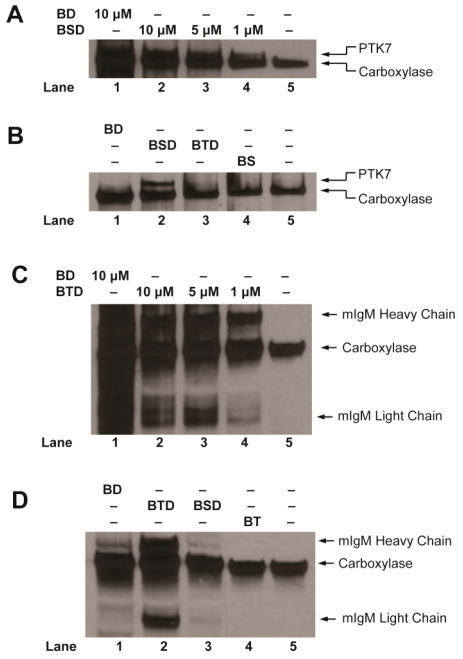

Figure 2. Dose dependence and selectivity of ligation. Jurkat or Ramos cells were incubated with Ac_4ManNAz for 3 d, washed, and then incubated with the indicated cyclooctyne conjugate (BD = biotin-DBCO, BSD = biotin-sgc8c-DBCO conjugate, BTD = biotin-TD05.1-DBCO conjugate, BT = biotin-TD05.1, BS = biotin-sgc8c). Conjugate concentration was 2.5 μM in panels B and D. Cells were washed, lysed, and then analyzed by Western blot with streptavidin-HRP. The blots in panels A and C represent glycoprotein labeling from a titration of the aptamer-cyclooctyne conjugate. The blots in panels B and D show labeling with the correct aptamer-cyclooctyne conjugate in comparison to two controls: the mismatched aptamer-cyclooctyne conjugate and an aptamer lacking a cyclooctyne. Loading controls and full-length blots are included in Figure S3. All images are representative blots from biological triplicates.

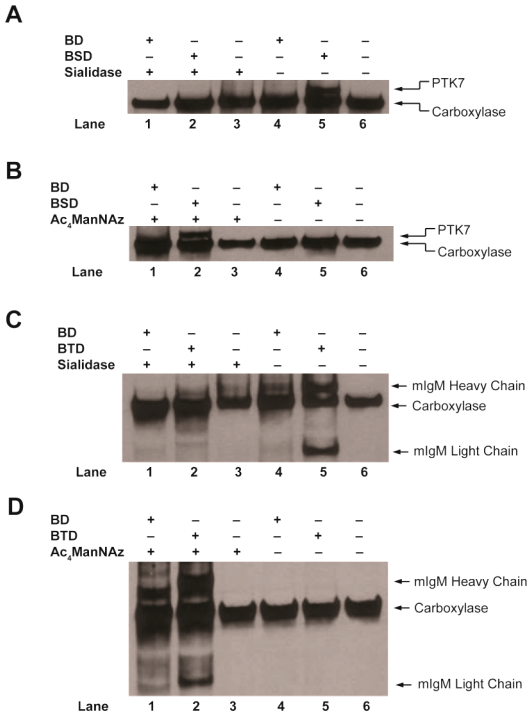

Figure 3. Sialidase sensitivity and SiaNAz dependence of labeling. Jurkat or Ramos cells were incubated with Ac_4ManNAz or DMSO control for 3 d and washed. Cells in panels C and D were then treated with sialidase as indicated. All cells were then treated with 2.5 μM of the appropriate conjugate, lysed, and then analyzed by Western blot with streptavidin-HRP (BD = biotin-DBCO, BSD = biotin-sgc8c-DBCO conjugate, BTD = biotin-TD05.1-DBCO). Blots in panels A and C show the loss of labeling upon cell-surface removal of sialic acids with a sialidase. Blots in panels B and D show the dependence of labeling upon the presence of azidoglycans on the cell surface. Loading controls and full-length blots are included in Figure S4. All images are representative blots from biological triplicates.

the cyclooctyne into fewer conformations that could be better positioned to react with the azide. Surprisingly, we found no noticeable difference between PEG_4 and no PEG linkers (Figure S2), suggesting that the glycans that the probes are labeling reside within a 1−3 nm distance (the length of a PEG_4 chain) from the 3′ terminus of the aptamer.

As strained alkynes have some background reactivity with biological nucleophiles[25] that might be enhanced through proximity effects, we wanted to confirm that labeling was dependent on the presence of SiaNAz. To test this, we pretreated cells with a broad spectrum sialidase from *Artherobacter ureafaciens* to remove SiaNAz residues.[26] The cells were then subjected to labeling with a cyclooctyne-aptamer conjugate. To confirm the molecular identity of the labeled species, we labeled cells with the appropriate biotinylated cyclooctyne-aptamer conjugates and enriched by pulldown with neutravidin beads. After extensive washing, isolated biotinylated proteins were trypsinized on bead, and their peptides were identified by mass spectrometry. PTK7 and mIgM were positively identified from experiments with Jurkat and Ramos cells, respectively (Tables S1 and S2). It is significant to note that despite the ubiquity of sialylated cell surface proteins, the only cell surface protein to appear from the Jurkat cell peptide ID list was PTK7. For the Ramos cell peptide ID experiment, mIgM heavy and light chains were identified as expected, in addition to a leukosialin, a highly abundant cell surface sialylated glycoprotein.[27] Together, these experiments provide the first evidence that PTK7 is sialylated.

Finally, we wanted to detect labeling of glycoproteins directly on live cells. We synthesized a cyclooctyne-aptamer conjugate based on a lower affinity version of TD05.1 (TD05) which is incapable of binding at 37 °C.[28] Aptamer conjugates that covalently bind should remain attached to the cell surface even after incubation at 37 °C, at which point noncovalently attached aptamers lift off. We treated Ramos cells with this temperature-sensitive conjugate, washed extensively at 37 °C, and then analyzed the cells via flow cytometry. Cells treated with Ac_4ManNAz showed higher signal and also retained more signal after washing (Figure 4). As a negative control, we treated Jurkat cells with the mismatched aptamer conjugate (TD05). A modest dose-dependent signal was observed in an azide-independent manner, reflecting nonspecific and noncovalent binding of the aptamer that was not removed in the washing step (Figure S5).

In conclusion, we have developed a novel chemical strategy to selectively modify sialylated glycoforms of proteins on live cells. A limitation of this technology is that it remains agnostic to the chemical linkage that anchors the glycan to the protein. That is, it does not reveal whether the sialylated glycan is N- or O-linked. It should be noted that this approach could be easily adapted to detect various other chemically addressable PTMs (other carbohydrates, lipids, etc.) by labeling with other azide-tagged metabolites. However, like all metabolic labeling strategies, it

DOI: 10.1021/jacs.5b04279
J. Am. Chem. Soc. 2015, 137, 10452−10455

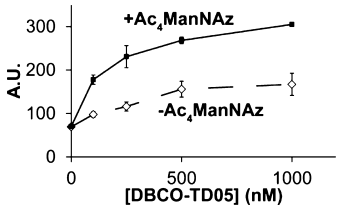

Figure 4. Flow cytometric analysis of live Ramos cells. Ramos cells were incubated with Ac_4ManNAz or DMSO vehicle for 3 d. Cells were then incubated with various concentrations of DBCO-TD05-Fluorescein conjugate for 30 min on ice. Noncovalently bound aptamer was removed by incubation at 37 °C for 1 h and serial washing. Cells were then analyzed by flow cytometry. A negative control with mismatched cell line did not show any detectable labeling above background (Figure S5). Error bars represent standard deviation from biological triplicates.

reports on newly synthesized molecules and is subject to the metabolism of the biological system. Furthermore, pilot attempts at applying this strategy to fluorescence microscopy were unsuccessful due to poor signal-to-noise after washing. We are exploring alternate probe designs and washing protocols to optimize the approach for this particular application.

Using this technology, we discovered a novel glycoform of PTK7. As dimerization-dependent co-receptor, this observation raises the possibility of glycosylation-dependent regulation of the Wnt pathway, analogous to the sialylation-sensitive dimerization of EGFR.[4] Notably, the Cell-SELEX technique offers a rich source of affinity reagents on which glycoform-specific variants can be based, enhancing the generalizability of the method.[29] As well, one can envision adaptations of the method to specifically block protein glycoforms from biological recognition events. We predict that these glycoform-selective reagents will greatly aid in the interrogation of glycoprotein function in living systems.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b04279.

Experimental procedures, characterization data, supporting figures, and schemes (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*crb@berkeley.edu

Author Contributions

‡These authors contributed equally.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Matthew Zhou, Samantha Keyser, Frances Rodriguez, Chelsea Gordon, and Lauren Wagner for critical reading of the manuscript and thoughtful conversations. GDAE was supported by an NIH Ruth Kirchstein Graduate Fellowship. This work was funded by NIH grant R37 GM058867 to C.R.B.

■ REFERENCES

(1) Marth, J. D.; Grewal, P. K. *Nat. Rev. Immunol.* **2008**, *8*, 874−887.
(2) Paszek, M. J.; DuFort, C. C.; Rossier, O.; Bainer, R.; Mouw, J. K.; Godula, K.; Weaver, V. M. *Nature* **2014**, *511* (7509), 319−325.
(3) Hudak, J. E.; Canham, S. M.; Bertozzi, C. R. *Nat. Chem. Biol.* **2013**, *10*, 69−75.
(4) Liu, Y. C.; Yen, H. Y.; Chen, C. Y.; Chen, C. H.; Cheng, P. F.; Juan, Y. H.; Wong, C. H. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108* (28), 11332−11337.
(5) Swindall, A. F.; Bellis, S. L. *J. Biol. Chem.* **2011**, *286* (26), 22982−22990.
(6) Anthony, R. M.; Nimmerjahn, F.; Ashline, D. J.; Reinhold, V. N.; Paulson, J. C.; Ravetch, J. V. *Science* **2008**, *320* (5874), 373−376.
(7) Katorcha, E.; Makarava, N.; Savtchenko, R.; Baskakov, I. V. *PLoS Pathog.* **2014**, *10* (9), e1004366.
(8) Pinto, R.; Carvalho, A. S.; Conze, T.; Magalhães, A.; Picco, G.; Burchell, J. M.; David, L. *J. Cell. Mol. Med.* **2012**, *16* (7), 1474−1484.
(9) Vichier-Guerre, S.; Lo-Man, R.; Bay, S.; Deriaud, E.; Nakada, H.; Leclerc, C.; Cantacuzene, D. *J. Pept. Res.* **2000**, *55* (2), 173−180.
(10) Brumbaugh, K.; Johnson, W.; Liao, W. C.; Lin, M. S.; Houchins, J. P.; Cooper, J.; Campos-Gonzalez, R. *Sig. Trans. Immunohist.* **2011**, *717*, 3−43.
(11) Mond, J. J.; Lees, A.; Snapper, C. M. *Annu. Rev. Immunol.* **1995**, *13* (1), 655−692.
(12) Van Elssen, C. H.; Clausen, H.; Germeraad, W. T.; Bennet, E. P.; Menheere, P. P.; Bos, G. M.; Vanderlocht, J. *J. Immunol. Methods* **2011**, *365* (1), 87−94.
(13) Teo, C. F.; Ingale, S.; Wolfert, M. A.; Elsayed, G. A.; Nöt, L. G.; Chatham, J. C.; Boons, G. J. *Nat. Chem. Biol.* **2010**, *6* (5), 338−343.
(14) Jewett, J. C.; Bertozzi, C. R. *Chem. Soc. Rev.* **2010**, *39*, 1272−1279.
(15) Baskin, J. M.; Prescher, J. A.; Laughlin, S. T.; Agard, N. J.; Chang, P. V.; Miller, I. A.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104* (43), 16793−16797.
(16) Laughlin, S. T.; Baskin, J. M.; Amacher, S. L.; Bertozzi, C. R. *Science* **2008**, *320*, 664−667.
(17) Belardi, B.; de la Zerda, A.; Spiciarich, D. R.; Maund, S. L.; Peehl, D. M.; Bertozzi, C. R. *Angew. Chem., Int. Ed.* **2013**, *52* (52), 14045−14049.
(18) Haga, Y.; Ishii, K.; Hibino, K.; Sako, Y.; Ito, Y.; Taniguchi, N.; Suzuki, T. *Nat. Commun.* **2012**, *3*, 907.
(19) Lin, W.; Du, Y.; Zhu, Y.; Chen, X. *J. Am. Chem. Soc.* **2014**, *136* (2), 679−687.
(20) Brody, E. N.; Willis, M. C.; Smith, J. D.; Jayasena, S.; Zichi, D.; Gold, L. *Mol. Diagn.* **1999**, *4* (4), 381−388.
(21) Shangguan, D.; Li, Y.; Tang, Z.; Cao, Z. C.; Chen, H. W.; Mallikaratchy, P.; Tan, W. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103* (32), 11838−11843.
(22) Mallikaratchy, P.; Tang, Z.; Kwame, S.; Meng, L.; Shangguan, D.; Tan, W. *Mol. Cell. Proteomics* **2007**, *6* (12), 2230−2238.
(23) Peradziryi, H.; Tolwinski, N. S.; Borchers, A. *Arch. Biochem. Biophys.* **2012**, *524* (1), 71−76.
(24) Papageorgiou, D. N.; Demmers, J.; Strouboulis, J. *Protein Expression Purif.* **2013**, *89* (1), 80−83.
(25) van Geel, R.; Pruijn, G. J.; van Delft, F. L.; Boelens, W. C. *Bioconjugate Chem.* **2012**, *23* (3), 392−398.
(26) Prescher, J. A.; Dube, D. H.; Bertozzi, C. R. *Nature* **2004**, *430* (7002), 873−877.
(27) Ma, X. B.; Zheng, Y.; Yuan, H.; Jiang, J.; Wang, Y. P. *Hum. Pathol.* **2015**, *46*, 593−599.
(28) Mallikaratchy, P.; Ruggiero, A.; Gardner, J. R.; Kuryavyi, V.; Maguire, W. F.; Heaney, M. L.; McDevitt, M. R.; Patel, D. J.; Scheinberg, D. A. *Nucleic Acids Res.* **2011**, *39*, 2458−2469.
(29) Sefah, K.; Shangguan, D.; Xiong, X.; O'Donoghue, M. B.; Tan, W. *Nat. Protoc.* **2010**, *5* (6), 1169−1185.

DOI: 10.1021/jacs.5b04279
J. Am. Chem. Soc. 2015, 137, 10452−10455