Instruction for use of endo-BCOT-(PEG)$_n$-Acid

Introduction

The BroadPharm endo-BCOT-(PEG)$_n$-Acid is a triple bond labeling reagent that reacts with primary and secondary amines. This cyclic triple bond reacts with the azide group simultaneously without needing of a metal catalyst; it is **non-metal catalyzed 'click' chemistry** functional group. Endo-BCOT-(PEG)$_n$-Acid must be first dissolved in a minimal amount of an organic solvent, such as dimethylsulfoxide (DMSO) or dimethylformamide (DMF) and then added to the buffer containing the protein or other molecule. The reagent forms an emulsion that allows the reaction to proceed. Unlike many conjugation reagents, endo-BCOT and azide activated biomolecules have long-term stability. The endo-BCOT to azide conjugation reaction is a truly chemoselective and bioorthogonal ligation reaction that can be performed in a complex mixture of biological molecules in aqueous media without reacting with any of them.

Important Product Information

• Avoid buffers containing primary amines (e.g., Tris or glycine) as these will compete with the reaction. If necessary, dialyze or desalt to exchange the protein sample into an amine-free buffer such as phosphate-buffered saline (PBS).

• After pegylation, the unreacted PEG linker is easily removed by size exclusion using either desalting columns or dialysis. A 10 mL desalting column is best suited for processing pegylation reactions involving 1-10 mg of protein in approximately 0.5-2 mL. For smaller amounts of protein and/or smaller reaction volumes, both the pegylation and subsequent buffer exchange may be performed in a single Thermo Scientific Slide-A-Lyzer MINI Dialysis Unit.

Additional Materials Required

• Water-miscible organic solvent (molecular sieve-treated) such as DMSO or DMF

• Small-volume, non-coring syringes for dispensing the reagent stock solution while minimizing exposure to air

• Buffer A: Phosphate-buffered saline, PBS (20mM sodium phosphate, 0.15M NaCl; pH 7.2, Product No. 28372) or other non-amine, lone-pair sulfur-free buffers

• Buffer B: MES-buffered saline (0.1M MES, 0.5M NaCl; pH 6.0 or 0.1M MES, 0.9% NaCl; pH 4.7; Product No. 28390) or other non-amine, non-carboxy, lone-pair sulfur-free buffers

• EDC

• NHS

• Hydroxylamine•HCl

• General Procedure for endo-BCOT-(PEG)$_n$-Acid /protein Conjugation

1. Equilibrate the endo-BCOT-(PEG)$_n$-Acid reagents to room temperature before opening bottles.

2. Prepare stock solutions by dissolving 100 mg of each reagent in the desired amount of DMF or DMSO. Cap, store and handle stock solutions as directed in the Important Product Information Section.

3. Prepare the appropriate amount of surface or Protein Buffer A.

4. The carboxylic acid groups on the PEG linker can be activated by adding appropriate amounts of EDC and NHS to the modified surface in small amount of Buffer B and reacting for 15 minutes at room temperature. For best results, perform this reaction at pH 5-6.

Note: The activation reaction with EDC and NHS is most efficient at pH 4.5-7.2; however, the reaction of NHS-activated molecules with primary amines is most efficient at pH 7-8.

5. Add the desired amine-containing substrate, prepared in Buffer A, to the activated surface and react for 2 hours at room temperature. For best results, raise the pH of the reaction solution to 7.2-7.5 with Buffer A immediately before adding the amine-containing substrate.

6. To quench the conjugation reaction, add hydroxylamine or another amine-containing buffer. Hydroxylamine hydrolyzes non-reacted NHS. Other quenching compounds include Tris, lysine, glycine or ethanolamine; however, these primary amine-containing compounds modify carboxylic acids.

•General Procedure for use endo-BCOT-$(PEG)_n$-Acid forPegylatingIgG and other Proteins

The following protocol typically results in approximately two triple bond molecules per IgG. The degree of endo-BCOT-$(PEG)_n$-Acid incorporation can vary depending on the parameters of the pegylation reaction, including protein concentration, endo-BCOT-$(PEG)_n$-Acid concentration, pH and time. Commonly used reaction conditions include incubation at 4-37°C, pH values from 7 to 9, and incubation times from a few minutes to overnight.

1. Dissolve 2 mg of IgG in 1 mL of Buffer B: MES-buffered saline (pH 6.0).

2. Immediately before use, dissolve 1 mg of endo-BCOT-$(PEG)_n$-Acid and 2 mg of EDCin 75 µL of DMF or DMSO. Add 25 µL of the endo-BCOT-$(PEG)_n$-Acid/EDC solution to the IgG solution.

3. Incubate the reaction on ice for two hours or at room temperature for 30 minutes.

4. Remove the unreacted reagent by dialysis or gel filtration.

5. Store the pegylated protein at at the specified conditions for unpegylated protein until ready for use.

•General Procedure for Chemoselective Ligation through Copper-free Click Chemistry

Our conjugation chemistry is based on the reaction of the so-called endo-BCOT-$(PEG)_n$-Acid reagent with an azide linker to form a stable triazole. The ‘click reaction’ is very fast at room temperature and creates stable triazole. This unique covalent bond is created when endo-BCOT, incorporated into one type of biomolecule reacts with an azide linker, incorporated into a second biomolecule.

This method requires a three-step reaction:

Step 1: Activation of Biomolecule #1 with endo-BCOT-$(PEG)_n$-Acid linker

Step 2: Activation of Biomolecule #2 with azide

Step 3: Mixing the two activated biomolecules to form a conjugate

Step 4: (optional): Removing excess of azide or endo-BCOT activated biomolecule with endo-BCOT or azide scavenger

Product Features and Benefits:

• Stable: forms a triazole

• Biocompatabile- catalyst not required (e.g. Cu(I))

• Specificity: azide reacts only with endo-BCOT in the presence of -NH_2, -SH,-COOH and other protein functionalities

• The reactive moieties do not interact with functionalities on biomolecules

• The chemistry can be performed in aqueous buffered media, yielding highlyefficienct conjugations

Important endo-BCOT Product Information:

• Avoid buffers that contain azides, which can react with endo-BCOT.

• Reactions with endo-BCOT and azides are more efficient at high concentration. Typical reaction times are less than 2 hours; however, incubating for longer can improve efficiency.

General Protocol: Copper-free Click Reaction

1. Prepare the azide containing protein in reaction buffer.

2. Add endo-BCOT bio molecule to azide containing sample.

Recommendation: Add 1 mol equivalent of the limiting reagent to 1.5-2 mol equivalents of the highest abundance reagent.

3. Incubate the reaction at room temperature for 2-6 hours or at 4 ºC for 6-12 hours.

4. The reaction is now ready for purification by size exclusion chromatography if desired.