Maleimide-PEG-DPPE Synthesis Protocol
2015

ABOUT

This is a technical report detailing the protocol for the synthesis of maleimide-PEG-dppe (MALPEGDPE) to be used for conjugation of a thiol-terminated molecule with phospholipid DPPE. For questions, contact Ian Hoffecker. To cite this report, use the following DOI: 10.13140/RG.2.1.4735.6002 and consider citing some of the following related work.

Ian Hoffecker  document author

Yuji Teramura  original method developer¹

Narufumi Kitamura  protocol improved for yield and safety²

Hiroo Iwata, Yusuke Arima, Toshiki Matsui  made other contributions to method³


### MATERIALS

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Manufacturer</th>
<th>Product No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHS-PEG-MAL&lt;sub&gt;α-N-hydroxysuccinimidyl-ω-maleimidy&lt;/sub&gt; poly(ethylene glycol) MW:5000)</td>
<td>NOF Corporation</td>
<td>SUNBRIGHT MA-050TS</td>
</tr>
<tr>
<td>Triethylamine 99%</td>
<td>WAKO</td>
<td>202-02646</td>
</tr>
<tr>
<td>DPPE (Dipalmitoyl-sn-glycero-3-phosphoethanolamine)</td>
<td>NOF Corporation</td>
<td>COATSOME®ME-6060</td>
</tr>
<tr>
<td>Chloroform, Super Dehydrated (containing 150 ppm Amylene as a stabilizer)</td>
<td>WAKO</td>
<td>039-21931</td>
</tr>
<tr>
<td>Triethylamine</td>
<td>WAKO</td>
<td>202-02641</td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>WAKO</td>
<td>052-01165</td>
</tr>
<tr>
<td>Benzene</td>
<td>Sigma Aldrich</td>
<td>401765-100ML</td>
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<tr>
<td>Liquid N&lt;sub&gt;2&lt;/sub&gt;</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Manufacturer</th>
<th>Product No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass syringe</td>
<td>Corning</td>
<td>9350-1</td>
</tr>
<tr>
<td>Round Bottom Flask</td>
<td>Iwaki</td>
<td>NASU-FK 50</td>
</tr>
<tr>
<td>11G4 Filter</td>
<td>Iwaki</td>
<td>-</td>
</tr>
<tr>
<td>Vacuum Flask</td>
<td>Iwaki</td>
<td>-</td>
</tr>
<tr>
<td>Rotavap</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lyophilizer/Vacuum freeze drying apparatus</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 1: Reagents and Equipment
Preparations

- Bake molecular sieves if not using super dehydrated chloroform.

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Day 1 - NHS Coupling of DPPE to PEGMAL

- Warm bottles of DPPE and NHSPEGMAL from -20°C to RT inside a dessicator bulb for 15-30 minutes.

- At the weighing station, de-static two eppendorf tubes and the NHSPEGMAL and DPPE inner contents.

- Weigh out 0.2 g of NHSPEGMAL and add to one of the tubes.

- Weigh out 0.022 g of DPPE and add to the other tube.
• Work in a fume hood and use a glass syringe to pipette an arbitrary amount of super dehydrated chloroform into each tube. Approximately 1 ml will be sufficient to dissolve DPPE or at least suspend it long enough to transfer it.\(^4\)

• Move the contents of both eppendorf tubes to a round bottom flask (50 ml) that has been thoroughly cleaned and dried.\(^5\)

• Add more chloroform to tubes to salvage as much reagent as possible. The final flask volume should be approximately (though not strictly) 3 ml.

• Making sure to work in a fume hood, transfer a few drops of triethylamine (about 100 µl).

• Add a stir bar, seal the flask so that it is air-tight, e.g. with rubber stopper.

• Use a pump with inlet tubing connected to a needle to pierce the rubber cap and suck out some air to reduce humidity inside the reaction flask.

• Inject a small amount of \(N_2\) gas.

• Repeat the last two steps another two times.

• Use a plastic fastener to tightly secure the rubber cap.

• Stir overnight or until transparent. \(^6\)

\(^4\) Note that chloroform containing ethanol stabilizers must be purified with pre-baked 4A molecular sieves (large pores) to remove ethanol as this can hydrolyze w NHS-PEG-MAL. Non-dehydrated chloroform must also be purified with pre-baked 3A molecular sieves (small pores) or distillation. Bake 60 deg in vacuum oven and add to reaction flask w chloroform prior to starting NHS-PEG-MAL steps.

\(^5\) Some DPPE will not be dissolved, but this is acceptable since it will dissolve as the reaction proceeds.

\(^6\) If the solution is not transparent by the next day, add more triethylamine.
Day 2 - Precipitation, Purification, and Drying

- Prepare a beaker of diethyl ether (200 ml) in an ice box, in the hood, and with strong stirring (enough to create a funnel/vortex).

- Use a glass pipette to slowly drip the contents of the MALPEGDPPE into the diethyl ether.\(^7\)

- Stop the stirring, cover with plastic wrap, and wait 30 minutes for precipitate to settle to the bottom.

\(^7\) Solution will become turbid as MALPEGDPPE precipitates.
• Meanwhile, set up a filtration system with an 11G4 filter. The 11G4 filter refers to the vessel capacity while G4 refers to the pore size. 17G4 filters can also be used. Note that the filter should be cleaned prior to use by flushing it with chloroform followed by diethyl ether to prevent MALPEGDPPE from redissolving in any residual chloroform.
• Fill the filter vessel with the precipitate-containing diethyl ether with the vacuum unconnected at first and allow gravity draining initially.\(^9\)

\(^9\) As draining begins to slow, you can create a weak pressure by cupping a gloved hand over the filter rim and pressing gently. This speed up the filtration process somewhat.
• Once gravity draining is no longer effective, attach the vacuum and carefully increase its strength to facilitate draining. ¹⁰

Too much vacuum will force precipitate through the filter and result in loss of product, so it is important to use just enough vacuum to speed up the process so that you will finish in a reasonable time span of about 30 or 45 minutes. You can monitor the loss of products by the turbidity of the bottom liquid, which should be transparent.
• Continue filtering the remaining mixture and leave the vacuum on for a few minutes after draining is complete in order to remove residual solvent. When the filter is dry, visually confirm that some powder has accumulated and that the bottoms are clear. Redo this process if too much precipitate has been lost.

• Transfer the filter vessel to the top of a clean round bottom flask, and pipette an arbitrary volume of chloroform over the precipitated MALPEGDPPE to dissolve it and collect it in the flask.
• As the chloroform drains, obtain some liquid $N_2$ for the rotavap pump solvent trap and begin setting up the rotavap by connecting components.
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• Attach the round bottom flask to the rotavap and remove the chloroform.\textsuperscript{11} 

\textsuperscript{11} Note that CF$_3$COOH and other contaminants in the rotavap, if not cleaned well, will have a deleterious effect on MALPEGDPPE activity. Also note that
• Redissolve the dessicated MALPEGDPPE by glass-pipetting a few ml of benzene into the round bottom flask, and transfer the solution to a small vial which will become the final receptacle for the MALPEGDPPE.
• Prepare an icebox with a layer of liquid $N_2$, and submerge the capped vial of MALPEGDPPE solution.
• After the benzene has solidified, remove the vial and loosen the cap. Place the vial into a lyophilizer and dessicate overnight.
Day 3 - Storage

• Store at -20°C
• Optional: Verify with HPLC/Methanol gradient.

12 The final color of MALPEGDPPE is a pale pink if it has decent activity. If the powder is too white, then either a step in the synthesis process is resulting in hydrolysis or the starting NHSPEGMAL may be old.

References
