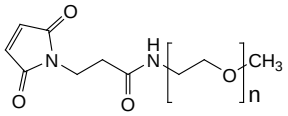
INSTRUCTIONS

$MM(PEG)_n$ Reagents

Branched and unbranched sulfhydryl-reactive PEGylation reagents

MAN0016376
Rev. A.0
Pub. Part No. 2161768.7

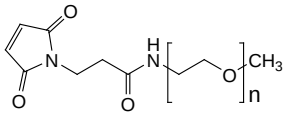
Number	Description
22711	**$MM(PEG)_{12}$,** 100mg
22712	**$MM(PEG)_{12}$,** 1g Form: Low melting point solid Molecular Weight: 710.81 Spacer Arm: 51.9Å



n = 12

Methyl-PEG_{12}-Maleimide

22713	**$MM(PEG)_{24}$,** 100mg Form: Low melting point solid Molecular Weight: 1239.44 Spacer Arm: 92.8Å

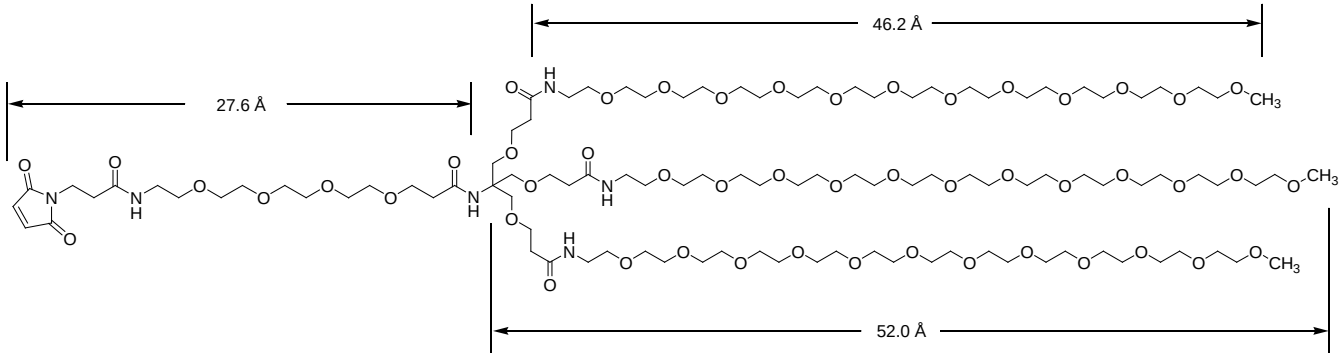


n = 24

Methyl-PEG_{24}-Maleimide

22361	**$TMM(PEG)_{12}$,** 100mg Form: Waxy, semi-translucent solid Molecular Weight: 2360.75 Spacer Arm: See structure

(Methyl-PEG_{12})$_3$-PEG_4-Maleimide



Storage: Upon receipt store desiccated at 4°C. Product is shipped at ambient temperature.

Introduction

Thermo Scientific™ $MM(PEG)_n$ and $TMM(PEG)_{12}$ enable simple and efficient modification of proteins and other molecules that have sulfhydryl groups. Modification adds polyethylene glycol (PEG) spacers (PEGylation) with terminal methyl groups. The PEG spacer is hydrophilic (water-soluble), and this property is transferred to the labeled macromolecule. Consequently, PEGylation of proteins and peptides can significantly increase water solubility and reduce aggregation, often without adversely affecting their biological activities. In addition, PEGylation can reduce immunogenicity of the labeled molecule.

Typical PEGylation reagents contain heterogeneous mixtures of different PEG chain lengths; however, Pierce PEGylation Reagents are homogeneous compounds of defined molecular weight and spacer arm length, providing precision in optimizing modification applications.

Maleimide-activated reagents are effective for protein modification of sulfhydryl groups. Maleimide groups react efficiently and specifically with free (reduced) sulfhydryls at pH 6.5-7.5 to form stable thioether bonds (Figure 1). Most proteins have cysteine residues whose side-chain sulfur atoms typically occur in pairs as disulfide bonds. Reduction of these disulfide

bonds exposes the sulfhydryl group required as a target for PEGylation with maleimide-activated reagents. Alternatively, sulfhydryl groups can be added to molecules using various modification reagents (See Important Product Information Section). The MM(PEG)$_n$ reagents are readily soluble in water or organic solvents such as dimethylsulfoxide (DMSO), methylene chloride or dimethylformamide (DMF).

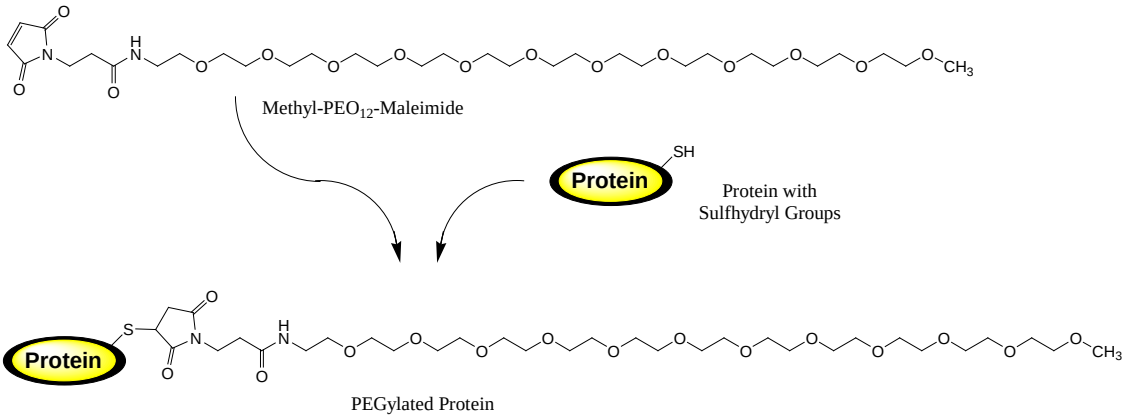


Figure 1. PEGylation of protein with MM(PEG)$_{12}$. Proteins are many times larger than the PEGylation reagent and may contain several sulfhydryl groups, each of which could be labeled.

Important Product Information

- The MM(PEG)$_n$ reagents are low melting-point solids that are difficult to weigh and dispense. To facilitate handling, make a stock solution immediately before first use by dissolving the reagent in dry (anhydrous, molecular sieve-treated) organic solvent, such as dimethylformamide (DMF, Product No. 20673) and dimethylsulfoxide (DMSO, Product No. 20688). Although the maleimide group is more stable than some other types of reactive groups, it can hydrolyze to form a non-reactive maleimic acid. Therefore, 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 removed with a syringe. With proper handling, the stock solution is stable for three months.
- Molecules to be reacted with the maleimide moiety must have free (reduced) sulfhydryls. Reduce peptide disulfide bonds with Thermo Scientific™ Immobilized TCEP Disulfide Reducing Gel (Product No. 77712). Reduce disulfide bonds in high molecular weight proteins using 5mM TCEP (1:100 dilution of TCEP Solution, Product No. 77720) for 30 minutes at room temperature, followed by TCEP removal using a desalting column (e.g., Thermo Scientific™ Zeba™ Spin Desalting Columns). Proteins (e.g., antibodies) can be inactivated by complete reduction of their disulfide bonds. Selective reduction of hinge-region disulfide bonds in IgG can 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.
- Avoid extraneous sulfhydryl-containing components in the reaction buffers during conjugation (e.g., DTT), as they react with the maleimide portion of the reagent, inhibiting and reducing conjugation efficiency of the intended target.
- The maleimide group reacts predominantly with free sulfhydryls at pH 6.5-7.5, forming stable thioether bonds. At pH values > 7.5, reactivity toward primary amines and hydrolysis of the maleimide groups can occur. At pH 7, the maleimide group is ~1000 times more reactive toward a free sulfhydryl than to an amine.
- If desired, excess nonreacted MM(PEG)$_n$ reagent can be removed by size exclusion using either desalting columns or dialysis units (See Related Thermo Scientific Products).

Additional Materials Required

- Water-miscible organic solvent (molecular sieve-treated) such as dimethylsulfoxide (DMSO, Product No. 20688) or dimethylformamide (DMF, Product No. 20673) for preparing reagent stock solution
- Small-volume, non-coring syringes for dispensing reagent stock solution while minimizing exposure to the air
- Phosphate-buffered saline (PBS) or other sulfhydryl-free buffer having pH 6.5-7.5 for use as reaction buffer (See Important Product Information and Related Thermo Scientific Products)
- Desalting columns or dialysis units for buffer exchange and removal of excess reagent following modification (e.g., Zeba Spin Desalting Columns or Thermo Scientific™ Slide-A-Lyzer™ Dialysis Units)

Procedure for PEGylating Proteins with MM(PEG)$_n$ Reagents

The amount of MM(PEG)$_n$ reagent to use for each reaction depends on the number of free sulfhydryls, the amount of modification desired, and the amount and concentration of the molecule to be labeled. By regulating the reagent-to-target molar ratio, the extent of labeling can be controlled. As a starting point use a 5- to 20-fold molar excess of PEGylation reagent for protein solutions > 2mg/mL. When labeling more dilute solutions, a greater relative molar fold excess of reagent may be necessary to achieve the same results. Optimal molar ratios for small molecule modification may differ significantly. Example calculations for IgG modification (molecular weight 150,000) are provided for convenience.

A. Calculate the Amount of Reagent Needed

1. Calculate the quantity in millimoles of the reagent to add to the reaction for a 20-fold molar excess:

$$\text{mL protein} \times \frac{\text{mg protein}}{\text{mL protein}} \times \frac{\text{mmol protein}}{\text{mg protein}} \times \frac{\text{20 mmol PEGylation Reagent}}{\text{mmol protein}} = \text{mmol PEGylation Reagent}$$

   Note: the value 20 in this equation corresponds to the suggested reagent molar fold excess for a 2mg/mL protein sample.

2. Calculate microliters of 250mM PEGylation reagent stock solution (prepared in Step B.1) to add to the reaction:

$$\text{mmol PEG} \times \frac{1{,}000{,}000\ \mu\text{L}}{\text{L}} \times \frac{\text{L}}{250\ \text{mmol}} = \mu\text{L PEGylation Reagent Stock Solution}$$

Example Calculation:

For 1mL of a 2mg/mL IgG (150,000 MW) solution, ~1µL of 250mM PEGylation Reagent will be added.

$$1\,\text{mL IgG} \times \frac{2\,\text{mg IgG}}{1\,\text{mL IgG}} \times \frac{1\,\text{mmol IgG}}{150{,}000\,\text{mg IgG}} \times \frac{20\,\text{mmol PEG}}{1\,\text{mmol IgG}} = 0.000266\,\text{mmol PEGylation Reagent}$$

$$0.000266\,\text{mmol PEGylation Reagent} \times \frac{1{,}000{,}000\,\mu\text{L}}{\text{L}} \times \frac{\text{L}}{250\,\text{mmol}} = 1.07\,\mu\text{l of 250 mM PEGylation Reagent Stock Solution}$$

B. Prepare 250mM Reagent Stock Solution

1. Read the Important Product Information (previous section) before preparing and storing this solution.
2. Remove vial of reagent from -20°C storage and fully equilibrate it to room temperature before opening.
3. Prepare a 250mM PEGylation Reagent Stock Solution by dissolving 100mg of reagent (i.e., entire contents of vial, approximately 100µL) in the following volume of dry water-miscible solvent (e.g., DMF or DMSO):
   - MM(PEG)$_{12}$: 463µL (add 4.63mL to Product No. 22712 to make total volume to 5.63mL)
   - MM(PEG)$_{24}$: 223µL
   - TMM(PEG)$_{12}$: 63µL (To make 125mM Stock, add 226µL instead of 63µL.)
4. Cap, store and handle stock solutions as directed in the Important Product Information Section.

C. Labeling Reaction

1. Dissolve protein to be modified in sulfhydryl-free buffer at pH 6.5-7.5, according to the calculations made in Section A.

   Note: Protein already in sulfhydryl-free buffer at pH 6.5-7.5 may be used without buffer exchange or dilution.

2. If the vial of PEGylation Stock Solution had been stored since preparation, remove it from -20°C storage and fully equilibrate it to room temperature before opening.
3. Using a syringe, remove an appropriate volume (See Calculations in Section A) of 250mM PEGylation Reagent Stock Solution, dispense it into the protein solution and mix well.
4. Incubate reaction on ice or room temperature for two hours to overnight.

   Note: Except for possible degradation or microbial growth, there is no harm in reacting longer than the specified time.

5. Labeling is complete at this point and, although excess nonreacted and hydrolyzed PEGylation reagent remains in the solution, it is often possible to perform preliminary tests of the labeled protein. Once proper function and labeling has been confirmed, the labeled protein may be purified from nonreacted $MM(PEG)_n$ by desalting or dialysis.

Related Thermo Scientific Products

22341	**$MS(PEG)_4$,** 100mg (amine-reactive Methyl-PEG_4-NHS Ester)
22509	**$MS(PEG)_8$,** 100mg (amine-reactive Methyl-PEG_8-NHS Ester)
22685	**$MS(PEG)_{12}$,** 100mg (amine-reactive Methyl-PEG_{12}-NHS Ester)
28372	**BupH™ Phosphate Buffered Saline Packs,** 40 pack
69576	**Slide-A-Lyzer MINI Dialysis Unit Kit**
66382, 66807	**Slide-A-Lyzer Dialysis Cassette Kits**
89889	**Zeba Spin Desalting Columns, 7K MWCO, 2mL,** 5/pkg
89891	**Zeba Spin Desalting Columns, 7K MWCO, 5mL,** 5/pkg

General Reference

Morar, A.S., *et al*. (2006). PEGylation of proteins: A structural approach. *BioPharm Int* **(4)**34-46.