

# Instructions for Bis-NHS-(PEG)<sub>n</sub> crosslinkers

## Introduction

BroadPharm Bis-NHS-(PEG)<sub>n</sub> Reagents are homobifunctional crosslinkers for covalent conjugation between amine-containing molecules. Crosslinkers having polyethylene glycol (PEG) spacers are convenient and useful alternatives to those with purely hydrocarbon spacer arms. PEG spacers improve water-solubility of reagent and the conjugate, reduce the tendency of conjugates to aggregate upon storage, and decrease immunogenic response to the spacer itself. By contrast to typical PEG reagents that contain heterogeneous mixtures of different PEG chain lengths, BroadPharm PEG reagents are homogeneous compounds of defined molecular weight and spacer arm length, providing greater precision in optimization and characterization of crosslinking applications. Homobifunctional crosslinkers containing *N*-hydroxysuccinimide (NHS) esters are often used for low-resolution 3-D studies of protein structure and protein interaction analysis. Accessible  $\alpha$ -amine groups at the N-termini of proteins and peptides and the  $\epsilon$ -amine of lysine residues react with NHS esters at pH 7-9 to form covalent amide bonds. The reaction results in the release of NHS. Hydrolysis of the NHS ester is the major competing reaction, the rate of which increases with pH and occurs more readily in dilute protein solutions. NHS ester crosslinking reactions are most commonly performed in phosphate, carbonate/bicarbonate, HEPES and borate buffers. Other buffers may also be used, provided they do not contain primary amines such as Tris or glycine. Using a large excess of Tris or glycine at neutral-to-basic pH can quench the reaction.

## Product Information

- Store the vial of the reagent at -20°C with desiccant. Bis-NHS-(PEG)<sub>n</sub> Reagents are viscous pale liquids or low melting point solid that are difficult to weigh and dispense. To facilitate handling, make a stock solution immediately before first use by dissolving the crosslinker in dry (anhydrous, molecular sieve-treated) organic solvent, such as dimethylsulfoxide (DMSO). Minimize reagent exposure to moisture because the NHS-ester reactive group is susceptible to hydrolysis. Store unused stock solution in a moisture-free condition (e.g., capped under an inert gas such as argon or nitrogen) at -20°C. Equilibrate reagent vial to room temperature before opening to avoid moisture condensation inside the container. Minimize exposure to air by keeping the stock solution capped by a septum through which reagent can be obtained with a syringe. With proper handling, the stock solution is stable for three months.
- Avoid buffers containing primary amines (e.g., Tris or glycine) during conjugation because they will compete with the intended reaction. If necessary, dialyze or desalt samples into a buffer such as phosphate-buffered saline (PBS).
- The crosslinker-to-protein molar ratio affects the modification extent of available amine groups and, therefore, crosslinking. This ratio requires optimization to yield the extent of crosslinking best for the specific application.

## Procedure for Crosslinking Proteins in Solution

Generally, a 10- to 50-fold molar excess of crosslinker over the amount of amine-containing protein(s) results in sufficient conjugation between proximal amino-groups. Empirical testing of reagent and protein concentrations is necessary to determine optimal conditions for the experiment.

### **A. Material Preparation**

- Conjugation Buffer: Phosphate-buffered saline (PBS) or other amine-free buffer at pH 7-8.
- Crosslinker Stock Solution: Read the Important Product Information (previous section) before preparing this solution. Prepare a 250mM Crosslinker Stock Solution by dissolving 100 mg of Bis-NHS-(PEG)<sub>n</sub> in following volumes of dry DMSO. Cap, store and handle the stock solution as directed in the previous section.
- (Optional) Quenching Buffer: 1M Tris•HCl, pH 7.5 (1M glycine or lysine also may be used.)
- (Optional) Desalting column

### **B. Procedure for Soluble Protein Crosslinking**

1. Dissolve the protein in Conjugation Buffer at 0.1mM (e.g., 5 mg in 1 mL for a 50kDa protein).
2. Add Bis-NHS-(PEG)<sub>n</sub> Reagent to the dissolved protein(s) at 1mM final concentration (10-fold molar excess for 0.1mM protein solution) by adding 4 µL of Crosslinker Stock Solution per milliliter of protein solution.
3. Incubate the reaction mixture for 30 minutes at room temperature or 2 hours at 4°C.
4. Quench reaction by adding Quenching Buffer at 20-50mM final and incubating for 15 minutes at room temperature. Alternatively, remove the unreacted reagent and reaction byproducts by desalting column or dialysis.

### **Procedure for Extra-Cellular Crosslinking**

Crosslinking may be performed on cells in suspension or on adherent cells in culture plates. In the latter situation, diffusion of the crosslinking reagent to all surfaces of the cells is limited and occurs predominately on the exposed surface. Culture media must be washed from the cells, otherwise amine-containing components will quench the reaction. Using a concentrated cell suspension is most effective to maximize reagent efficiency. Generally, a final concentration of 1-5mM reagent is effective. The NHS reaction speed increases with increasing pH; therefore, pH 8.0 is used in the following example so the reaction can be completed quickly. Bis-NHS-(PEG)<sub>n</sub> Reagents are not membrane-permeable and crosslink molecules only on the cell surface; for intracellular crosslinking experiments, use DSS ( Thermo Fisher Product No. 21655).

#### **A. Material Preparation**

- B.** • Conjugation Buffer: Phosphate-buffered saline (PBS, 20mM sodium phosphate, 0.15M NaCl, pH 8). HEPES, bicarbonate/carbonate or borate buffer may be used as alternatives.
- Crosslinker Stock Solution: Prepare as directed in Material Preparation Section of the previous procedure. Cap, store and handle the stock solution as directed in the Important Product Information Section.
- (Optional) Quenching Buffer: 1M Tris•HCl, pH 8.0 (1M glycine or lysine also may be used.)

#### **B. Procedure**

1. Suspend cells at  $\sim 25 \times 10^6$  cells/mL in PBS (pH 8).
2. Wash cells three times with ice-cold PBS (pH 8) to remove amine-containing culture media and proteins from the cells.
3. Add Bis-NHS-(PEG)<sub>n</sub> Reagent to the suspended cells at 1-5mM final by adding 4-20 $\mu$ L of Crosslinker Stock Solution per milliliter of cell suspension.
4. Incubate the reaction mixture for 30 minutes at room temperature. (To decrease active internalization of Bis-NHS-(PEG)<sub>n</sub> Reagent, perform this incubation at 4°C or on ice.)
5. Add Quench Solution at 10-20mM final and incubate for 10 minutes.

**Procedure for Chemistry Coupling a Bis-NHS-(PEG)<sub>n</sub> Linker to a Small Molecule with a Primary Amine**

- Using 1.2 to 1.5 e.q. of Bis-NHS-(PEG)<sub>n</sub> compare to each primary amine on the small molecule, adding 4 to 10 equivalents of DIPEA or TEA. Stir at room temperature

Note: the use of solvents such as THF, DCM, ACN is acceptable. For better solubility of the Bis-NHS-(PEG)<sub>n</sub>, use DMF or DMSO, but these require extractive work-up to remove these solvents.