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TITLE:

Cell-free Scaled Production and Adjuvant Addition to a Recombinant Major Outer Membrane Protein from *Chlamydia muridarum* for Vaccine Development

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KEYWORDS:

Cell-free production, Nanolipoprotein particle, Telodendrimer, Chlamydia, Major outer membrane protein, Vaccine

SUMMARY:

This protocol describes using commercial, cell-free protein expression kits to produce membrane proteins supported in nanodisc that can be used as antigens in subunit vaccines.

ABSTRACT:

Subunit vaccines offer advantages over more traditional inactivated or attenuated whole-cell-derived vaccines in safety, stability, and standard manufacturing. To achieve an effective protein-

based subunit vaccine, the protein antigen often needs to adopt a native-like conformation. This is particularly important for pathogen-surface antigens that are membrane-bound proteins. Cell-free methods have been successfully used to produce correctly folded functional membrane protein through the co-translation of nanolipoprotein particles (NLPs), commonly known as nanodiscs.

This strategy can be used to produce subunit vaccines consisting of membrane proteins in a lipid-bound environment. However, cell-free protein production is often limited to small scale (<1 mL). The amount of protein produced in small-scale production runs is usually sufficient for biochemical and biophysical studies. However, the cell-free process needs to be scaled up, optimized, and carefully tested to obtain enough protein for vaccine studies in animal models. Other processes involved in vaccine production, such as purification, adjuvant addition, and lyophilization, need to be optimized in parallel. This paper reports the development of a scaled-up protocol to express, purify, and formulate a membrane-bound protein subunit vaccine.

Scaled-up cell-free reactions require optimization of plasmid concentrations and ratios when using multiple plasmid expression vectors, lipid selection, and adjuvant addition for high-level production of formulated nanolipoprotein particles. The method is demonstrated here with the expression of a chlamydial major outer membrane protein (MOMP) but may be widely applied to other membrane protein antigens. Antigen effectiveness can be evaluated *in vivo* through immunization studies to measure antibody production, as demonstrated here.

INTRODUCTION:

Prokaryotic or eukaryotic lysates for cell-free expression of proteins are readily available as commercial products for synthesizing proteins of interest (for a complete review, see [1]). These expression systems are available at various scales and utilize lysates from various organisms, including *E. coli*, tobacco plants, and mammalian cultures. Cell-free lysates offer multiple benefits over traditional recombinant protein production approaches, including ease of use and robust, rapid protein production. While these approaches are primarily used to produce soluble proteins, this group has pioneered an approach for their use to express membrane proteins.

This novel approach makes minor modifications to existing cell-free expression systems by including DNA encoding two protein products for expression, an apolipoprotein and the membrane protein of interest. The expressed apolipoprotein (derivatives of either ApoA1 or ApoE4) interacts with lipids added to the cell-free lysate to spontaneously assemble (~20 nm) NLPs. When co-translated with a membrane protein of interest, the NLP and membrane protein form a soluble nanoparticle complex wherein the membrane protein is embedded within the NLP lipid bilayer. Thus, the membrane protein is more accessible for downstream applications, as it is contained within soluble, discrete particles. This approach can produce functional oligomeric protein complexes within the NLP bilayer[2] and can produce the antigen component of a subunit vaccine, which is subsequently mixed with lipophilic adjuvants to form a nanoparticle vaccine featuring co-localized antigen and adjuvant suitable for *in vivo* assessment.

This current method is modified from a previously published protocol[3]. Key modifications are

focused on the scale-up of the cell-free reaction and subsequent purification of the protein-NLP complex. A further modification includes the addition of an amphiphilic polymer known as a telodendrimer, which is first mixed with the lipids before addition to the cell-free reaction. Co-translation of the plasmids in the presence of the telodendrimer and the lipids produces a telodendrimer NLP (tNLP). The addition of the telodendrimer also helps modulate the size and monodispersity of the resulting tNLP nanoparticles[4]. This protocol is specifically optimized for for large-scale vaccine studies to produce a membrane-bound subunit antigen protein, chlamydial MOMP[5,6]. The method produces recombinant MOMP associated with tNLP to form a highly soluble MOMP-tNLP complex that retains MOMP oligomerization. A typical 3 mL scale-up production yields >1.5 mg of purified MOMP. The cell-free produced MOMP-tNLP is amenable to rapid adjuvant addition for *in vivo* immunogenicity testing.

PROTOCOL:

All animal studies were performed at the University of California, Irvine, in Public Health Service (PHS)-assured facilities in accordance with the guidelines set by the Institutional Animal Care and Use Committee.

1. Glassware preparation

NOTE: All materials used in producing vaccine-grade formulations for animals are endotoxin-free.

1.1. To destroy contaminating endotoxin, bake cleaned glassware that will hold buffers in an oven at 180 °C for 4 h.

2. Buffer preparation

2.1. Prepare 250 mL of the Ni affinity purification buffers listed in **Table 1**. Store them at 4 °C for up to 6 months.

3. Reaction preparation

3.1. Weigh out 20 mg of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) into an endotoxin-free, 1.5 mL centrifuge tube. Dissolve it in 1 mL of endotoxin-free water, probe-sonicate at least four times at 6 A for 1 min, with 1 min pauses in between, until clear. Remove any contaminant metal from the probe by centrifugation at 13,000 × *g* for 2 min at 22 °C and then transfer the solubilized lipid to a new 1.5 mL endotoxin-free tube.

3.2. Weigh out 1 mg of PEG5k-CA8 telodendrimer into a 1.5 mL endotoxin-free tube. Dissolve in endotoxin-free water to a concentration of 20 mg/mL. Vortex until fully dissolved and dilute to 2 mg/mL.

3.3. In a new endotoxin-free tube, combine 210 µL of 20 mg/mL DMPC solution with 210 µL of 2 mg/mL telodendrimer solution.

4. Cell-free production of MOMP-tNLPs for subunit vaccine formulations

4.1. Prepare MOMP-tNLPs using cell-free methods modified from a previously published protocol[5].

4.1.1. Two hours before setting up the cell-free reaction, open the prokaryotic cell-free protein expression kit and thaw one of the reconstitution buffers. Once thawed, add one tablet of EDTA-free protease inhibitor cocktail and let it dissolve fully.

4.2. Follow this protocol using a kit designed to run 5 x 1 mL reactions.

4.2.1. For each 1 mL reaction, add 525 µL of reconstitution buffer to the *E. coli* lysate bottle and gently roll to dissolve. Add 250 µL of reconstitution buffer to the bottle containing reaction additives (e.g., ATP, GTP) and gently roll to dissolve.

4.2.2. Add 8.1 mL of reconstitution buffer to the reaction feed bottle, recap with a rubber stopper (take care not to touch the inside of the rubber stopper), and invert/roll gently to dissolve.

4.2.3. Add 3 mL of reconstitution buffer to the amino acid mixture bottle, recap with a rubber stopper, and invert/roll gently to dissolve.

4.2.4. Add 1.8 mL of reconstitution buffer to the methionine bottle, roll gently to dissolve, and then store on ice until use.

4.3. Prepare the reaction solution.

4.3.1. To the *E. coli* lysate bottle, add 225 µL of reconstituted Reaction Mix, 270 µL of reconstituted amino acid mix without methionine, and 30 µL of reconstituted methionine. Additionally, add 400 µL of the DMPC/telodendrimer mixture, 15 µg of MOMP plasmid, and 0.6 µg of Δ49ApoA1 plasmid. Roll/gently shake to mix.

4.3.2. Take 20 µL of the total solution and set it aside in a 1.5 mL tube for the GFP-expressing control reaction (see below).

4.4. Prepare the feed solution. To the feed mix bottle, add 2.65 mL of reconstituted amino acid mix without methionine and 300 µL of reconstituted methionine. Roll/gently shake to dissolve.

4.5. Transfer 1 mL of the reaction solution to the inner reaction chamber provided in the cell-free reaction kit and seal when filled. Transfer 10 mL of the feed solution to the outer chamber of the reaction vessel and seal.

4.6. Add 0.5 µL of the GFP control plasmid (0.5 mg/mL) to the previously aliquoted 20 µL reaction mixture.

4.7. Place the reaction in a shaker at 300 rpm, 30 °C for up to 18 h. To verify that the reaction was successful, use a UV light source to check for fluorescence due to the synthesis of the control GFP (**Figure 1A**) after as little as 15 min of incubation.

5. MOMP-tNLP purification

5.1. Use immobilized nickel affinity chromatography to purify the MOMP-tNLP nanoparticle complex from the cell-free reaction mixture using the His-tag on the Δ49ApoA1 protein.

5.1.1. Transfer 1 mL of a 50% slurry of His-Tag Purification Resin to a disposable 10 mL chromatography column and equilibrate it with 3 mL of Binding buffer.

5.1.2. Let the buffer drain, cap the outlet, and add 250 µL of Binding buffer to the resin.

5.1.3. Before adding the cell-free reaction to the column, save 20 µL for later analysis by SDS-PAGE. Mix the cell-free reaction with the equilibrated resin and incubate it on a laboratory rocker at 4 °C for 1 h.

5.2. Uncap the column, wash the cap with 500 µL of additional Binding buffer, and add this liquid to the rest of the column.

5.3. Collect the liquid flow-through from the column for later analysis by SDS-PAGE.

5.4. Wash the column with 1 mL of Wash buffer containing 20 mM imidazole six times and collect fractions. Take care not to let the resin dry out between washes. On the second wash, vigorously agitate the resin by pipetting up and down using a 1 mL pipette.

5.5. Elute the MOMP-tNLPs in six 300 µL fractions of Elution buffer 1 (containing 250 mM imidazole), followed by one final elution with 300 µL of Elution buffer 2 (containing 500 mM imidazole). On the second elution, vigorously agitate the resin by pipetting up and down using a 1 mL pipette.

5.6. Image the gels using a gel imager at 600 nm (**Figure 2**). Use SDS-PAGE to quantify the amount of individual protein in the nanoparticle solution if there is a protein standard for comparison.

5.7. Generate a standard curve using the densities of the MOMP bands. Resolve the MOMP-tNLP samples on the same SDS-PAGE gel and calculate the MOMP component of the particles using the MOMP standard curve (**Figure 3**).

6. Western and dot blots and storage

6.1. For western blotting, resolve the samples by SDS-PAGE and transfer the gels using a commercial dry blotting system with standard settings according to the manufacturer's protocol.

6.1.1. Remove the blots from the stack after the transfer is complete, and incubate each blot overnight at 4 °C in a suitable blocking buffer containing 0.2% Tween 20 and either 0.5 mg/mL MAb40 or 0.2 mg/mL MAbHIS anti-His-tag antibody directed against the His-tag from Δ49ApoA1 protein.

6.1.2. Wash each blot 3 times for 5 min with PBS-T (1x PBS, 0.2% Tween 20, pH 7.4).

6.1.3. Incubate the blots for 1 h in blocking buffer containing secondary antibody conjugated to a fluorophore (e.g., IRDye) at a 1:10,000 dilution.

6.1.4. Rewash the blots 3 times for 5 min with PBS-T. Use a fluorescence imager to image the blots after the final wash.

6.2. For dot blots, blot 3 µg of purified MOMP-tNLP and empty tNLP using a dot blot apparatus. Block and develop the blots using the same methods described above for western blotting.

6.3. Freeze the mixed solution on dry ice and lyophilize it overnight using a lyophilizer. Store the dried formulations at -20 °C until needed.

6.4. Reconstitute lyophilized tNLPs using endotoxin-free water. Gently roll until the lyophilized cake is fully dissolved and rehydrated. To remove trehalose, dialyze the solution against PBS using a 3.5 kDa cutoff dialysis membrane.

7. Adjuvant addition

NOTE: These and other similar NLP-based sub-unit vaccine formulations can readily incorporate lipophilic adjuvants such as CpG-ODN1826 and FSL-1. CpG-ODN1826 is a modified Class B CpG oligonucleotide (5'-tccatgacgttcctgacgtt-3') with a full phosphorothioate backbone featuring a 5' cholesterol moiety (5'-chol-C6). The conjugation of CpG-ODN1826 to tNLPs is mediated by the hydrophobic interactions between the cholesterol moiety and the phospholipid bilayer of the tNLP and has been demonstrated and well-characterized, as previously reported[9,10].

7.1. Prior to incorporation into these formulations, purify the cholesterol-modified CpG by reversed-phase chromatography to remove contaminating endotoxin as well as any unmodified CpG molecules.

7.1.1. Upon receipt from the vendor, rehydrate the lyophilized CpG material in endotoxin-free water and purify it on a preparative C4 RP-HPLC column using a separation gradient consisting of 10 mM triethylammonium acetate (TEAA) (mobile phase A) and acetonitrile (mobile phase B).

NOTE: Additional details are available in **Table 2**.

7.1.2. Pool and lyophilize the fractions containing cholesterol-modified CpG. To ensure complete

removal of residual TEAA, reconstitute the CpG with 15 mL of endotoxin-free water and re-lyophilize it three times.

7.1.3. After the final lyophilization, reconstitute CpG in endotoxin-free water (>20 mg/mL final CpG concentration), aliquot, and store it at -80 °C until needed. For addition to formulations, dilute the CpG to a concentration of 1–2.5 mg/mL.

NOTE: FSL-1 is available as a vaccine-grade, lyophilized powder. This is reconstituted using sterile and endotoxin-free water at a concentration of 1 mg/mL. The vaccine is administered intramuscularly (i.m.), with each dose containing 10 µg of MOMP in a total volume of 50 µL.

7.2. To achieve the desired formulation dose, dialyze the nanoparticles into PBS and concentrate them using a centrifugal vacuum concentrator before adjuvant addition. Take care when doing this to prevent complete drying of the sample—check the sample volume every 20–30 min during centrifugation.

7.3. Add the adjuvant under sterile conditions in a biosafety cabinet. To assess successful incorporation, analyze the final formulations and their components by analytical size-exclusion chromatography (SEC).

NOTE: For these preparations, an SEC column was used in PBS buffer (0.5 mL/m in flow rate), and elution was detected using a UV-vis diode array detector. Incorporation was assessed by comparing the absorption of the adjuvanted particles to that of the unadjuvanted particles at 214 and 280 nm.

7.4. Store the adjuvanted MOMP-tNLP and empty tNLP at 4 °C prior to animal use for a period of up to 14 days. To fully assess the stability of a new tNLP formulation, periodically analyze the stored tNLPs by SEC.

NOTE: Stability will vary from formulation to formulation.

8. Serum testing

8.1. Obtain female 3-week-old mice (BALB/c, n = 6).

8.2. Vaccinate the mice intramuscularly (i.m.) in each hindlimb with 10 µg of MOMP in the form of MOMP-tNLP adjuvanted with 5 µg of CpG and 1 µg FSL-1 (total volume per injection = 50 µL).

8.3. Four weeks after the initial vaccination (prime), vaccinate the animals a second time (boost) with 10 µg of MOMP in the form of MOMP-tNLP adjuvanted with 5 µg of CpG and 1 µg FSL-1 (total volume per injection = 50 µL).

8.4. On day 56 after the initial vaccination, collect blood to assess antibody titers. Begin by anesthetizing the mice by injecting i.p. a solution of xylazine (0.3 mg/20 g body weight) and

ketamine (3.0 mg/20 g body weight). Pinch the front and hind legs to make sure no jerking occurs. Apply petroleum jelly around the eyes to prevent eye dryness during anesthesia.

8.5. Using a micro-hematocrit capillary tube, puncture the retro-orbital plexus. Collect 100 μL of blood in a microcentrifuge tube.

8.6. After blood collection, observe the mice until they recover from anesthesia and can maintain sternal recumbency.

8.7. Let the blood clot at room temperature for 30 min and then spin down at 2,000 × *g* for 10 min. Collect the serum and freeze at -80 °C.

8.8. At this time, challenge the animals with *Chlamydia muridarum* or euthanize them. Euthanize the mice by first injecting i.p. a solution of xylazine (0.3 mg/20 g body weight) and ketamine (3.0 mg/20 g body weight) followed by cervical dislocation.

8.9. Test serum antibodies specific for MOMP using western blotting techniques as described above. Pool mouse sera from all immunized mice and use the pooled serum in place of a primary antibody at 1:5,000 dilution.

REPRESENTATIVE RESULTS:

The SDS-PAGE profile of the Ni affinity purification of MOMP-tNLP from a 1 mL cell-free reaction is shown in **Figure 1B**. The reaction resulted in high levels of expression for both the MOMP and the Δ49ApoA1 protein. Previous results showed that the cell-free expression of Δ49ApoA1 in the presence of DMPC and telodendrimer resulted in the formation of telodendrimer nanolipoprotein particles (tNLPs)[4]. The co-elution of MOMP with Δ49ApoA1 indicated that MOMP is associated with tNLPs, as the His-tag is only present on the tNLP scaffold Δ49ApoA1 and not on MOMP. MOMP is a highly insoluble protein that can only be eluted through complexing with tNLPs, which have been shown to facilitate the solubilization of membrane proteins.

The elution fractions containing MOMP-tNLPs were pooled and the total protein concentration determined using a fluorescence-based quantitation device, or a device that measures concentration through absorbance at 280 nm, following the manufacturer's instructions for protein quantitation. To allow for precise dosing of the MOMP vaccine, it is also important to determine the concentration of MOMP in the purified complexes. We developed a method to quantify MOMP based on gel densitometry (**Figure 2**) wherein a purified recombinant MOMP with known concentration was used as the standard. By establishing the standard curve and comparing it to the MOMP-tNLP sample, the MOMP concentration can be quantified accurately. The determination of MOMP concentration in the purified sample enabled the estimation of the yield of MOMP in cell-free reactions at various scales, which is important for planning the reaction setup appropriate to downstream studies (**Table 3**).

MOMP needs to form oligomers to elicit a robust immune response[11]. To test the oligomeric state

of MOMP, MOMP-tNLP was analyzed in the presence and absence of both heat and the reducing agent dithiothreitol (DTT, 50 mM, **Figure 3A**). Higher-order oligomers of MOMP were identified through SDS-PAGE when samples were not treated with heat and DTT. In comparison, samples treated with heat in the presence of DTT showed primarily two distinct bands on the gel, corresponding to MOMP and Δ49ApoA1 (approximately 40 kDa and 22 kDa, respectively). These results closely resemble the gel banding pattern attributed to oligomer formation of MOMP, which is critical to its effectiveness.

Further western blot analysis using MAb40, an antibody against the linear epitope on the variable domain of MOMP protein, showed a similar banding pattern, confirming the oligomer formation by MOMP protein in its non-denatured state (**Figure 3B**). An important factor impacting MOMP oligomer formation is the ratio between the MOMP plasmid and the Δ49ApoA1 plasmid during the cell-free reaction setup. **Table 4** lists the ratio of plasmids and the resulting insertion rate of MOMP into tNLPs. Previous studies indicated that chlamydial MOMP and other outer membrane proteins may exist primarily as trimers[12]. To maximize the trimer formation in the cell-free reaction, it is desirable to have the insertion rate close to three MOMP proteins per NLP, which corresponds to a ~25:1 MOMP-to-Δ49ApoA1 plasmid ratio.

A dot blot assay was used as a more streamlined method to detect the presence of MOMP and tNLP. The MAb40 antibody was used to detect total MOMP. The MAbHIS antibody targeted to the His-tag on the Δ49ApoA1 scaffold of the tNLP was used to assess the presence of tNLP. The co-signaling of MAb40 and MAbHIS antibodies indicated MOMP-tNLP formation. The control reaction produced empty tNLP, which only showed a positive signal from MAbHIS (**Figure 3C**). To test the immunogenicity of MOMP-tNLPs produced in the cell-free reaction, we adjuvanted MOMP-tNLP with CpG + FSL-1 and injected intramuscularly (i.m.) into mice in a prime-boost regimen as described above. Sera were collected from the immunized mice, and MOMP-specific IgG antibody was measured using a western blot assay (**Figure 4**). The sera from mice injected with adjuvanted MOMP-tNLP showed strong MOMP binding, indicating that MOMP-tNLP could elicit an immune response *in vivo*.

FIGURE AND TABLE LEGENDS:

Figure 1: Expression and purification of MOMP-tNLP. (**A**) Image of tubes containing small aliquots of a cell-free reaction that successfully expressed GFP controls luminating under UV light source (right) compared to lysates without GFP plasmid (left). (**B**) Protein Gel stained with SYPRO Ruby after SDS-PAGE shows the purification profile of MOMP-tNLP. MOMP migrates at 40 kDa and the Δ49ApoA1 migrates at 22 kDa. Abbreviations: MOMP = chlamydial major outer membrane protein; tNLP = telodendrimer nanolipoprotein particle; MOMP-tNLP = MOMP-tNLP complex; GFP = green fluorescent protein-encoding plasmid; MW = Molecular weight marker; T = total cell-free lysate; FT = flow-through; R1–R3 = cell-free reaction aliquots; W1, W6 = Washes 1 and 6; E1–E7 = Elutions 1 through 7; Δ49ApoA1 = His-tagged mouse ApoA1 derivative.

Figure 2: Quantification of MOMP in MOMP-tNLP samples. (**A**) SDS-PAGE gel stained with SYPRO Ruby for the quantification of MOMP. Recombinant MOMP with known concentration was loaded onto the gel to obtain the standard curve. Each lane contained 0.1 µg, 0.2 µg, 0.5 µg, 1

µg, and 2 µg of MOMP. MOMP-tNLP samples that were being quantified were loaded on the same gel. (**B**) The MOMP concentration standard curve was generated using densitometry. An equation relating normalized band density and the amount of MOMP was established. The equation was used to calculate the MOMP content in the unknown samples. Abbreviations: MOMP = chlamydial major outer membrane protein; tNLP = telodendrimer nanolipoprotein particle; MOMP-tNLP = MOMP-tNLP complex; SDS-PAGE = sodium dodecylsulfate polyacrylamide gel electrophoresis.

Figure 3: Cell-free-produced MOMP-tNLP allow MOMP to form higher-order structures. (**A**) SDS-PAGE gel of MOMP-tNLP with and without treatment of heat and reducing agent DTT, stained with SYPRO Ruby. With heat and DTT, MOMP primarily appeared as a monomer band at ~40 kDa, as heat and the reducing agent broke down the majority of higher-order MOMP structure. In the absence of heat and DTT, the higher-order bands were present, indicating MOMP oligomer conformation. (**B**) Western blot of MOMP-tNLP and MOMP alone, untreated and treated with heat and DTT. After transfer, the membrane was probed with MAb40 (1:1,000 dilution). A banding pattern similar to the SYPRO Ruby-stained gel was observed, confirming that the higher molecular weight bands were indeed MOMP oligomers. (**C**) Dot blot of MOMP-tNLP and empty tNLP samples (in duplicate) probed with MAb40 and MAbHIS. Abbreviations: MOMP = chlamydial major outer membrane protein; tNLP = telodendrimer nanolipoprotein particle; MOMP-tNLP = MOMP-tNLP complex; SDS-PAGE = sodium dodecylsulfate polyacrylamide gel electrophoresis; DTT = dithiothreitol.

Figure 4: Cell-free-produced MOMP-tNLP is highly immunogenic. Serum from immunized mice showed strong anti-MOMP IgG signal. MOMP-tNLP adjuvanted with CpG + FSL-1 was used to immunize mice. Sera from six immunized mice were collected, pooled, and used to probe MOMP-tNLP. The serum was able to bind to MOMP in a western blotting assay and showed strong IgG signal (left). The western blot using MAb40 as primary antibody (right) showed similar bands, indicating that the serum contained MOMP-specific IgG. Abbreviations: MOMP = chlamydial major outer membrane protein; tNLP = telodendrimer nanolipoprotein particle; MOMP-tNLP = MOMP-tNLP complex; CpG = cholesterol-modified CpG adjuvant; FSL-1 = lipophilic adjuvant.

Table 1: List of buffers needed for nickel affinity purification detailing concentrations of each component and pH.

Table 2: Conditions for reversed phase HPLC purification of cholesterol-modified CpG. Abbreviations: TEAA = triethylammonium acetate; MeCN = acetonitrile.

Table 3: The quantity of lipids, telodendrimer, and plasmids used for differently scaled cell-free reactions and the corresponding yields. Abbreviations: MOMP = chlamydial major outer membrane protein; DMPC = 1,2-dimyristoyl-sn-glycero-3-phosphocholine.

Table 4: The plasmid ratios in a cell-free reaction and the resulting MOMP insertion rates. Abbreviations: MOMP = chlamydial major outer membrane protein; tNLP = telodendrimer nanolipoprotein particle; Δ49ApoA1 = His-tagged mouse ApoA1 derivative.

DISCUSSION:

Chlamydia is the most common sexually transmitted infection that affects both men and women. Although vaccine research on Chlamydia spans decades, a safe and effective vaccine that can be scaled to mass production has remained elusive[13]. The chlamydial MOMP is considered the lead candidate as a protective vaccine antigen; however, MOMP is highly hydrophobic and prone to incorrect folding[14,15]. Further study has revealed that MOMP exists in oligomeric states that are essential for its immunogenicity[11]. Detailed here is a validated, cell-free co-expression method that produces oligomeric MOMP formed within tNLP nanoparticle as a vaccine, with yields of approximately 1.5 mg of purified MOMP per 3 mL of lysate. This fully collated procedure can be further scaled for industrial production, increasing its prospects as a useful approach for generating vaccines.

We have previously published on using cell-free expression to produce membrane proteins embedded within NLPs[3,16], as well as expression into telodendrimer-stabilized discs. However, this latter technique produced membrane-protein particles with greater heterogeneity and lower solubility.[4] Additionally, the immunogenicity of MOMP-telodendrimer particles is unclear compared to MOMP-tNLP particles[17].

This procedure can be adapted to scale up the expression of bacterial membrane proteins that are promising candidates as antigens for use in subunit vaccines. Not only does this procedure produce solubilized bacterial membrane protein, but the overall nanoparticle structure is amenable to further modification using a variety of lipophilic vaccine adjuvants including, but not limited to, CpG conjugated to a cholesterol moiety or FSL-1. Expression of other candidate antigens from bacteria is feasible, although parameters such as expression temperature, lipid choice, and type of expression system may need to be explored to achieve optimal yields.

Additionally, plasmid choice and ratio are critical in this process. Both plasmids used should be constructed from the same backbone. If the inserts are approximately the same length, the ratios can be based on the mass of the plasmid added, as described here. However, ratioing based on moles will give more reproducible results, particularly when scaling the reactions. Ratios that work well in screen-scale reactions (< 0.5 mL) may not be applicable to larger reactions and may require additional optimization. Non-membrane proteins can still be expressed using cell-free kits but may not require the lipid nanoparticle (co-expression) to produce a soluble product. Additionally, while this protocol describes adjuvanting with CpG and FSL-1, this system is amenable to formulation with other lipophilic adjuvants or admixing with soluble adjuvants as desired.

It is essential to avoid contamination when setting up the cell-free expression reaction as this can affect yields. Any additives to the reaction, including the plasmids themselves, should be highly pure. Additionally, the expressed proteins should only be in contact with materials and solutions that are free of endotoxin contamination. Endotoxin contamination in candidate formulations can lead to inconsistent and spurious results of immunological assays and can be harmful in sufficient quantities. While not described here, additional purification following nickel affinity

chromatography may be necessary if many contaminants are observed in subsequent analysis steps such as through SDS-PAGE. This could be accomplished with SEC, although conditions may require optimization on a formulation by formulation basis.

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DISCLOSURES:

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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3 Cappuccio, J. A. et al. Cell-free co-expression of functional membrane proteins and apolipoprotein, forming soluble nanolipoprotein particles. *Molecular & Cellular Proteomics: MCP.* **7** (11), 2246–2253 (2008).

4 He, W. et al. Controlling the diameter, monodispersity, and solubility of ApoA1 nanolipoprotein particles using telodendrimer chemistry. *Protein Science.* **22** (8), 1078–086 (2013).

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14 Findlay, H. E., McClafferty, H., Ashley, R. H. Surface expression, single-channel analysis and membrane topology of recombinant *Chlamydia trachomatis* Major Outer Membrane Protein. *BMC Microbiology.* **5**, 5 (2005).
15 Sun, G., Pal, S., Weiland, J., Peterson, E. M., de la Maza, L. M. Protection against an intranasal challenge by vaccines formulated with native and recombinant preparations of the *Chlamydia trachomatis* major outer membrane protein. *Vaccine.* **27** (36), 5020–5025 (2009).
16 He, W. et al. Cell-free expression of functional receptor tyrosine kinases. *Scientific Reports.* **5** (1), 12896 (2015).
17 Tifrea, D. F. et al. Induction of protection in mice against a *Chlamydia muridarum* respiratory challenge by a vaccine formulated with the major outer membrane protein in nanolipoprotein particles. *Vaccines.* **9** (7), 755 (2021).

Figure 1

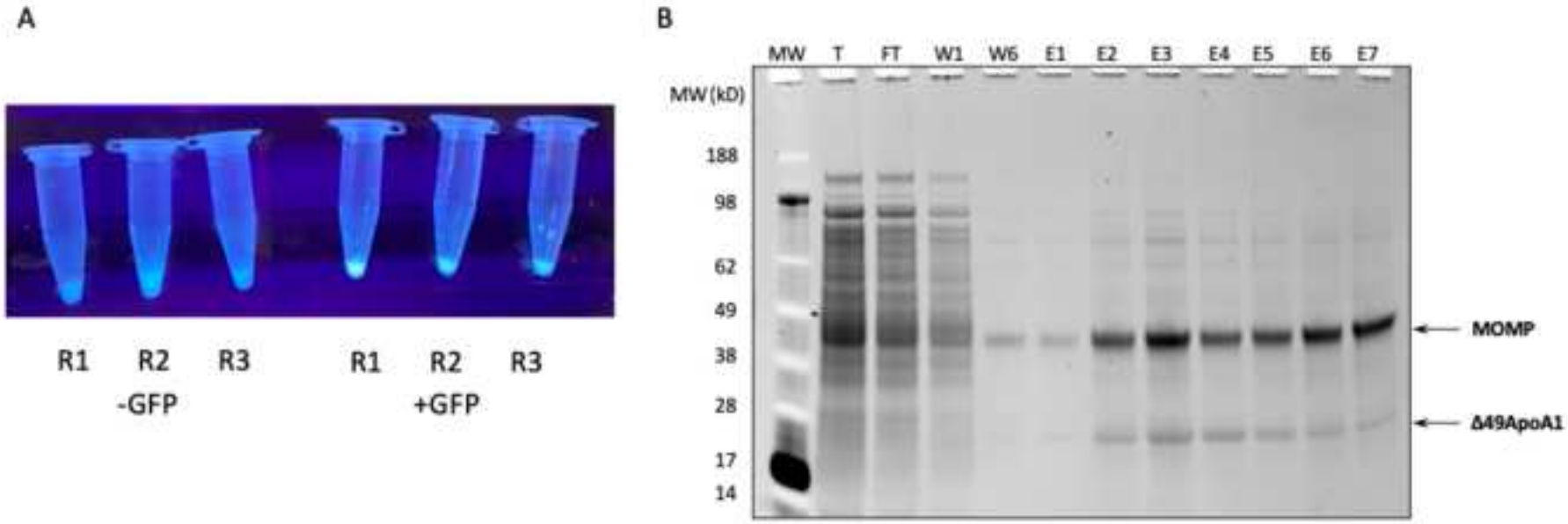


Figure 2

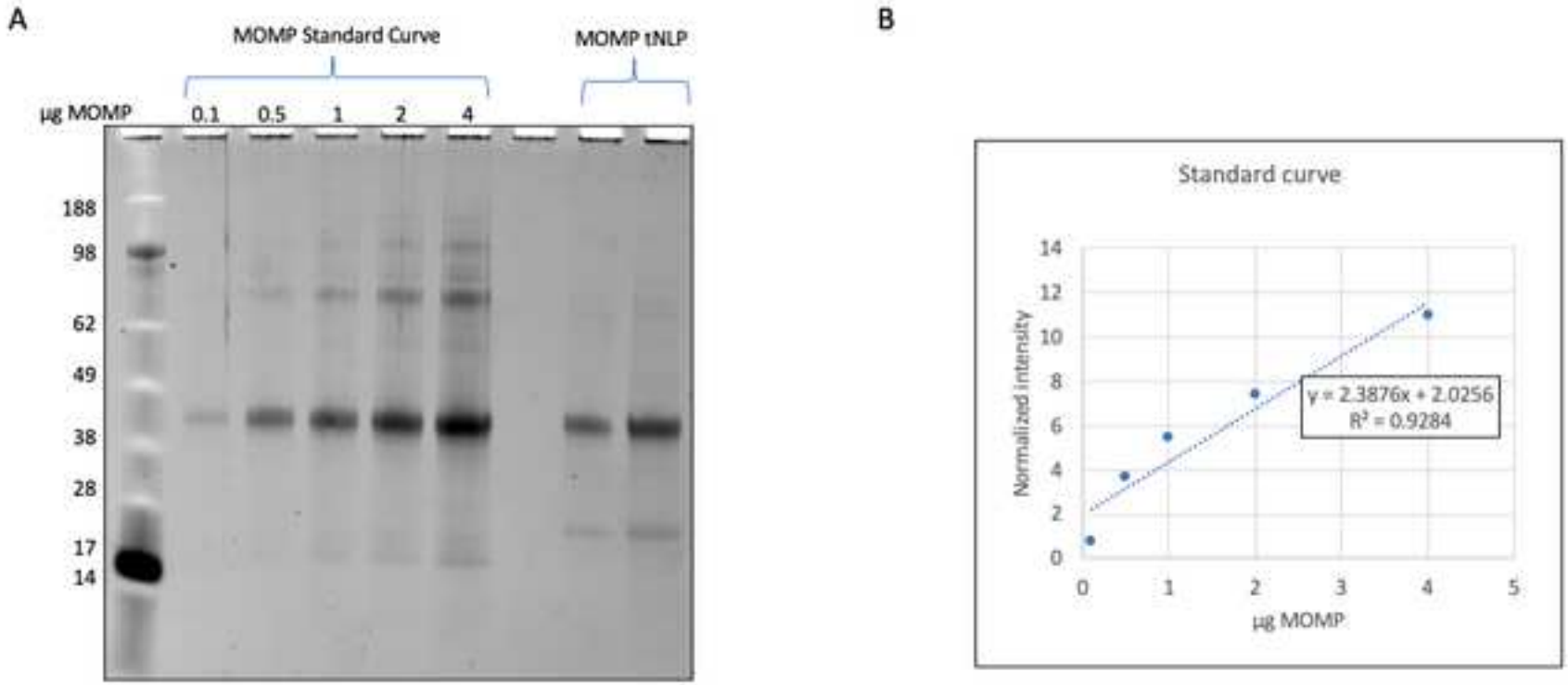


Figure 3

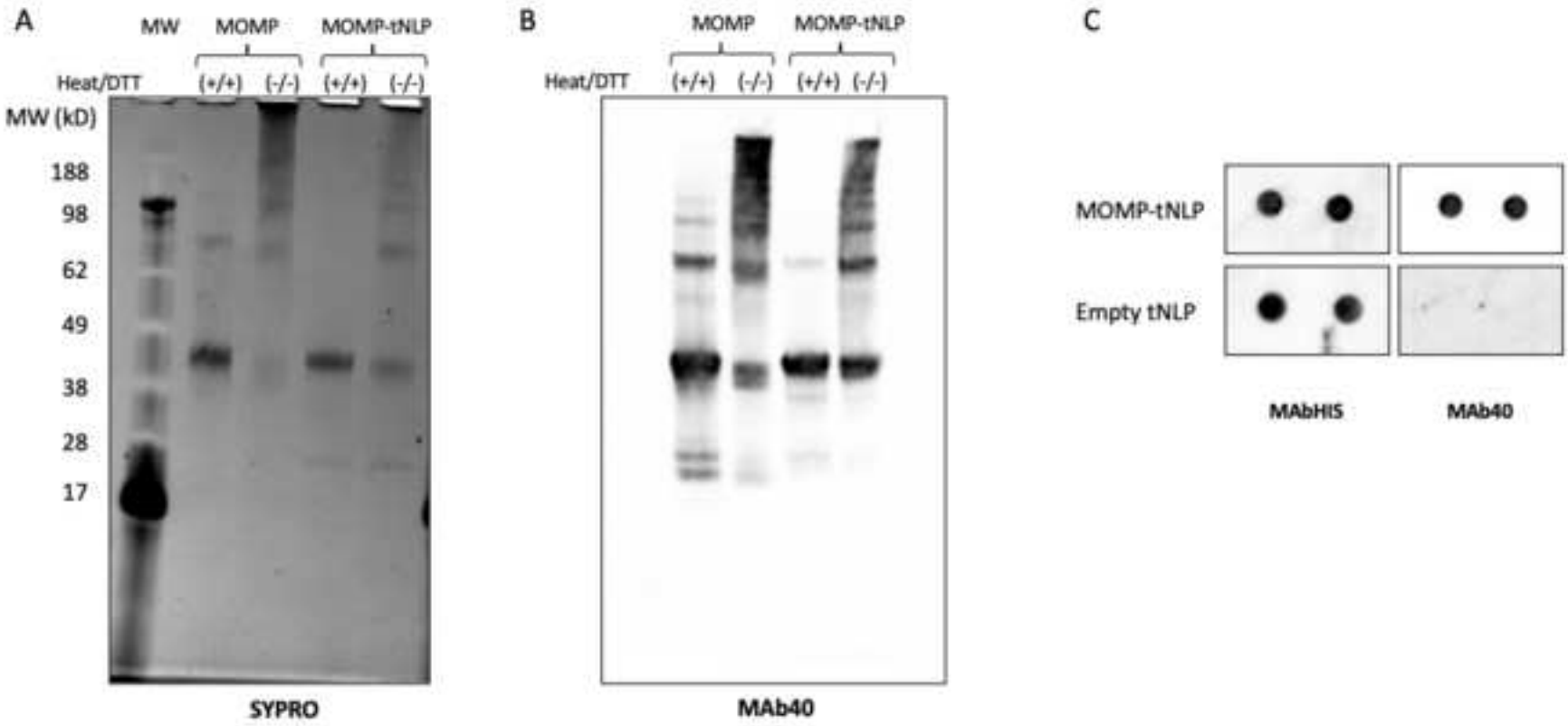
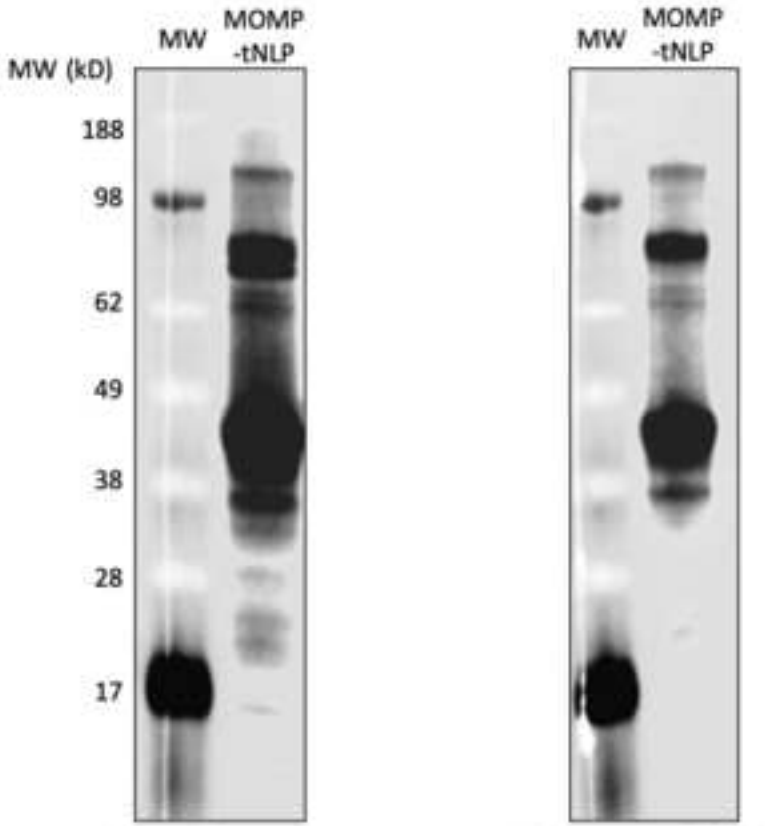


Figure 4



Primary Ab = serum from mice immunized with MOMP-tNLP, 1:5,000.

Primary Ab = MAb40, 1:1,000.

Table 1

Buffer name	NaH_2PO_4	NaCl	Imidazole	pH
Binding Buffer	50 mM	300 mM	10 mM	8.0
Wash Buffer	50 mM	300 mM	20 mM	8.0
Elution Buffer 1	50 mM	300 mM	250 mM	8.0
Elution Buffer 2	50 mM	300 mM	500 mM	8.0

Table 2

Runtime	50 min
Flow rate	6.0 mL/min
Gradient type	Binary
Buffer A	10 mM TEAA in H_20
Buffer B	MeCN

Gradient	**% Buffer B**
0 min	25%
30 min	60%
30.5 min	100%
40 min	100%
40.5 min	25%
50 min	25%

Table 3

Cell-free lysate (mL)	DMPC lipid (mg)	Telodendrimer (mg)	MOMP plasmid (µg)	Purified MOMP yield (mg)
1	4	0.4	15	0.5
2	8	0.8	30	1.1
3	12	1.2	45	1.6
5	20	2	75	2.7

Table 4

Ratios of plasmid input, MOMP : Δ49ApoA1	**1:1**	**5:1**
Ratios of the amount of protein produced, MOMP : Δ49ApoA1	0.02	0.32
Estimated number of MOMP insertion per tNLP	0.03	0.37

10:1	25:1	50:1	100:1
0.64	3.46	6.55	20.04
0.75	4.04	7.65	23.39

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Table of Materials

Materials table (2) (1).xlsx

Lawrence Livermore National Laboratory

Amit Krishnan, Ph.D.
Review Editor,
Journal of Visualized Experiments
Manuscript ID: JoVE63028
TITLE: Cell-free scaled production and adjuvant addition to a recombinant major outer membrane protein from Chlamydia muridarum for vaccine development

Dear Dr. Krishnan,
Please consider our revised manuscript titled “Cell-free scaled production and adjuvant addition to a recombinant major outer membrane protein from *Chlamydia muridarum* for vaccine development” for publication in the Journal of Visualized Experiments. We thank the reviewers for their thorough assessment and helpful comments, and we appreciate the opportunity to revise our manuscript in oreder to address concerns raised by the reviewers. Below please find our responses to the reviewers’ comments (which have been bolded for clarity). We have also made other minor modifications to the text to enhance the clarity of the overall message.
Kindest Regards,

Matthew Coleman, PhD
Senior Scientist
Physical and Life Sciences Directorate
Lawrence Livermore National Laboratory
7000 East Ave., Livermore, CA 94551
office: 925.423.7687
email: coleman16@llnl.gov

Editor's comments:

1. **Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.**
   All authors have reviewed the manuscript and we have corrected any errors found.

2. **Please provide an institutional email address for each author.**
   Done.

3. **Please reword the lines to avoid previously published works:191-193, 194-195, 197-199, 330-333. Please refer to the iThenticate report attached.**
   We have reviewed the report and made an effort to avoid overlap of text with previous works.

4. **Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).**
   We have reduced the use of pronouns in the manuscript.

5. **JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.**
   Use of commercial language has been removed.

6. **Line 45-53: Please include the lines as paragraph.**
   Corrected.

7. **Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution (Please move the lines 293-296)**
   We have moved the ethics statement to the correct position.

8. **Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.**
   The protocol section has been edited to use the imperative tense when possible.

9. **Line 95-96: The Protocol should contain only action items that direct the reader to do something. Consider revising the lines to direct the reader to do something.**

We have changed this step to direct the reader:

"1.1. All material used in producing vaccine-grade formulations for animals are endotoxin-free. To prepare glassware for this process, bake cleaned glassware in an oven at 180ºC for four hours."

10. **For SI units, please use standard abbreviations when the unit is preceded by a numeral throughout the protocol. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL, 7 cm2**
    Corrected.

11. **For time units, please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral throughout the protocol. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks**
    Corrected.

12. **Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.**

13. **Line 297-298: Please specify the volume injected.**
    We have added this information:

    "Total volume per injection is 50 µL.**"**

14. **Line 299-300; How much was the booster dose.**
    We have added this information:

    "11.2. Four weeks after the initial vaccination (prime), animals are vaccinated a second time (boost) with 10 µg of MOMP in the form of MOMP-tNLP adjuvanted with 5 µg of CpG and 1 µg FSL-1. Total volume per injection is 50 µL."

15. **Line 301: Please specify the volume of blood drawn. What was used to draw blood.**
    We have added this information:

"11.6. On day 56 after the initial vaccination, collect blood to assess antibody titers. Using a micro-hematocrit capillary tube, puncture the retro-orbital plexus. Collect 100 μL blood in a microcentrifuge tube."

16. **Being a video based journal, JoVE authors must be very specific when it comes to the humane treatment of animals. Regarding animal treatment in the protocol, please add the following information to the text:**
    a) **Please include an ethics statement before all of the numbered protocol steps indicating that the protocol follows the animal care guidelines of your institution.**
    b) **Please specify the euthanasia method, if any.**
    c) **Please mention how animals are anesthetized and how proper anesthetization is confirmed.**
    d) **Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia.**
    e) **For survival strategies, discuss post-surgical treatment of animal, including recovery conditions and treatment for post-surgical pain.**
    f) **Discuss maintenance of sterile conditions during survival surgery.**
    g) **Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency.**
    h) **Please specify that the animal that has undergone surgery is not returned to the company of other animals until fully recovered.**
    i) **Please do not highlight any steps describing euthanasia.**

    We have added in the pertinent details to section 11 of the protocol.

17. **Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.**
    We have indicated the essential sections.

18. **Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.**
    Corrected.

19. **As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:**
    a) **Critical steps within the protocol**
    b) **Any modifications and troubleshooting of the technique**
    c) **Any limitations of the technique**
    d) **The significance with respect to existing methods**
    e) **Any future applications of the technique**

We have revised the discussion section to address these points.

20. **Please do not use the &-sign or the word "and" when listing authors in the references. Authors should be listed as last name author 1, initials author 1, last name author 2, initials author 2, etc. Title case and italicize journal titles and book titles. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.**
Corrected.

21. **Use Uppercase letters to label the figures in a multipaneled image (replace "a, b, c" by "A, B,C")**
Corrected.

22. **Figure 1: Please label the figure to make it more informative**
Corrected.

23. **Graphical abstract: Please add the figure as a schematic (e.g., Figure 1) and include a title and description in the Figure Legends.**
We have added a description in the legends.

24. **Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.**
Done.

<u>Reviewer #1:</u>

1. What is the novelty of the current work compared to the previous ones? For example, a similar work has already been published in journal of biochemical chemistry, 2017, J Biol Chem. 2017 Sep 8;292(36):15121-15132. doi: 10.1074/jbc.M117.784561, Cell-free production of a functional oligomeric form of a Chlamydia major outer-membrane protein (MOMP) for vaccine development cited as ref.5.

We concur that similar work has been published with a focus on production, function and immunogenicity of the Chlamydia major outer-membrane protein (MOMP). However, those previous publications did not focus on replicating and visually illustrating the minute details related to the methodology for co-translation with ApoA1 in the presence of lipids solubilized by the telodendrimer using the continuously fed cell-free lysate. Publication of these details in JOVE will reach a far broader audience and allow other groups to apply these methodologies to wider range of membrane bound proteins.

2. If the goal was to enhance the yield of protein production, 1.5mg/ml reported by

others suffice for vaccine purposes. Also, the yield of protein production as the novelty of current work is an important factor yet is not mentioned throughout the paper, more specifically in the abstract part.

We totally agree with the reviewer and have tried to further highlight the scale of the protein production from a few mLs of cell free lysate. We have added text to the abstract and discussion to further make this point.

3. Another key issue is that subunit vaccines are incapable of eliciting full protection against all serotypes/serovars due to the nature of C.t. as an intracellular bacterium and the complicated β-barrel transmembrane structure of MOMP. Thus using a subunit vaccine in the current work compared to other recent attempts deems fruitful. see the recent study https://www.nature.com/articles/s41541-021-00312-9 which is much better that the current work and also ref.5.

Our current manuscript is focused on presenting the collated details for a cell-free protein production protocol that can produce a fully functional beta-barrel such as the MOMP pore (He et al., 2017) that is capable of being protective in an animal challenge study (Tifrea et al., 2021). The methods we have detailed for JOVE cover several other factors regarding lipids, adjuvants and other possible additives that may improve expression as well as potentially protection in future studies. We cannot comment on the reviewers suggested work given the antigen, adjuvants, experimental methods as well as animal model systems are very different.

Reviewer #2:

P3, L135: "w/o" change to "without"
Corrected.

P3, L141: "w/o" change to "without"
Corrected.

P5, L183: How much amount of eluted MOMP-tNLP?
We now specify 5-15 µl of the eluted MOMP-tNLP is loaded.

"Aliquots of the eluted MOMP–tNLPs, washes, flowthrough, and total lysate are mixed with 4X SDS-PAGE sample loading buffer."

P6, L211: Information about PVDF could be added to the table of materials
The PVDF transfer stacks are listed in the table of materials, catalog number IB24001.

P6, L218: "5 minutes" change it to "5 minutes each"
Corrected.

P6, L222: "5 minutes" change it to "5 minutes each"
Corrected.

P7, L244: "DI water" Please provide the full name (i.e., deionized water) before using the acronym
Corrected.

P7, L246: "at on dry ice" change to "on dry ice"
Corrected.

P8, L288: Detected at specific wavelength?
We now specify the wavelengths of 214 and 280 nm.

"Incorporation is assessed through comparing absorption of the adjuvanted particles to the unadjuvanted particles at 214 and 280 nm."

P9, L310: "previous results" Is this referring to the previous study or this study? This is referencing a previous article by He et al. We have added the reference number here.
This refers to a previous article and we have added the reference number here.

P9, L316-318: This info could be added to the protocol.
We have made a note to follow the instructions provided by the manufacturer for protein quantitation.

"The elution fractions containing MOMP-tNLPs were pooled and the total protein concentration were determined using a fluorescence-based quantitation device, or a device that measures concentration through absorbance at 280 nm, following manufacturers instructions for protein quantitation.**"**

P9, L328: To include DTT in the table of materials.
The DTT solutions used are purchased pre-made and these are listed as catalog number NP0009.

P11, L326-328: The DTT was the one added in the sample buffer? What was the concentration? (10X was the stock?) I wonder this experiment should also be described in the protocol?
We have added the diluted concentration of DTT for clarity.

"To test the oligomeric state of MOMP, we analyzed MOMP-tNLP in the presence and absence of both heat and the reducing agent DTT (50 mM, Figure 3a)."

P9, L339: Since authors are referring to the "Previous studies", perhaps authors could provide at least 2 or more references here.
We have added additional references supporting this claim, see references 12-15.

P12, L407: If this is the authors' previous paper, please include the citation.
We have inserted references 3 and 19 here clarifying this.

P11, L399: Perhaps authors could also discuss or compare their findings with other similar studies and highlight the amount of purified MOMP produced from this study?
We described the MOMP yield in the introduction, but reiterate it here.
"Detailed here is a validated cell-free co-expression method that produces oligomeric MOMP formed within tNLP nanoparticle as a vaccine, with yields of approximately 1.5 mg of purified MOMP per 3 mL of lysate.**"**

Figure 1a: Image without color? Is this for GFP color?
We have replaced this with a color image that shows the GFP color more clearly.

Reviewer #3:
1. Lane 108 in the protocol section, is the DMPC lipid can be used for different membrane antigens?
Yes, DMPC can be used to prepare other membrane protein formulations, but there may be a benefit from screening other lipids. We have made a note of this in the discussion:

"Expression of other candidate antigens from bacteria is feasible, although parameters such as expression temperature, lipid choice, and type of expression system may all need to be explored in order to achieve optimal yields."

2. Lane 111 in the protocol section, the authors need to specify the temperature of the centrifugation.
Corrected.

"Remove any contaminant metal from the probe by centrifugation at 13k for 2 mins at 22°C and then transfer the solubilized lipid to a new 1.5 mL endotoxin-free tube.**"**

3. Lane 156 in the protocol section, what kind of shaker has been used? The shaking speed (to be included) and the temperature (30°C) are suitable for the production of the chlamydial MOMP antigen but are they appropriate for the overexpression of other membrane proteins?
The shaker is listed in the materials as being from New Brunswick. We make a note that conditions may need to be optimized for other proteins.

*"*Place the reaction in a shaker at 300 RPM, 30°C for up to 18 hours. These conditions, particularly temperature, may need to be optimized for expression of other membrane proteins.*"*

4. Lane 164, please mention the reference of the chromatography column.
These columns are listed in the materials table as "Disposable, polypropylene fritted columns 10 ml capacity".

5. Lane 301, please give the protocol for obtaining the whole blood from vaccinated mice.
We have added additional detail for this procedure:

*"*11.5. On day 56 after the initial vaccination, collect blood to assess antibody titers. Begin by anesthetizing mice by injecting i.p. a solution of Xylazine (0.3mg/20g body weight) and Ketamine (3.0 mg/20g body weight). Front and hind legs are pinched to make sure no jerking occurrs. Apply vaseline jelly around the eyes to prevent eye dryness during anesthezia.

11.6. Using a micro-hematocrit capillary tube, puncture the retro-orbital plexus. Collect 100 μL blood in a microcentrifuge tube.*"*

6. Figure 1, several contaminants are visible in the elution fractions. At an industrial scale, it would be interesting to know whether these contaminants are presents in the different batches of the recombinant vaccine preparations and the potential toxicity of these contaminants. How can the authors may characterize these contaminants? A specific protocol to remove these contaminants has to be added in the protocol section.

We have made a note at the end of the discussion that size exclusion chromatography can be used to further purify formulations if desired purity is not achieved after nickel affinity chromatography:

"While not described here, additional purification following nickel affinity chromatography may be necessary if many contaminants are observed in subsequent analysis steps such as through SDS-PAGE. This could be accomplished with SEC, but conditions may require optimization on a formulation by formulation basis."

Reviewer #4:

1. How to prepare the highly insoluble recombinant MOMP protein.

Our protocol is designed to produce a scaffold supported MOMP that is soluble without the need for the use of detergents in a single step, where co-translation produces both the MOMP and ApoA1 protein in the presence of lipids. Another approach is the use of preformed NLPs added to cell-free reaction with plasmids encoding MOMP (Katzen et al., 2008). The protocol could also potentially work with insoluble recombinant MOMP by including detergents and lipids as a means to solubilize MOMP. The process could then just focus on the translational system for expressing ApoA1 protein to form the supporting nanodisc scaffold within the reaction.

2. What micropipette and pipette tips are used.

We have added the manufacturer and catalog information for the pipettes and tips used to the materials table.

3. What type of glass vials were used for lyophilization.

Polypropylene tubes were used as containers during lyophilization and this is now specified in the text:

"Make note of the final volume and aliquot into endotoxin-free 15 mL or 50 mL polypropylene tubes as desired."