Video Article

Chemoselective Modification of Viral Surfaces via Bioorthogonal Click Chemistry

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Abstract

The modification of virus particles has received a significant amount of attention for its tremendous potential for impacting gene therapy, oncolytic applications and vaccine development.[1,2,3] Current approaches to modifying viral surfaces, which are mostly genetics-based, often suffer from attenuation of virus production, infectivity and cellular transduction.[4,5] Using chemoselective click chemistry, we have developed a straightforward alternative approach which sidesteps these issues while remaining both highly flexible and accessible.[1,2]

The goal of this protocol is to demonstrate the effectiveness of using bioorthogonal click chemistry to modify the surface of adenovirus type 5 particles. This two-step process can be used both therapeutically[1] or analytically,[2,6] as it allows for chemoselective ligation of targeting molecules, dyes or other molecules of interest onto proteins pre-labeled with azide tags. The three major advantages of this method are that (1) metabolic labeling demonstrates little to no impact on viral fitness,[1,7] (2) a wide array of effector ligands can be utilized, and (3) it is remarkably fast, reliable and easy to access.[1,2,7]

In the first step of this procedure, adenovirus particles are produced bearing either azidohomoalanine (Aha, a methionine surrogate) or the unnatural sugar *O*-linked *N*-azidoacetylglucosamine (*O*-GlcNAz), both of which contain the azide (-N_3) functional group. After purification of the azide-modified virus particles, an alkyne probe containing the fluorescent TAMRA moiety is ligated in a chemoselective manner to the pre-labeled proteins or glycoproteins. Finally, an SDS-PAGE analysis is performed to demonstrate the successful ligation of the probe onto the viral capsid proteins. Aha incorporation is shown to label all viral capsid proteins (Hexon, Penton and Fiber), while *O*-GlcNAz incorporation results in labeling of Fiber only.

In this evolving field, multiple methods for azide-alkyne ligation have been successfully developed; however only the two we have found to be most convenient are demonstrated herein – strain-promoted azide-alkyne cycloaddition (SPAAC) and copper-catalyzed azide-alkyne cycloaddition (CuAAC) under deoxygenated atmosphere.

Video Link

The video component of this article can be found at https://www.jove.com/video/4246/

Protocol

Refer to **Table 1** for preparation of all media, buffers and solutions referenced in this protocol.

1. Production of Aha-Labeled Adenovirus

1. Prepare eleven 100-mm tissue culture dishes of human embryonic kidney cells (HEK 293) maintained in HEK 293 cell growth medium (see **Table 1**) at 37 °C until they have reached 80 - 90% confluency. (Note: Cultures with confluency over 90% may be difficult to infect and may not produce viruses as desired.)
2. Loosen the cells in one of the dishes by first removing the growth medium, rinsing once with 5 ml of TD buffer, then incubating the cells for one min in 1 ml of trypsin-EDTA (1×).
3. Add 9 ml of HEK 293 cell growth medium, and use a hemocytometer to count the number of harvested HEK 293 cells from this dish. Use this number to calculate the amount of adenovirus type 5 (Ad5) required for infection using a multiplicity of infection (MOI) value of 10 PFU / cell. Account for the fact that for normal Ad5, the infectivity index (ratio of infectious particles to total number of particles) is typically 1:20.
4. Prepare 10 ml of infection buffer.
5. Slowly remove the HEK 293 cell growth medium from the ten remaining culture dishes, then wash with 5 ml of TD buffer. Add 1 ml of infection buffer to each dish and incubate at 37 °C for 1 hr. During this infection period, gently rock each culture dish side to side at 15-min intervals.
6. After infection, add 9 ml of fresh HEK 293 cell growth medium and incubate at 37 °C for 18 hr.

7. Prepare 100 ml of Aha labeling medium (or Met control medium for the control). Be sure to filter the concentrated stock solutions with a 0.2-µm sterile syringe filter before preparing the labeling media.
8. Carefully (so as not to wash away the infected cells) remove the HEK 293 cell growth medium after 18 hr and wash each culture dish with 5 ml of TD buffer.
9. Remove the TD buffer wash solution and add 10 ml of Aha labeling medium to each culture dish. For a control, methionine control medium should be used instead of the Aha labeling medium at this step.
10. Incubate the infected cells in labeling medium at 37 °C for 6 hr. Labeling can be carried out anytime between 12 - 24 hr post-infection but should not be longer than 6 hr in duration. This interval has been determined to maximize overlap with viral structural protein translation while ensuring infectivity is not significantly diminished. [7]
11. Carefully remove the labeling medium so as not to disrupt the cells. Add 10 ml of fresh HEK 293 cell growth medium to each dish and incubate the cells at 37 °C for another 18 hr. To minimize loss of infected cells during this step, it is not necessary to remove the labeling solution entirely. The HEK 293 cell growth medium contains an excess of Met which will outcompete any residual Aha.
12. Proceed to step 3 for purification.

2. Production of Azido-Sugar-Labeled Adenovirus

1. Follow the procedure titled Production of Aha-Labeled Adenovirus up to and including step 1.5.
2. Prepare 100 ml of Azido-sugar labeling medium.
3. Cover the cells with 10 ml of fresh Azido-sugar labeling medium and incubate at 37 °C for 44 hr.

3. Purification of Azide-Labeled Adenoviral Particles

1. Remove the media along with the infected cells from each culture dish and transfer to conical tubes. Centrifuge at 2,000 g for 10 min at 4 °C. Remove the supernatant and resuspend the pellet in TD buffer (using 8 ml of TD buffer per 10 dishes).
2. Lyse the infected cells by repeated (3 or more) freeze-thaw cycles using a Dewar flask of liquid nitrogen and a 37 °C water bath. Centrifuge at 2,000 g for 10 min at 4 °C to separate cell debris from the supernatant.
3. Prepare a CsCl density gradient centrifuge tube by first pipetting 2.5 ml of 1.25 g/ml CsCl solution into an Ultra Clear centrifuge tube. Next, use a pipette with its tip positioned at the bottom of the tube to slowly add 2.0 ml of 1.4 g/ml CsCl solution, taking care not to disturb the gradient interface.
4. Pipette the supernatant from step 3.2 on top of the CsCl density gradient tube, and if necessary add TD buffer to fill the tube. Centrifuge the samples in a swinging bucket rotor at 125,000 g for 1 hr at 15 °C in an ultracentrifuge.
5. After completion of ultracentrifugation, the virus particles should be visible as a thick white band at the interface of the two CsCl solutions. Make a small perforation at the bottom of the tube with a sterile needle to allow the solution to drain dropwise.
6. Collect the thick white band, which should contain the labeled Ad5 particles, taking care to exclude excess CsCl solution. Avoid collecting cellular proteins which will band above the virus layer. Additionally exclude empty virion capsids which form a lighter layer slightly above the thick white virus band.
7. Optionally, perform a final purification on the labeled Ad5 particles. Add the collected fraction from step 3.6 to a 4-ml ultracentrifuge tube and fill the tube with 1.35 g/ml CsCl solution. Centrifuge at 125,000 g for 18 hr at 15 °C and collect the thick white band which forms in the middle of the tube as described in steps 3.5 and 3.6. (Note that if the viral titer is low, a white band may not be visible after the second centrifugation, and it will not be possible to recover the labeled virus).
8. For storage of the virus particles over long periods of time, dialysis of the collected virus extract should be performed against virus storage buffer. Store the dialyzed virus samples at -80 °C.

4. Ligation of Modified Adenoviral Particles with Alkyne Probes Using Strain-Promoted Azide-Alkyne Cycloaddition (SPAAC)

1. To a solution of 50 µl of azide-modified Ad5 in virus storage buffer, add 0.25 µl of SPAAC labeling solution. (Refer to the table of reagents for a list of venders of commercially available strained alkynes).
2. Allow the strain-promoted cycloaddition reaction to proceed overnight at room temperature. If a light-sensitive probe (*e.g.* TAMRA) is used, cover the reaction vessel with aluminum foil.
3. Purify the labeled virus according to step 6.

5. Ligation of Modified Adenoviral Particles with Alkyne Probes Using Copper(I)-Catalyzed Azide-Alkyne Cycloaddition (CuAAC) in Deoxygenated Atmosphere

1. Place 7.2 mg of copper(I) bromide (CuBr) powder in an Eppendorf tube, covered with aluminum foil.
2. Pipette 1 ml of DMSO into a second Eppendorf tube.
3. Prepare 50 µl of CuAAC labeling solution in a third Eppendorf tube. Cover with aluminum foil if the alkyne probe is light-sensitive (*e.g.* TAMRA-Alkyne).
4. Place the three Eppendorf tubes, uncapped, in a deoxygenated glove bag for 6 hr. All samples should remain inside the glove bag until the completion of the CuAAC reaction.
5. Prepare a 50× stock solution of CuBr by adding 1 ml of DMSO to 7.2 mg of CuBr powder, both of which have been deoxygenated according to step 5.4.
6. Initiate the CuAAC reaction by pipetting 1 µl of this CuBr stock solution to 50 µl of the CuAAC labeling solution, which has been deoxygenated according to step 5.4. Allow the reaction to proceed for 12 hr inside the glove bag.

7. Purify the labeled virus according to step 6.

6. Purification and Storage of Labeled Virus Particles

1. Purify the labeled virus samples using Centri-Sep gel filtration spin columns equilibrated with Virus Storage Buffer, following the manufacturer's guidelines.
2. Alternatively, purification can be carried out by dialysis against Virus Storage Buffer.
3. The labeled virus particles should be stored at -80 °C.

7. SDS-PAGE of Labeled Adenovirus & Fluorescent Gel Scanning

1. Add 20 µl Laemmli sample buffer (2×) to 20 µl of purified labeled virus and boil for 10 min. Centrifuge the samples for 30 secs to remove any insoluble precipitates. If residual copper is present after purification, boiling the sample can result in thick streaks of protein on the SDS-PAGE gel, causing difficulty during visualization. In this case, forgo boiling of the sample in this step.
2. Attach the gel cassette to the electrophoresis cell and add running buffer. Load the required number of wells with the viral protein samples. Use one well to load pre-stained molecular weight markers. Run the gel for 1 hr at 200 V. Keep the electrophoresis cell covered with aluminum foil during the entire period to reduce photobleaching of the fluorophore label.
3. For best resolution and to remove all free fluorophore, allow the tracking dye to run out of the gel during electrophoresis. Stop the electrophoresis and quickly transfer the gel to a fluorescent gel scanner and scan the gel for fluorescence emission at the appropriate wavelength (574 nm for TAMRA).
4. The number of dye molecules conjugated to each viral particle can be quantified by measuring the fluorescence of several standard TAMRA concentrations and comparing with the sample from step 7.3.

8. Representative Results

The observed results of SDS-PAGE & fluorescent gel scanning of TAMRA-conjugated Ad5 are shown in **Figure 3**. Although these fluorescent gel scans were performed on samples modified via CuAAC, results should be comparable when probes are ligated via SPAAC. These gel scans indicate that Aha labeling results in modification of the three Ad5 capsid proteins (Hexon, Penton, and Fiber), while sugar labeling modifies only the Fiber capsid protein. Furthermore, an increase in Aha concentration (4 mM vs. 32 mM) results in a greater degree of Aha incorporation, but it also has been shown to decrease infectivity. Prior results[7] indicate that for Ad5 produced in 4mM Aha, roughly 5% of exposed methionine sites are TAMRA-modified, with this number increasing to 10% when 32 mM Aha is used. This corresponds to approximately 280 and 510, respectively, dye-labeled sites per Ad5 particle. Analysis of sugar-labeling[1] has indicated that roughly 22 of the 36 glycosylation sites on Ad5 are occupied by an azido sugar and subsequently labeled with TAMRA.

If ten tissue culture plates are used to produce azide-modified Ad5 as described in this protocol, 200 - 300 µl of azide-modified virus should be obtained from the white band collected in step 3.6, with a titer of approximately 1.5×10^{12} particles / ml. A common difficulty of this procedure is the absence of a viral band at this purification step, which can be attributed to low viral titer. This is generally a result of infecting cells which are either over- or under-confluent, or by washing away cells - which become loosened from the plate after infection - while rinsing during step 1.

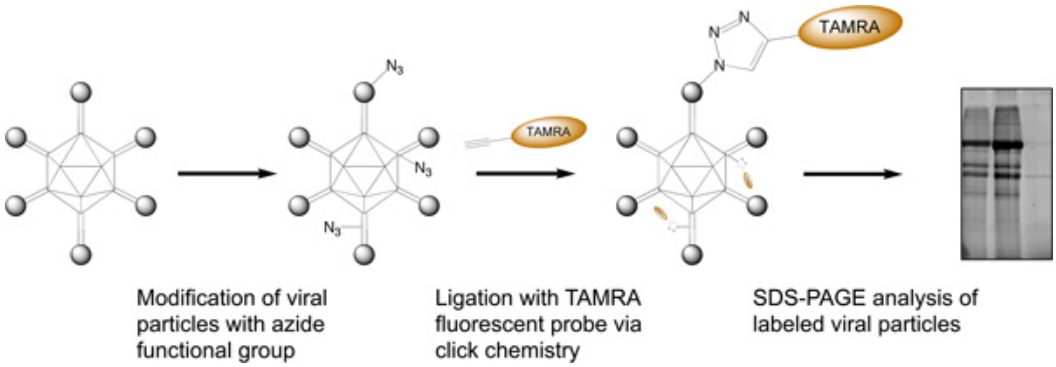

Figure 1. Protocol overview. Azide-containing Aha or GalNAz is first incorporated into adenovirus particles. Ligation with a fluorescent alkyne probe is then performed via "click" chemistry. Finally, SDS-PAGE is used to demonstrate ligation of the fluorescent probe.

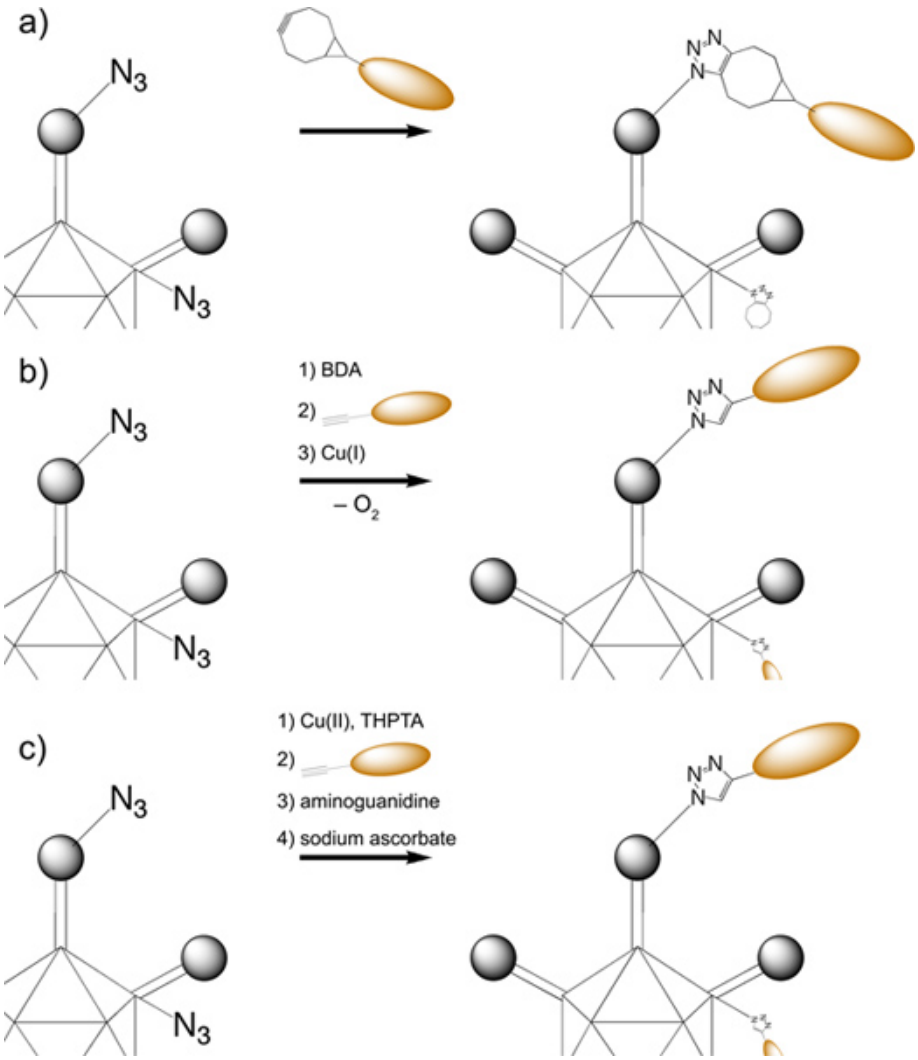

Figure 2. Ligation of alkyne probe onto azide-modified virus via (a) SPAAC, (b) CuAAC under de-oxygenated and (c) oxygenated atmosphere.

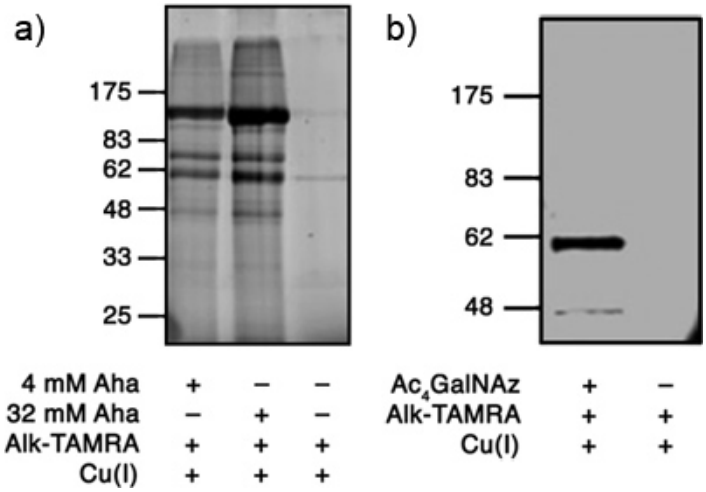

Figure 3. Fluorescent scans of SDS-PAGE of azide-labeled adenovirus type 5 (Ad5) after CuAAC conjugation with TAMRA-Alkyne. (a) Aha-labeled Ad5, using two concentrations of Aha. Labeling appears to occur on adenovirus Hexon, Penton and Fiber capsid proteins, with the extent of labeling increasing with increased Aha concentration. (b) Ac_4GalNAz-labeled adenovirus appears to be labeled only on the Fiber capsid protein.

Solution	Preparation	Notes

Ad5 storage buffer	5 mM Tris-HCl buffer (pH 8.0) containing 0.5 mM $MgCl_2$, 50 mM NaCl, 25% (v/v) glycerol, and 0.05% (w/v) Bovine Serum Albumin (BSA)	Store at -20 °C
Ad5 infection buffer	2% (v/v) bovine calf serum, 1× TC solution, Ad5 in storage buffer (volume determined using an MOI of 10 PFU / cell and an infectivity index of 1:20). Bring the solution to the final volume using TD buffer	Ad5 concentration may be determined via UV absorption.
TC solution (200×)	136 mM $CaCl_2$, 100 mM $MgCl_2$	Store at 4 °C
TD buffer	137 mM NaCl, 5 mM KCl, 0.07 mM Na_2HPO_4, and 25 mM Tris-HCl, pH 7.5	Store at 4 °C
HEK 293 cell growth medium	Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) bovine calf serum (BCS) and 1% (v/v) Pen Strep	Store at 4 °C
Cys stock solution (100×)	DMEM (-Met / -Cys) supplemented with 200 mM L-Cysteine	Prepare fresh and filter with a 0.2-µm sterile syringe filter before use.
Aha stock solution (25×)	DMEM (-Met / -Cys) supplemented with 100 mM L-Azidohomoalanine	Prepare fresh and filter with a 0.2-µm sterile syringe filter before use.
Aha labeling medium	DMEM (-Met / -Cys) supplemented with 10% BCS, Aha stock solution (1×), 2 mM L-Cysteine	Prepare fresh before use. This corresponds to a 4 mM concentration of Aha, although different concentrations may be used (see **Representative Results**).
Met stock solution (25×)	DMEM (-Met / -Cys) supplemented with 100 mM L-Methionine	Prepare fresh and filter with a 0.2-µm sterile syringe filter before use.
Met control medium	DMEM (-Met / -Cys) supplemented with 10% BCS, Met stock solution (1×), 2 mM L-Cysteine	Prepare fresh before use.
Ac_4GalNAz stock solution (1,000×)	50 mM peracetylated *N*-azidoacetylgalactosamine (Ac_4GalNAz) in methanol	Store at 4 °C. Ac_4GalNAz is a metabolic precursor to GlcNAz.
Azido-sugar labeling medium	100 µl Ac_4GalNAz stock solution, 100 ml HEK 293 cell growth medium	Allow methanol to evaporate prior to adding HEK 293 cell growth medium.
Cesium chloride solutions	CsCl dissolved in TD buffer: 1.25 g/ml (18.08 g / 50 ml H_2O) 1.35 g/ml (25.60 g / 50 ml H_2O) 1.40 g/ml (31.00 g / 50 ml H_2O)	Density gradients for ultracentrifugation
Virus storage buffer	Phosphate buffered saline (PBS), pH 7.2, containing 0.5 mM $CaCl_2$, 0.9 mM $MgCl_2$, and 10% (v/v) glycerol	Store at -20 °C
TAMRA-BCN (SPAAC) labeling solution	TAMRA-BCN in DMSO: 1. Use UV absorbance (OD_{260} Assay) to determine the titer of the virus solution in step 4.1 2. Determine the total number of viral particles in the 50 µl aliquot (N_{Ad5}) 3. The TAMRA-BCN molarity (in M) is determined using: $c_{BCN} = 4 \times 10^8 \times (N_{Ad5} / N_{Avo})$ $N_{Avo} = 6.022 \times 10^{23}$ (so that the reaction mixture prepared in Step 4 contains 100 alkyne probes per virion)	Strain-promoted alkyne probe For SDS-PAGE, a viral titer of approximately 10^{12} particles / ml is suggested
TAMRA-Alkyne (CuAAC) labeling solution	50 µl of azide-modified Ad5 in virus storage buffer (N_{Ad5} determined as above), 1.5 mM bathophenanthroline disulfonate (*i.e.* 1.5 × final CuBr concentration), TAMRA-Alkyne. TAMRA-Alkyne molarity (in M) is determined using: $c_{Alk} = 2 \times 10^6 \times (N_{Ad5} / N_{Avo})$	Tris buffer is known to be a competitive and inhibitory ligand of copper.[8]

	(so that the reaction mixture prepared in Step 5 contains 100 alkyne probes per virion)	
Laemmli sample buffer (2×)	125 mM Tris-HCl (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, and 0.004% (w/v) bromophenol blue	
Running buffer	187 mM glycine, 19 mM Tris-HCl, 3.5 mM SDS in H_2O	Store at 4 °C

Table 1. Preparation of the buffers and solutions required by this protocol.

Discussion

The development of chemoselective and bioorthogonal click reactions, including those of azides, is a rapidly-evolving area of research, and subsequently there is a growing number of these reactions to choose from for bioconjugation applications. We have restricted the scope of this protocol to include only two methods which were chosen because of their usefulness in our own lab and commercial availability of all reagents.

In the first such route - strain-promoted azide-alkyne cycloaddition (SPAAC) - the alkyne is contained within a cyclooctyne ring (**Figure 2a**). This recently-developed method can be conducted under oxygenated atmosphere and without the need for a copper catalyst.[9,10] Until recently, one drawback of this method was the laborious synthesis and reduced commercial availability of these strained alkynes compared with the terminal alkynes used in copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC); however, a growing library of these probes is now commercially available (see table of reagents). For these reasons, we have chosen SPAAC as the method of choice in this protocol.

Also described in this protocol is the ligation of azide-modified adenoviral particles via CuAAC in deoxygenated atmosphere using bathophenanthroline disulfonic acid disodium salt (BDA) as a chelating agent (**Figure 2b**). This method is convenient in that the BDA catalyst is both commercially available and inexpensive. Furthermore, reported yields are generally highest of the available methods, and complications arising from the generation of reactive oxygen species (cf. oxygenated CuAAC, below) in solution are avoided.[8] However, the copper(I) catalyst is known to be cytotoxic,[11] and the specialized equipment required to implement this procedure may deter some groups from using it. Nonetheless, we find this method efficient and useful for adenovirus modification.

A third useful method of CuAAC has been recently developed to work under oxygenated atmospheric conditions (**Figure 2c**).[8] This method enjoys the same benefits as the BDA-chelated approach but uses instead THPTA as a chelating agent to accommodate atmospheric oxygen without oxidative destruction of the substrate. One small drawback of this approach is the current lack of commercial availability of THPTA, although straightforward syntheses of this chelating agent have been reported.[8] Although we have not performed this ligation method on our own system, THPTA / CuAAC would be expected to produce comparable results to BDA / CuAAC, with the added convenience of oxygenated atmosphere tolerance.

Despite intentionally developing this protocol around commercially available materials, we still wish to recognize the cost advantages and increased flexibility of synthesizing the azides and alkyne probes used herein. Azidohomoalanine can be prepared in as little as three days and for significantly less than commercial sources.[6,12] Reported strained alkyne preparations are more difficult[11] but afford greater freedom of probe selection.

Disclosures

No conflicts of interest declared.

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