INSTRUCTIONS

Thermo SCIENTIFIC

SM(PEG)$_n$ Crosslinkers

Amine-to-sulfhydryl crosslinkers with soluble polyethylene glycol (PEG) spacer arms

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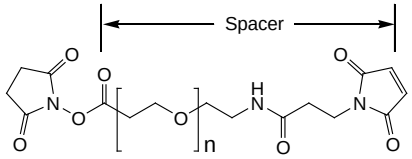
Number	Description	
		
22102 **22103**	**SM(PEG)$_2$,** 100mg **SM(PEG)$_2$,** 1g Form: Low-melting point solid Spacer Arm: 17.6Å Molecular Weight: 425.39 Net Mass Addition: 310.12	n = 2 NHS-PEG$_2$-Maleimide (succinimidyl-[(*N*-maleimidopropionamido)-diethyleneglycol] ester)
22104 **22107**	**SM(PEG)$_4$,** 100mg **SM(PEG)$_4$,** 1g Form: Viscous liquid or solid Spacer Arm: 24.6Å Molecular Weight: 513.50 Net Mass Addition: 398.17	n = 4 NHS-PEG$_4$-Maleimide (succinimidyl-[(*N*-maleimidopropionamido)-tetraethyleneglycol] ester)
22105	**SM(PEG)$_6$,** 100mg Form: Viscous liquid or solid Spacer Arm: 32.5Å Molecular Weight: 601.60 Net Mass Addition: 486.20	n = 6 NHS-PEG$_6$-Maleimide (succinimidyl-[(*N*-maleimidopropionamido)-hexaethyleneglycol] ester)
22108	**SM(PEG)$_8$,** 100mg Form: Viscous liquid Spacer Arm 39.25Å Molecular Weight: 689.71 Net Mass Addition: 574.27	n = 8 NHS-PEG$_8$-Maleimide (succinimidyl-[(*N*-maleimidopropionamido)-octaethyleneglycol] ester)
22112 **22113**	**SM(PEG)$_{12}$,** 100mg **SM(PEG)$_{12}$,** 1g Form: Viscous liquid Spacer Arm: 53.4Å Molecular Weight: 865.92 Net Mass Addition: 750.38	n = 12 NHS-PEG$_{12}$-Maleimide (succinimidyl-[(*N*-maleimidopropionamido)-dodecaethyleneglycol] ester)
22114	**SM(PEG)$_{24}$,** 100mg Form: Viscous liquid Spacer Arm: 95.2Å Molecular Weight: 1394.55 Net Mass Addition: 1279.01	n = 24 NHS-PEG$_{24}$-Maleimide (succinimidyl-[(*N*-maleimidopropionamido)-tetracosaethyleneglycol] ester)

Storage: Upon receipt store desiccated at -20° C. Product is shipped at ambient temperature.

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Introduction

The SM(PEG)$_n$ reagents are heterobifunctional crosslinkers with *N*-hydroxysuccinimide (NHS) ester and maleimide groups that allow covalent conjugation of amine- and sulfhydryl-containing molecules. Crosslinkers having polyethylene glycol (PEG) spacers are convenient alternatives to reagents with purely hydrocarbon spacer arms. PEG spacers improve water solubility of reagent and conjugate, reduce the potential for aggregation of the conjugate, and increases flexibility of the crosslink, resulting in reduced immunogenic response to the spacer itself. By contrast to typical PEG reagents that contain heterogeneous mixtures of different PEG chain lengths, Pierce™ PEGylation Reagents are homogeneous compounds of defined molecular weight and spacer arm length, providing greater precision in optimization and characterization of crosslinking applications.

N-hydroxysuccinimde (NHS) esters react with primary amines at pH 7-9 to form amide bonds, while maleimides react with sulfhydryl groups at pH 6.5-7.5 to form stable thioether bonds (Figure 1). In aqueous solutions, hydrolytic degradation of the NHS ester is a competing reaction whose rate increases with pH. The maleimide group is more stable than the NHS-ester group but will slowly hydrolyze and also lose its reaction specificity for sulfhydryls at pH values greater than 7.5. For these reasons, conjugation experiments involving this type of heterobifunctional crosslinker are usually performed at pH 7.2-7.5, with the NHS-ester (amine-targeted) reaction being accomplished before or simultaneous with the maleimide (sulfhydryl-targeted) reaction.

NHS/maleimide crosslinkers can be used to prepare antibody-enzyme and hapten-carrier protein conjugates in a two-step reaction scheme. First, the amine-containing protein is reacted with a several-fold molar excess of the crosslinker, followed by removal of excess (nonreacted) reagent by desalting or dialysis; finally, the sulfhydryl-containing molecule is added to react with the maleimide groups already attached to the first protein.

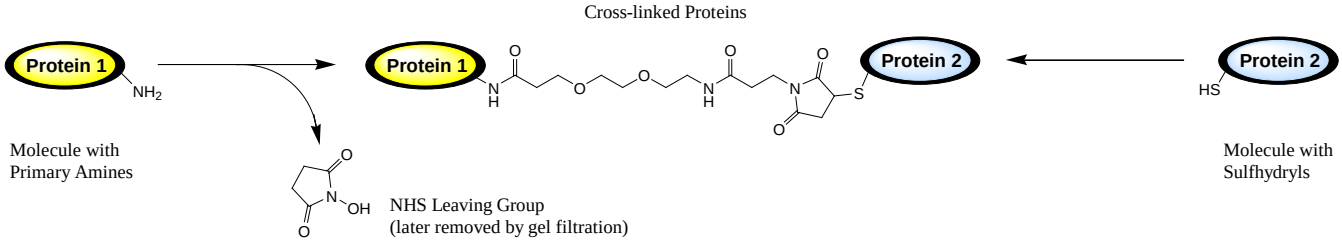

Figure 1. Structure of crosslink formed by reaction of SM(PEG)$_2$ with amine and sulfhydryl molecules.

Important Product Information

- SM(PEG)$_n$ reagents are viscous pale liquids 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, Product No. 85190). 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 aliquots may 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) and sulfhydryls during conjugation because they will compete with the intended reaction. If necessary, dialyze or desalt samples into an appropriate buffer such as phosphate buffered saline (PBS).
- Molecules to be reacted with the maleimide moiety must have free (reduced) sulfhydryls. Reduce peptide disulfide bonds with Immobilized TCEP Disulfide Reducing Gel (Product No. 77712). Reduce disulfide bonds in high molecular weight proteins using 5 mM TCEP (1:100 dilution of TCEP Solution, Product No. 77720) for 30 minutes at room temperature, followed by two passes through an appropriate desalting column (e.g., Thermo Scientific™ Zeba™ Spin Desalting Columns). Be aware that proteins (e.g., antibodies) can be inactivated by complete reduction of their disulfide bonds. Selective reduction of hinge-region disulfide bonds in IgG may be accomplished with 2-Mercaptoethylamine•HCl (2-MEA, Product No. 20408). Sulfhydryls can be added to molecules using *N*-succinimidyl *S*-acetylthioacetate (SATA, Product No. 26102 or SAT(PEG)$_4$, Product No. 26099) or 2-iminothiolane•HCl (Traut's Reagent, Product No. 26101), which modify primary amines.

Procedure for Two-step Protein Crosslinking

Generally, a 10- to 50-fold molar excess of crosslinker over the amount of amine-containing protein results in sufficient maleimide activation to enable several sulfhydryl-containing proteins to be conjugated to each amine-containing protein. Dilute protein solutions require a high molar excess of reagent to achieve adequate activation. Empirical testing is necessary to determine activation levels and final conjugation ratios that are optimal for the intended application.

A. Material Preparation

- Conjugation Buffer: Phosphate buffered saline (PBS, pH 7.2; e.g., Product No. 28372) or other amine- and sulfhydryl-free buffer at pH 6.5-7.5 (see Important Product Information) – adding EDTA to 1-5mM chelates divalent metals, thereby preventing metal-catalyzed disulfide formation.
- Crosslinker Stock Solution: Read the Important Product Information (previous section) before preparing this solution. Prepare a 250mM Crosslinker Stock Solution by dissolving 100mg of crosslinker (entire contents of vial, approximately 100µL) in the following volume of dry DMSO:
    - $SM(PEG)_2$: 840µL (For Product No. 22103 add ~8.4mL to make total volume to 9.4mL.)
    - $SM(PEG)_4$: 680µL (For Product No. 22107 add ~6.8mL to make total volume to 7.8mL.)
    - $SM(PEG)_6$: 564µL
    - $SM(PEG)_8$: 480µL
    - $SM(PEG)_{12}$: 360µL (For Product No. 22113 add ~3.6 mL to make total volume to 4.6mL.)
    - $SM(PEG)_{24}$: 187µL

  Cap, store and handle stock solutions as directed in the Important Product Information.
- Desalting column to separate modified protein from excess crosslinker and reaction byproducts (e.g., Zeba Spin Desalting Columns)
- Amine-containing protein (Protein-NH_2) and sulfhydryl-containing protein (Protein-SH) to be conjugated

B. Protocol

Note: For best results, ensure that Protein-SH is prepared (see Important Product Information) and ready to combine with Protein-NH_2 in step 5.

1. Dissolve Protein-NH_2 in Conjugation Buffer at 0.1mM (e.g., 5mg in 1mL for a 50kDa protein).
2. Add crosslinker to dissolved Protein-NH_2 at 1mM final concentration (= 10-fold molar excess for 0.1mM protein solution) by adding 4µL of the 250mM Crosslinker Stock Solution per milliliter of Protein-NH_2 solution.
3. Incubate reaction mixture for 30 minutes at room temperature or 2 hours at 4°C.
4. Remove excess crosslinker using a desalting column equilibrated with Conjugation Buffer.

   Note: Follow the desalting column product instructions to determine which fractions contain Protein-NH_2. Alternatively, locate the protein by measuring for fractions having peak absorbance at 280nm; however, be aware that the NHS-ester leaving group also absorbs strongly at 280nm.
5. Combine and mix Protein-SH and desalted Protein-NH_2 in a molar ratio corresponding to that desired for the final conjugate and consistent with the relative number of sulfhydryl and activated amines that exist on the two proteins.
6. Incubate the reaction mixture at room temperature for 30 minutes or 2 hours at 4°C.

   Note: Generally, there is no harm in allowing the reaction to proceed for several hours or overnight, although usually the reaction will be complete in the specified time. To stop the conjugation reaction before completion, add buffer containing reduced cysteine at a concentration several times greater than the sulfhydryls of Protein-SH.

   Note: Conjugation efficiency may be estimated by electrophoretic separation and subsequent protein staining.

Related Products

Table 1. Other NHS/Maleimide crosslinkers.

Crosslinker Name	Spacer Arm Length (Å)	Spacer Arm Composition (between ester and maleimide)	Product No. (NHS)	Product No. (Sulfo-NHS)
AMAS	4.4	Alkane	22295	NA
BMPS	5.9	Alkane	22298	NA
GMBS	7.3	Alkane	22309	22324
MBS	7.3	Aromatic	22311	22312
SMCC	8.3	Cyclohexane	22360	22322
EMCS	9.4	Alkane	22308	22307
SMPB	11.6	Alkane/Aromatic	22416	22317
SMPH	14.2	Alkane/Amide	22363	NA
LC-SMCC	16.2	Alkane/Amide/Cyclohexane	22362	NA
KMUS	16.3	Alkane	NA	21111

28372 **BupH™ Phosphate Buffered Saline Packs,** 40 pack, each pack yields 500mL of 0.1M sodium phosphate, 0.15M sodium chloride, pH 7.2 when reconstituted with 500mL water.

69576 **Slide-A-Lyzer™ MINI Dialysis Unit Kit,** for 10-100µL sample volumes, 10 units plus float

66382, 66807 **Slide-A-Lyzer Dialysis Cassette Kits,** for 0.5-3mL and 3-12mL sample volumes, respectively

89889 **Zeba Spin Desalting Columns, 7K MWCO, 2mL**

89891 **Zeba Spin Desalting Columns, 7K MWCO, 5mL**

31490 **Horseradish Peroxidase,** 10mg

77600 **Imject™ mcKLH (in PBS),** 5 × 20mg

77150 **Imject SuperCarrier™ Immune Modulator (in PBS),** 10mg

77140 **Imject Freund's Complete Adjuvant,** 5 × 10mL

77145 **Imject Freund's Incomplete Adjuvant,** 5 × 10mL

22582 **Ellman's Reagent,** 5g, for determining free sulfhydryl content in peptides and proteins

XP04200BOX **Novex™ Tris-Glycine protein gels** (see thermofisher.com/proteingels for a complete listing)

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