# Generation of Protein-Phosphorodiamidate Morpholino Oligomer Conjugates for Efficient Cellular Delivery via Anthrax Protective Antigen

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## Abstract

Phosphorodiamidate morpholino oligomers (PMOs) offer great promise as therapeutic agents for translation blocking or splice modulation due to their high stability and affinity for target sequences. However, in spite of their neutral charge as compared to natural oligonucleotides or phosphorothioate analogs, they still show little permeability for cellular membranes, highlighting the need for effective cytosolic delivery strategies. In addition, the implementation of strategies for efficient cellular targeting is highly desirable to minimize side effects and maximize the drug dose at its site of action. Anthrax toxin is a three-protein toxin of which the pore-forming protein anthrax protective antigen (PA) can be redirected to a receptor of choice and lethal factor (LF), one of the two substrate proteins, can be coupled to various cargoes for efficient cytosolic cargo delivery. In this protocol, we describe the steps to produce the proteins and protein conjugates required for cytosolic delivery of PMOs through the cation-selective pore generated by anthrax protective antigen. The method relies on the introduction of a unique cysteine at the C-terminal end of a truncated LF (aa 1–254), high-yield expression of the (truncated) toxin proteins in *E. coli*, functionalization of a PMO with a maleimide group and coupling of the maleimide-functionalized PMO to the unique cysteine on LF by maleimide-thiol conjugation chemistry. Through co-administration of PA with LF-PMO conjugates, an efficient cytosolic delivery of PMOs can be obtained.

**Key words** Antisense, Anthrax toxin, Protective antigen, Phosphorodiamidate morpholino oligomers, DNA analog, Drug delivery, Cellular internalization, Bioconjugate chemistry

## 1 Introduction

Phosphorodiamidate morpholino oligomers (PMOs) are uncharged DNA analogs with therapeutic potential due to their ability to specifically bind to target sites on RNA. By steric inhibition of translation initiation complexes, PMOs can block translation. Alternatively, by targeting sites associated with splicing of pre-mRNAs, PMOs can mediate splice modulation and thereby correct the consequences of splicing mutations at the pre-mRNA level, for instance those in inherited retinal dystrophies [1]. PMOs

have several qualities that are excellent for therapeutic development, including nuclease-resistance, long-term activity, low toxicity, and high specificity [2, 3]. However, a major challenge remains, which is achieving an efficient cellular delivery, particularly in vivo. PMOs are neutral molecules that because of their size are impermeable to cellular membranes. Delivery approaches that have been developed up to now include scraping of cells, particle-based approaches, and cell-penetrating peptide (CPP)-based delivery [3–5]. Cell scraping cannot be translated to in vivo applications and particle-based approaches suffer from delivery challenges in vivo such as poor tissue penetration and liver enrichment [6]. CPP-mediated delivery has demonstrated potential, but still does not target specific cell-surface receptors, indicating the need for a novel approach.

Recently, several groups have demonstrated that anthrax toxin, a sophisticated protein-based molecular machine that has evolved to efficiently deliver toxic catalytic proteins into the cytosol, can be employed for the functional delivery of various types of cargoes, including antisense oligonucleotides (AON) [7, 8]. The full anthrax toxin consists of three proteins: a pore-forming protein, called protective antigen (PA), that generates cation-selective pores and two enzyme components [9], called lethal factor (LF) and edema factor (EF). LF and EF in turn consist of two domains: the first domain binds the protein pore and the second domain is the enzymatically active domain and is thus responsible for the actual toxicity. For the delivery of PMOs via this mechanism, only PA and the PA pore-binding domain of LF are needed as protein components. For LF, this means that the enzymatic (toxicity-causing) domain of LF is replaced with a PMO.

In this chapter, we describe the preparation of the components needed to mediate cytosolic delivery of PMOs by the anthrax toxin translocation mechanism (Fig. 1a). The individual protein components, PA and LF (1–254) are produced in high quantity in soluble form in the cytosol of *E. coli*. LF (1–254) is by itself cysteine-free, so through the introduction of a unique C-terminal cysteine, a site-specific conjugation via maleimide-thiol conjugation chemistry can be achieved. Maleimide-thiol conjugation chemistry is useful for coupling biologically active molecules because it is fast, highly selective and it can be done in physiological buffers at 37 °C or at 4 °C [10].

To enable the conjugation of the PMO to the protein, PMOs containing a primary amine at the 3′ end are functionalized with a maleimide moiety through a bifunctional linker containing an NHS-ester and a maleimide group separated by a cyclohexane spacer. After coupling maleimide-functionalized PMOs with LF, uncoupled PMO is removed via dialysis, producing LF-PMO conjugates that can be delivered to the cytosol via anthrax protective antigen (Fig. 1b).
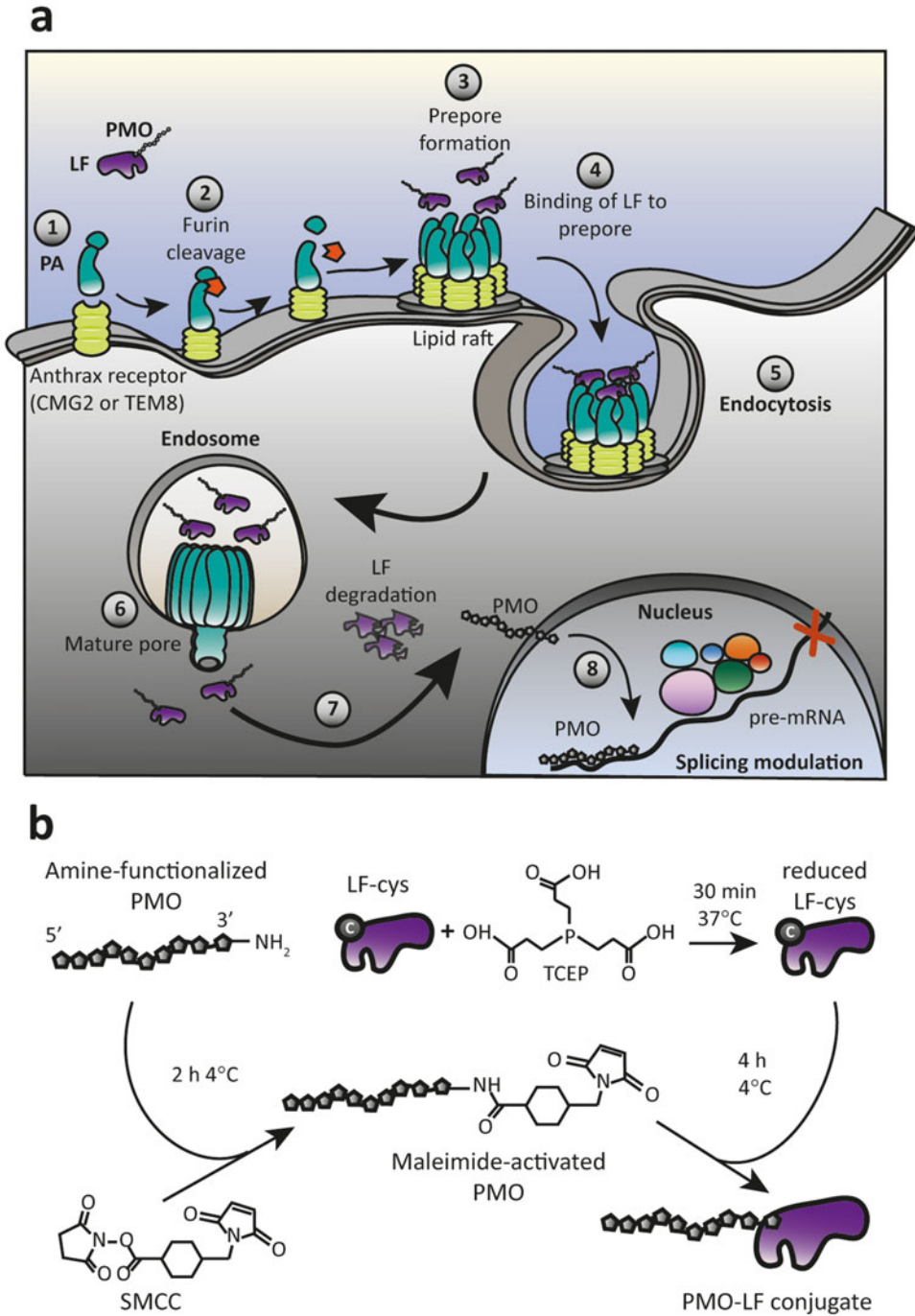
**Fig. 1** Cytosolic delivery of PMO using the anthrax toxin mechanism. (**a**) Schematic representation of the cytosolic delivery of the LF-PMO conjugate via anthrax protective antigen. Numbers indicate the distinct steps in the delivery process. (**b**) Schematic representation of the coupling of a PMO to LF-cys. *LF* lethal factor, *PA* protective antigen, *PMO* phosphorodiamidate morpholino oligomer, *SMCC* succinimidyl 4-(*N*-maleimido-methyl)cyclohexane-1-carboxylate, *TCEP* tris(2-carboxyethyl)phosphine

## 2    Materials

All buffers should be prepared with double-distilled water to ensure highly pure buffers. It is not necessary to work under sterile conditions. However, to perform experiments in the absence of antibiotics, make sure that the final conjugates are filter-sterilized before use.

### 2.1    Protein Expression

1. BL21(DE3) competent cells (see **Note 1**).

2. *E. coli* expression vector coding for anthrax protective antigen (see **Note 2**).

3. *E. coli* expression vector coding for LF (1–254) with a C-terminal cysteine (LF-cys) (see **Note 3**).

4. LB agar plates: add demi water to 10 g peptone, 5 g yeast, 8 g NaCl, and 15 g agar until a volume of 1 L is reached. Mix well, autoclave and store at room temperature. To make plates, heat the LB agar in the microwave until fully liquid, allow to cool until it is lukewarm before adding the antibiotic of choice. Pour liquid into a Petri dish until you reach a thickness of approximately 0.5 cm. Wait until the agar solidifies and store upside down at 4 °C.

5. 2× YT medium: For 1 L, add demi water to 16 g peptone, 10 g yeast, and 5 g NaCl until a volume of 1 L is reached. Mix well, autoclave, and store at room temperature.

6. Terrific Broth (TB): 12 g/L tryptone, 24 g/L yeast extract, and 4 mL/L glycerol, 17 mM $KH_2PO_4$ and 72 mM $K_2HPO_4$. Store at room temperature (see **Note 4**).

7. Autoclaved 20% (w/v) glucose solution in demi water.

8. Isopropyl β-D-1-thiogalactopyranoside (IPTG): 1 M stock solution in double-distilled water. Aliquot and store at −20 °C.

9. High-speed centrifuge.

10. Incubator shaker.

### 2.2    Protein Purification

1. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered saline (HBS)-wash: 50 mM HEPES, pH 8.0, 150 mM NaCl, 1 mM $MgCl_2$, 20 mM imidazole. Adjust the pH with NaOH to reach pH 8.0. Store at 4 °C. The buffer can be stored at 4 °C for up to 12 months.

2. HBS-resuspension buffer: HBS wash containing 250 μg/mL lysozyme and 1× complete EDTA-free protease inhibitor cocktail. Prepare fresh on the day of the experiment.

3. HBS-low salt: 50 mM HEPES, pH 8.0, 20 mM NaCl, 20 mM imidazole. Adjust the pH with NaOH to reach pH 8.0. Store at 4 °C. The buffer can be stored at 4 °C for up to 12 months.

4. HBS-high salt: 50 mM HEPES, pH 8.0 1 M NaCl, 20 mM imidazole. Adjust the pH with NaOH to reach pH 8.0. Store at 4 °C. The buffer can be stored at 4 °C for up to 12 months.

5. PBS-elution buffer: PBS, pH 7.4, 300 mM imidazole. Adjust the pH with HCl to reach pH 7.4. Store at 4 °C. The buffer can be stored at 4 °C for up to 12 months.

6. Ni-NTA superflow resin (e.g. Qiagen) equilibrated with HBS wash.

7. His-tagged tobacco etch virus (TEV) protease (commercially available).

8. PBS-EDTA-DTT: PBS containing 0.5 mM ethylenediamine-tetraacetic acid (EDTA) and 1 mM dithiothreitol (DTT). Prepare fresh for each experiment.

9. Sonicator.

10. Low-protein binding syringe filter for small volumes (<1 mL) with 0.22 µm pore size.

11. Äkta Pure or alternative chromatography system with UV detector.

12. Superdex 200 10/300 GL column or equivalent column.

13. 4 mL centrifugal filter with a 30 kDa cut-off.

**2.3 Protein-PMO Conjugation**

1. Tris-buffered saline (TBS): 20 mM Tris-HCl, pH 7.4, 150 mM NaCl. Adjust the pH with NaOH to reach pH 7.4 Store at 4 °C. The buffer can be stored at 4 °C for up to 12 months.

2. 7 kDa MWCO ZEBA spin columns (Thermo Fisher Scientific).

3. Degassed HBS: 50 mM HEPES, pH 7.2, 150 mM NaCl (*see* **Note 5**). Store at 4 °C. The buffer can be stored at 4 °C for up to 12 months. Degas again for 5–10 min before every use.

4. Phosphate-buffered saline (PBS): 10 mM phosphate, pH 7.2, 137 mM NaCl, 2.7 mM KCl. Store at room temperature. Adjust the pH with HCl to set the pH to 7.2 upon dilution from a 10× PBS stock solution prepared with 14.4 g/L $Na_2HPO_4$, 2.4 g/L $KH_2PO_4$, 80 g/L NaCl, and 2 g/L KCl, which gives a pH of ~6.8. The buffer can be stored at least for 1 year at room temperature.

5. 1 M lysine solution: For 10 mL, add double-distilled water to 1.46 g lysine until the volume reaches 10 mL. Store at −20 °C. The solution can be kept at −20 °C for at least 1 year.

6. 0.5 M tris(2-carboxyethyl)phosphine (TCEP) solution in double-distilled water, pH 7.0 (*see* **Note 6**) Store aliquots at −20 °C.

7. PMO with a 3′ primary amine modification (Gene tools).

8. Succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) linker (Thermo Fisher Scientific).

9. Dialysis membrane (e.g. 20 kDa MWCO).

# 3 Methods

### 3.1 Expression of PA and LF-cys

1. Mix 20 μL of BL21(DE3) competent *E. coli* bacteria with 0.5 μL of pure plasmid encoding either PA or LF-cys in a centrifuge tube and place on ice for 15–30 min.

2. Heat shock for 45 s in a water bath at 42 °C, place back on ice for 2 min and subsequently add 200 μL of LB medium. Place horizontally in an incubator shaker at 37 °C for 45 min and plate 10 μL out on an LB agar plate containing the appropriate type and concentration of antibiotic. After an overnight growth, confirm a proper density of colonies and place the plate upside down at 4 °C until starting the next step. It is recommended to start the following step on the same day.

3. Prepare a starter culture by picking a single colony and inoculating 50 mL of 2× YT medium containing 1% (w/v) glucose and antibiotic. Let the bacteria grow overnight at 37 °C with shaking at 150 rpm, orbit diameter 50 mm.

4. Use the starter culture to inoculate 1 L of TB supplemented with 0.8% (w/v) glucose and antibiotic to a starting $OD_{600}$ of 0.1.

5. Allow the bacteria to grow at 37 °C with shaking at 150 rpm, orbit diameter 50 mm, until an $OD_{600}$ of 0.7–0.8 is reached. At this point the expression can be induced by adding 100 μM IPTG. Decrease the temperature to 25 °C (*see* **Note 7**).

6. After 4 h of expression, centrifuge the cells for 10 min at $5000 \times g$ in 500 mL containers. Discard the supernatant and resuspend the pellets in 20 mL of medium. Transfer the resuspended cells to 50 mL conical tubes and centrifuge again for 10 min at $5000 \times g$. Discard the medium and snap-freeze the pellets in liquid nitrogen. Store at −80 °C until purification is commenced.

### 3.2 Purification of PA and LF-cys

PA and LF are susceptible to denaturation or aggregation when exposed to higher temperatures, so it is recommended to perform all of the subsequent steps at 4 °C.

1. Resuspend bacteria pellets from 1 L expression culture in 20 mL HBS-resuspension buffer.

2. Lyse the bacteria on ice by sonication (60 W with 15 pulses of 10 and 30 s pause between pulses in order to avoid excessive heat or formation of foam (*see* **Note 8**).
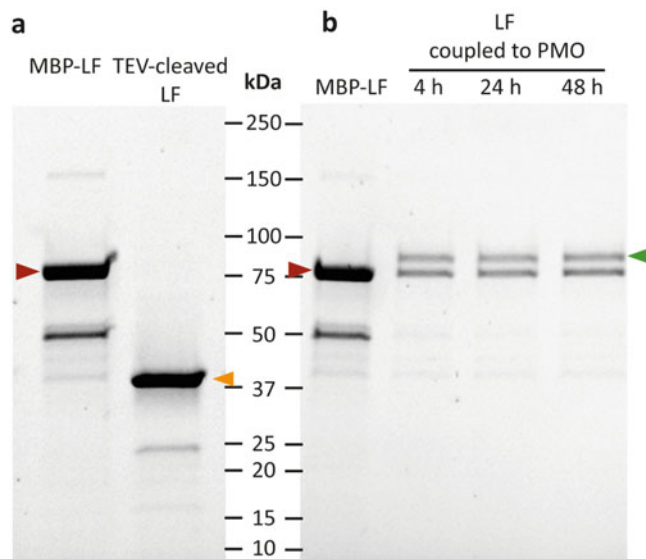
**Fig. 2** TEV cleavage of MBP-LF and time dependency of coupling efficiency of LF to PMO. (**a**) SDS-PAGE gel showing LF fused to MBP before TEV cleavage (red arrow) and LF after cleavage and purification via reverse IMAC (orange arrow). (**b**) SDS-PAGE gel illustrating the effect of incubation time on the conjugation efficiency of coupling LF to the PMO. Red arrow indicates 86 kDa band corresponding to MBP-LF before coupling. Green arrow shows a band with increased molecular weight at approximately 92 kDa corresponding to the coupled fraction (MW of PMO: 6.8 kDa). No marked differences are seen between the incubation time and the coupling efficiency, indicating that 4 h incubation is sufficient to achieve 50% coupling. Proteins on gels were visualized by stain-free imaging technology (Bio-Rad). *IMAC* immobilized metal ion affinity chromatography, *LF* lethal factor, *MBP* maltose-binding protein, *PMO* phosphorodiamidate morpholino oligomer, *TEV* tobacco etch virus

3. Centrifuge the bacterial lysates at $28{,}000 \times g$ for 40 min and filter the supernatant using 0.22 μm pore size filters.

4. Incubate the supernatants containing the His-tagged proteins with 1 mL of HBS wash-equilibrated Ni-NTA superflow resin for 1 h on a roller shaker.

5. Transfer the resin-containing solution to a column containing a bottom filter and wash with 20 column volumes (CV) of HBS-low salt, followed by 20 CV of HBS-high salt and 20 CV of HBS wash.

6. Pre-elute the proteins with 1 CV of PBS-elution buffer, followed by eluting with $4 \times 1$ CV. Each elution step should be for 10 min on a roller shaker (*see* **Note 9**).

7. Dialyze the eluted proteins overnight against 2 L 1× PBS. In the morning, exchange for fresh PBS and dialyze for two more hours.

8. The purified proteins His$_6$-MBP-LF-cys and His$_6$-MBP-PA can be cleaved with TEV protease to generate LF-cys and PA, respectively (Fig. 2a). To cleave off the His$_6$-MBP add a His-tagged TEV protease at a 1:20 (w/w) ratio in PBS-EDTA-DTT.

9. In order to separate the His$_6$-MBP and the His-tagged TEV from LF-cys or PA, a reverse immobilized metal ion affinity chromatography (IMAC) purification step must be done in which the *unbound* fraction is kept. Because the Ni-NTA resin is incompatible with high concentrations of EDTA and DTT, solutions must first be dialyzed against a 100-fold excess of HBS wash for at least 2 h to sufficiently remove the EDTA and DTT.

10. Incubate the dialyzed samples with 1 mL equilibrated Ni-NTA superflow resin for 1 h on a roller shaker.

11. Collect the unbound fraction, which should contain only LF-cys or PA. An SDS-PAGE gel can be run at this stage in order to confirm correct cleavage (Fig. 2a). To prepare LF-cys for reduction, perform a dialysis against HBS (*see* **Note 10**). Dialyze PA against PBS, after which it will be ready for further purification via size-exclusion chromatography (SEC) (Subheading 3.3).

12. LF needs to be reduced in order to generate the free thiol groups required for maleimide-thiol conjugation chemistry. To reduce LF-cys add a 100-fold molar excess of TCEP, incubate for 30 min at 37 °C, and remove excess TCEP using a 7 kDa MCWO ZEBA spin column. To calculate the amount of TCEP needed, determine the protein concentration using the absorption at 280 nm and the extinction coefficient ε280 of the protein (i.e. 91,680 M$^{-1}$ cm$^{-1}$ for MBP-containing LF and 23,840 M$^{-1}$ cm$^{-1}$ for cleaved LF).

**3.3 Purification of PA by Size-Exclusion Chromatography (SEC)**

After purification of PA via IMAC, we typically see some co-purification of degraded fragments, which can be removed via SEC (*see* **Note 11**). We generally purify our PA or PA fusion constructs via SEC using an Äkta pure chromatography system equipped with an S200 column (*see* **Note 12**), which yields pure and active proteins [11, 12].

1. Concentrate the sample to a volume of less than 500 μL, which is the maximal volume that can be loaded on an S200 column. Make sure that the protein solution is sufficiently concentrated so that in the maximally 500 μL several milligrams of proteins can be loaded (ideally 2–5 mg). For lower amounts, the separation of individual peaks can become problematic. Filtration of the protein using a low protein-binding syringe filter (0.22 μm pore size) before loading is recommended since protein

aggregates may clog the column. Alternatively, centrifugation of the sample at high speed (~20,000 × $g$) for 5 min and loading the supernatant can be done.

2. Connect the column to the chromatography system drop to drop (i.e. without introducing air) and equilibrate the column with PBS. Connect one or multiple injection loops (i.e. depending on the system) that can hold twice the volume of the sample to the chromatography system. For example, for a 500 μL sample, use a 1 mL injection loop. Wash the loop extensively with PBS before loading the sample.

3. When the sample has been loaded, start the run. A typical speed is 0.5 mL/min. Start fraction collection after the dead volume (~7–8 mL on an S200 column connected to an Äkta Pure system). A suitable fraction size is 0.5 mL. PA should elute at around 12 mL, which should give a major peak detectable by the UV detector.

4. Run an SDS-PAGE gel with collected fractions of any major peaks to determine the molecular weight and purity of the collected proteins. Full-length PA will have a size of approximately 83 kDa.

5. Combine all fractions that contain pure PA, concentrate with a 4 mL centrifugal filter (cut-off: 30 kDa) until a concentration of 2–5 mg/mL is obtained and freeze in aliquots. Aliquots should be snap-frozen in liquid nitrogen and kept at −80 °C. Thawed aliquots of PA can refrozen in liquid nitrogen at least three times without a noticeable decrease in activity.

### 3.4 Functionalizing PMO with a Maleimide Moiety

1. Dissolve PMO containing a primary amine in double-distilled water to a concentration of 1 mM. The PMO can be stored either at room temperature or aliquoted and stored at −80 °C for long-term storage (*see* **Note 13**).

2. Calculate the amount needed of the SMCC linker for the PMO coupling, taking into account that a 20-fold molar excess of the linker is needed for the reaction. Weigh the desired amount of the SMCC linker and dissolve in anhydrous DMF (e.g. 1.5 mg SMCC in 100 μL of DMF to make a stock solution of 45 mM). Freeze aliquots at −20 °C and only thaw briefly for use. The NHS moiety of the linker is very reactive and may hydrolyze already through the presence of trace amounts of water.

3. Mix the PMO with a 20-fold molar excess of the SMCC linker with minimal dilution (e.g. 900 μM PMO and 18 mM SMCC linker) and incubate for 2 h at 4 °C (*see* **Note 14**).

4. Quench unreacted linker by adding lysine to a final concentration of 100 mM using a 1 M lysine solution.

5. Separate the reacted PMO from the unreacted linker using a buffer-exchange procedure (e.g. 7 kDa ZEBA spin column, depending on the molecular weight of the PMO).

**3.5 Coupling of Anthrax Lethal Factor to Maleimide-Functionalized PMO**

1. Assuming full recovery of the PMO after the ZEBA spin column and equal volume, add a tenfold molar excess of the maleimide-functionalized PMO to the reduced LF-cys proteins (e.g. 800 μM PMO to 80 μM LF) and incubate for 4 h at 4 °C (Fig. 2b) (*see* **Note 15**).

2. Dialyze overnight to remove the unreacted PMO. Exchange dialysis buffer the next morning and dialyze for two more hours. Upon complete removal of the excess uncoupled PMO, the concentration of the PMO-conjugate can be calculated using the absorption coefficient of the PMO at 265 nm, while correcting for the absorption of the protein at 265 nm (*see* **Note 16**). The conjugates can be snap-frozen in liquid nitrogen and stored at −80 °C.

# 4 Notes

1. There are many *E. coli* strains suitable for high-level protein expression of the proteins described in this chapter, but we have good experience with the strain BL21(DE3). For the proteins in question, BL21(DE3) achieves high-level *and* soluble protein expression upon IPTG-mediated induction of T7 polymerase from a *lacUV5* promoter.

2. We typically express full-length PA fused to an N-terminal $His_6$-maltose-binding protein (MBP) sequence in a pQIq backbone [11]. MBP serves in this context as a solubility enhancer, leading to tens of mg of soluble protein produced in the cytosol of *E. coli* per liter expression culture in shake flasks. We include a TEV protease site between MBP and protective antigen that can be used to cleave off $His_6$-MBP and generate native PA.

3. Similar to the expression of PA, we express truncated LF (aa 1–254) fused to an N-terminal $His_6$-MBP sequence. For maleimide-thiol conjugations, we introduce a single cysteine at the most C-terminal position (LF-cys). Similar to PA, this construct gives us very high levels of soluble expression in the cytosol of *E. coli*.

4. 1 L Terrific broth is prepared by autoclaving 12 g tryptone, 24 g yeast extract, and 4 mL glycerol in 800 mL distilled water, followed by the addition of 100 mL of a separately autoclaved (or filter sterilized) solution of 0.17 M $KH_2PO_4$ and 0.72 M

$K_2HPO_4$. Adjust volume to 1 L. Mixing the separately autoclaved solutions can be done at the day of the experiment.

5. Degassing should be done at least for several minutes, which generally gives us good results. We flush with nitrogen gas at a speed which generates some, but not excessive, bubbling. Extending the degassing time to 1 h can be considered for optimal results.

6. Dissolving TCEP hydrochloride at 0.5 M in double-distilled water will result in an acidic solution with a pH between 2 and 3. Adjust the pH of the 0.5 M TCEP solution with concentrated NaOH or KOH to pH 7.0.

7. For expression of PA and LF constructs, we decrease the temperature to 25 °C in order to express maximal amounts of soluble proteins. The growth is much slower at 25 °C than at 37 °C, so it is normal that during 4 h of expression, bacteria will not grow much denser (final $OD_{600}$ between 2 and 3). Increasing the shaking speed improves aeration and can give better yields.

8. Even though the protocol describes lysing of cells by sonication, we typically obtain equally good results when we use French press bacterial cell lysis. With both methods, it is important to avoid excessive heat that may quickly denature or aggregate the proteins.

9. During the preelution, very little His-tagged protein will be eluted since the purpose is to equilibrate the resin with the elution buffer. Nevertheless, we prefer not to discard the preelution fractions since they still may contain *some* protein. Upon measuring the concentration, we make the decision to discard it and/or take it along for dialysis (our typical threshold is an A280 of 0.4).

10. Since the reduction in this protocol is done with TCEP, we prefer to use HBS over PBS because TCEP is more stable in HBS.

11. Next to SEC, PA can also be further purified via ion-exchange chromatography or both sequentially if a very high purity is needed.

12. For purification by SEC, we prefer to use a Superdex 200 10/300 GL column (GE Healthcare), which is suitable for separating proteins ranging in molecular weight from 10 to 600 kDa. We get a good separation when separating PA from its degraded fragments with this column. We have not compared with other comparable columns, which may perform equally well. Similarly, while we work with an Äkta pure system, alternative systems for SEC are likely to work equally well and the protocol described would only require small modifications.

13. PMOs can be dissolved in double-distilled water by pipetting up and down. The solution can be frozen at −80 °C or kept at room temperature. We have not seen any differences in coupling efficiency following freezing and rethawing.

14. The reaction can be performed at room temperature for 30 min or at 4 °C for 2 h. We get slightly better results when performing the latter protocol.

15. We observed an already high efficiency of coupling after 4 h (Fig. 2b), which did not noticeably increase during longer incubation times. It is possible that longer reaction times may still be better for some protein-PMO conjugations, in which case reaction times up to 48 h can be attempted. Higher excesses of PMO lead to greater efficiency but can be cost limiting. Furthermore, removal of much greater excesses of PMO using dialysis will take significantly longer. Additionally, more concentrated solutions of proteins (up to 5 mg/mL) and PMO are desired, taking into account the solubility of each.

16. PMOs absorb much more strongly than proteins at 265 and 280 nm, implying that absorption measurements at either wavelength will be dominated by the absorption of the PMO. For an accurate estimation of the protein-PMO conjugate, it is therefore important to completely remove the uncoupled PMO, which can be achieved either by extensive dialysis or by ion-exchange chromatography. By measuring the absorption of the conjugate at 265 nm and correcting for the absorption of the protein at 265 nm (for this, measure the pure protein and estimate the ε265 nm as follows: [(A265 nm/A280 nm)*ε280]), one can use the extinction coefficient of the PMO at 265 nm (provided by the manufacturer) to calculate the concentration of the protein-PMO conjugate. For a proper correction, take into account the labeling efficiency of the protein through analysis of conjugates by SDS-PAGE.

## Acknowledgments

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