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Cell-free scaled production and adjuvant addition to a recombinant major outer membrane protein from Chlamydia muridarum for vaccine development

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1 **TITLE:**
2 Cell-free scaled production and adjuvant addition to a recombinant major outer membrane
3 protein from *Chlamydia muridarum* for vaccine development
4
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14
15 **KEYWORDS:**
16
17 - *Cell-free production*
18 - *Nanolipoprotein particle*
19 - *Nanodisc*
20 - *Chlamydia*
21 - *Major outer membrane protein*
22 - *Vaccine*
23
24 **SUMMARY:**
25 This protocol describes using commercial, cell-free protein expression kits for the purpose of
26 producing membrane proteins that can be used as antigens in subunit vaccines.
27
28 **ABSTRACT:**
29 Subunit vaccines offer advantages over more traditional inactivated or attenuated whole-cell-
30 derived vaccines in safety, stability and standard manufacturing. In order to achieve an effective
31 protein-based subunit vaccine, the protein antigen often needs to adopt a native-like
32 conformation. This is particularly important for pathogen-surface antigens that are membrane-
33 bound proteins. Cell-free methods have been successfully used to produce correctly folded
34 functional membrane protein through co-translation of nanolipoprotein particles (NLPs),
35 commonly known as nanodiscs. This strategy can be used to produce subunit vaccines consisting
36 of membrane proteins in a lipid bound environment. However, cell-free protein production is
37 often limited to small scale (< 1 ml). The amount of protein produced in small scale production
38 runs is usually sufficient for biochemical and biophysical studies, however, in order to get enough
39 protein for vaccine studies in animal models, the cell-free process needs to be scaled-up,
40 optimized and carefully tested. Other processes involved in vaccine production, such as
41 purification, adjuvant addition, and lyophilization, need to be optimized in parallel. Here we
42 report the development of a scaled-up protocol to express, purify, and formulate a membrane-
43 bound protein subunit vaccine.

44
45 *1. Scaled-up cell-free reactions require optimization of plasmid concentrations and*
46 *ratios when using multiple plasmid expression vectors, lipid selection, and adjuvant*
47 *addition for high level production of formulated nanolipoprotein particles.*
48 *2. A chlamydial major outer membrane protein (MOMP) antigen demonstrates the*
49 *method's utility, which may be widely applied to other membrane protein antigens.*
50 *3. The antigen effectiveness can be evaluated in vivo through immunization studies*
51 *(antibody production, T-cell activation) followed by efficacy testing typically*
52 *performed by challenging vaccinated animals with different dosages of the pathogen*
53 *of interest.*
54
55 **INTRODUCTION:**
56 Prokaryotic or eukaryotic lysates for cell-free expression of proteins are readily available as
57 commercial products for synthesizing proteins of interest (for a complete review see: Carson et
58 al. 2012[1]). These expression systems are available at a variety of scales and utilize lysates from
59 various organisms including *E. coli*, tobacco plants, and mammalian cultures. Cell-free lysates
60 offer multiple benefits over traditional recombinant protein production approaches, including
61 ease of use and robust, rapid protein production. While these approaches are primarily used for
62 the production of soluble proteins, our group has pioneered an approach for their use to express
63 membrane proteins. This novel approach makes minor modifications to existing cell-free
64 expression systems by including DNA encoding two protein products for expression, an
65 apolipoprotein and the membrane protein of interest. The expressed apolipoprotein (derivatives
66 of either ApoA1 or ApoE4) interacts with lipids supplemented to the cell-free lysate to
67 spontaneously assemble nanoscale (~20 nm) lipid membrane particles (NLPs). When co-
68 translated with a membrane protein of interest, the NLP and membrane protein form a soluble
69 nanoparticle complex wherein the membrane protein is embedded within the NLP lipid bilayer.
70 Thus, the membrane protein is more accessible for downstream applications, as they are
71 contained within soluble, discrete particles. More recently, we have demonstrated that this
72 approach can produce functional oligomeric protein complexes within the NLP bilayer[2].
73 Furthermore, we have demonstrated that this approach can be used to produce the antigen
74 component of a subunit vaccine, which is subsequently mixed with lipophilic adjuvants to form a
75 nanoparticle vaccine featuring co-localized antigen and adjuvant suitable for *in vivo* assessment.
76 This current method is modified from a previously published protocol[3]. Key modifications
77 are focused on the scale-up of the cell-free reaction and subsequent purification of the protein-
78 NLP complex. A further modification includes the addition of an amphiphilic polymer known as a
79 telodendrimer, which is first mixed with the lipids then added to the cell-free reaction. Co-
80 translation of the plasmids in the presence of telodendrimer and lipids produces a telodendrimer
81 NLP (tNLP). Addition of the telodendrimer also helps modulate the size and monodispersity of
82 the resulting tNLP nanoparticles[4]. This protocol is specifically optimized for producing a
83 membrane-bound subunit antigen protein, chlamydial MOMP[5] for large scale vaccine studies.
84 The method produces recombinant MOMP associated with tNLP to form a highly soluble MOMP-
85 tNLP complex that retains MOMP oligomerization. A typical 3 ml scale-up production yields > 1.5

86 mg of purified MOMP. The cell-free produced MOMP-tNLP is amenable to rapid adjuvant
87 addition for *in vivo* immunogenicity testing.
88
89 **PROTOCOL:**
90 Enter text here (10-page maximum, 2.75 pages of highlighted text for filming).
91
92
93 **1. Glassware preparation**
94
95 1.1. All material used in producing vaccine-grade formulations for animals are endotoxin-free. To
96 prepare glassware for this process, bake cleaned glassware in an oven at 180ºC for four hours.
97
98 **2. Buffer preparation**

99 2.1. Prepare 250 milliliters of the following Ni affinity purification buffers. These may be stored
100 at 4ºC for up to 6 months.

101 2.1.1. Binding buffer, 50mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidazole, pH 8.0
102 2.1.2. Wash Buffer, 50mM NaH_2PO_4, 300 mM NaCl, 20 mM Imidazole, pH 8.0
103 2.1.3. Elution Buffer 1, 50mM NaH_2PO_4, 300 mM NaCl, 250 mM Imidazole, pH 8.0
104 2.1.4. Elution Buffer 2, 50mM NaH_2PO_4, 300 mM NaCl, 500 mM Imidazole, pH 8.0
105
106 **3. Reaction Preparation**
107
108 3.1. Weigh out 20 mg of DMPC into an endotoxin-free, 1.5 ml centrifuge tube. Dissolve into 1 ml
109 of endotoxin-free water, probe sonicate at least four times at 6 amps for 1 min, with 1 min pauses
110 in between, until clear. Remove any contaminant metal from the probe by centrifugation at 13k
111 for 2 mins and then transfer the solubilized lipid to a new 1.5 ml endotoxin-free tube.

112 3.2. Weigh out 1 mg of PEG5k-CA8 telodendrimer into a 1.5 ml endotoxin-free tube. Dissolve in
113 endotoxin-free water to a concentration of 20 mg/ml. Vortex until fully dissolved, and dilute to 2
114 mg/ml.

115 3.3. In a new endotoxin-free tube, combine 210 µl of 20 mg/ml DMPC solution with 210 µl of 2
116 mg/ml telodendrimer solution.

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

118 4.1. MOMP-tNLPs are prepared using cell-free methods modified from a previously published
119 protocol[5]. Two hours prior to setting up the cell-free reaction, open the RTS 500 ProteoMaster *E.*
120 *coli* HY cell-free reaction kit (Biotechrabbit GmbH) and thaw one of the reconstitution buffers.
121 Once thawed, add one tablet of cOmplete, EDTA-free Protease Inhibitor Cocktail (Roche) and

122 fully dissolve.

123 4.2. These systems are designed to run 5 x 1ml reactions. A typical scale-up production is 3 x 1ml.

124 4.2.1. For each 1 ml reaction, add 525 µl of reconstitution buffer to the *E. coli* lysate bottle and
125 gently roll to dissolve. Add 250 µl of reconstitution buffer to the Reaction Mix bottle and gently
126 roll to dissolve.

127 4.2.2. Add 8.1 ml of reconstitution buffer to the Feed Mix, recap with rubber stopper (take care
128 not to touch), and invert/roll gently to dissolve.

129 4.2.3. Add 3 ml of reconstitution buffer to Amino Acid Mix bottle, recap with rubber stopper (take
130 care not to touch the inside of the rubber stopper), and invert/roll gently to dissolve.

131 4.2.4. Add 1.8 ml of reconstitution buffer to Methionine bottle, roll gently to dissolve, and then
132 store on ice until use.

133 4.3. Prepare the reaction solution.

134 4.3.1. To the *E. coli* lysate bottle, add 225 µl reconstituted Reaction Mix, 270 µl of reconstituted
135 Amino Acid Mix w/o Methionine, and 30 µl of reconstituted Methionine. Additionally, add 400 µl
136 of the DMPC/telodendrimer mixture, 15 µg of MOMP plasmid, and 0.6 µg of Δ49ApoA1 plasmid.
137 Roll/gently shake to mix. Do not vortex.

138 4.3.2. Take 20 µl of the total solution and set aside in a 1.5 ml tube for the GFP expressing control
139 reaction (See below).

140 4.4. Prepare the feed solution. To the feed mix bottle, add 2.65 ml of reconstituted Amino Acid
141 Mix w/o Methionine and 300 µl of reconstituted Methionine. Roll/gently shake and do not
142 vortex.

143 4.5. At this time, the unused reconstitution buffer and methionine can be returned to the freezer
144 for storage.

145 4.6. Transfer 1 ml of the reaction solution to the inner reaction chamber that is provided in the
146 RTS 500 ProteoMaster *E. coli* HY cell-free reaction kit and seal when filled. Transfer 10 ml of the
147 feed solution to the outer chamber of the reaction vessel and seal. Do not over fill the chambers!
148 The presence of air bubbles at the top of both the inner reaction chamber and the inner feed
149 chamber will adversely affect the reaction.

150 4.7. Any remaining reaction solution can be placed in a 1.5 ml tube and allowed to mix alongside
151 the main vessel.

152 4.8. Add 0.5 µl of GFP control plasmid (0.5 mg/ml) to the 20 µl reaction mixture previously
153 aliquoted. The plasmid is normally supplied in the RTS 500 ProteoMaster *E. coli* HY cell-free
154 reaction kit. Most GFP expressing plasmid with a T7 promoter and *E.coli* ribosome binding site
155 (RBS) can also be used as the control plasmid.

156 4.9. Place the reaction in a shaker at 30 ºC for up to 18 hours. To verify that the reaction was
157 successfully prepared, synthesis of the control GFP can be checked for fluorescence using a UV
158 light source (Figure 1a) after as little as 15 minutes of incubation.

159 **5. MOMP-tNLP purification**

160 5.1. Immobilized nickel affinity chromatography can be used to purify the MOMP-tNLP
161 nanoparticle complex from the cell-free reaction mixture using the His-tag on the ∆49ApoA1
162 protein.

163 5.1.1. Transfer 1 ml of a 50% slurry of cOmplete His-Tag Purification Resin (Roche Molecular
164 Diagnostics) to a disposable 10-ml chromatography column and equilibrate with 3 ml of Binding
165 buffer.

166 5.1.2. Let the buffer drain, cap the outlet, and add 250 µl of Binding buffer to the resin.

167 5.1.3. Before adding the cell-free reaction to the column, save 20 µl for later analysis by SDS-
168 PAGE. The cell-free reaction is then mixed with the equilibrated resin and nutated at 4°C for 1
169 hour.

170 5.2. Uncap the column and wash the cap with 500 µl of additional Binding buffer and then add
171 this liquid to the rest of column.

172 5.3. Collect the liquid flow through from the column for later analysis by SDS-PAGE analysis.

173 5.4. Wash the column with 1 ml of Wash buffer containing 20 mM imidazole six times and collect
174 fractions. Take care not to let the resin dry out between washes. On the second wash, vigorously
175 agitate the resin by pipetting up and down using a 1 ml pipette.

176 5.5. Elute the MOMP-tNLPs in six 300 µl fractions of Elution buffer 1 (containing 250 mM
177 imidazole), followed by one final elution with 300 µl of Elution buffer 2 (containing 500 mM
178 imidazole). On the second elution, vigorously agitate the resin by pipetting up and down using a

1 ml pipette.

6. Analysis by SDS-PAGE

6.1. All elutions should be analyzed by SDS-PAGE to screen for quantity and purity of the protein of interest.

6.1.1. An aliquot of 5-15 µl of the eluted MOMP–tNLPs is mixed with NuPAGE™ LDS Sample Buffer (4X) (Life Technologies). Samples are also mixed with NuPAGE™ Sample Reducing Agent (10X) (Life Technologies) and heat-denatured unless otherwise indicated.

6.1.2. Fractions are then analyzed by gel electrophoresis using 1.0 mm NuPAGE™ 4 to 12%, Bis-Tris SDS-PAGE gels (Life Technologies) with 1X MES-SDS running buffer, along with the molecular weight standard SeeBlue Plus2 (Life Technologies). Gels are run for 35 min at 200 V.

6.2. Gels are subsequently stained with SYPRO™ Ruby Protein Gel Stain (Life Technologies) using the rapid protocol according to manufacturer's instructions.

6.2.1. Briefly, remove the gel from cassette and put in 60 ml of SYPRO™ Ruby gel stain. Microwave the gel with the solution for 30 seconds, agitate 30 seconds to distribute heat evenly, microwave another 30 seconds to 80–85°C, and agitate on an orbital shaker for 5 minutes.

6.2.2. Reheat the gel by microwaving a third time for 30 seconds, and then agitate on an orbital shaker for 23 minutes.

6.2.3. Next, transfer the gel to a clean container and wash in 100 ml of wash solution (10% methanol, 7% acetic acid) for 30 minutes. The transfer step is necessary to avoid heating the wash solution, which may reduce stain sensitivity, and also helps minimize background staining irregularities and stain speckles on the gel.

6.2.4. After washing, rinse the gel in ultrapure water twice for 5 minutes each.

6.3. Gels are then imaged using a LI-COR Odyssey Fc imager (LI-COR Biosciences) at a 600nm wavelength (Figure 2). If you have a protein standard to compare against, SDS-PAGE can also be used to quantify the amount of individual protein in the nanoparticle solution. In this example, serial dilutions of recombinantly expressed MOMP are resolved by SDS-PAGE and the densities of the bands are quantified using Image Studio V2.0 software (LI-COR Biosciences).

6.4. Generate a standard curve using the densities of the MOMP bands. MOMP-tNLP samples are resolved on the same SDS-PAGE gel and the MOMP component of the particles are calculated using the MOMP standard curve (Figure 3).

7. Western and Dot Blot

210 7.1. For Western blotting, samples are resolved by SDS-PAGE, then the gels are transferred using
211 the iBlot™ 2 Transfer Stacks, PVDF (Life Technologies) using an iBlot™ 2 Dry Blotting System (Life
212 Technologies), and the standard settings according to manufacturer's protocols.

213 7.1.1. Remove blots from the stack after the transfer is complete, and incubate the blot overnight
214 at 4°C in Odyssey® Blocking Buffer in TBS (LI-COR Biosciences) containing 0.2% Tween 20 and
215 either 0.5 mg/ml MAb40 or 0.2 mg/ml MAbHIS Penta-His antibody (Qiagen), which is directed
216 against the His-tag from Δ49ApoA1 protein. The antibodies dilutions used for blotting are 1:1,000
217 for MAb40 and 1:500-1,000 for MAbHIS antibody.

218 7.1.2. Wash blots 3 times for 5 minutes with PBS-T (1xPBS, 0.2% Tween 20, pH 7.4).

219 7.1.3. Blots are then incubated for 1 h in Odyssey® Blocking Buffer in TBS (LI-COR Biosciences)
220 containing 1 mg/ml IRDye 800CW goat (polyclonal) anti-mouse IgG (heavy and light) (LI-COR
221 Biosciences, 1:10,000).

222 7.1.4. Wash blots again 3 times for 5 minutes with PBS-T. After the final wash the blots are imaged
223 with a LI-COR Fc imager (LI-COR Biosciences) at wavelength 800nm.

224 7.2. For dot blots, 3 µg of purified MOMP-tNLP and empty tNLP are blotted using a Bio-Dot
225 apparatus (Bio-Rad) according to the manufacturer's instructions. The blots are then blocked and
226 developed using the same methods described above for Western blotting.

227 **8. Endotoxin assessment**

228 8.1. Endotoxin levels can be quantified using the Endosafe®-PTS™ (Charles River, Charleston, SC)
229 endotoxin testing system based on the Limulus Amebocyte Lysate (LAL) assay. Prepare
230 endotoxin-free 25mM Tris, pH 7.4 sample buffer using 1M Trizma hydrochloride solution (Sigma)
231 and Hyclone HyPure culture-grade water (VWR).

232 8.2. Typically, samples need to be diluted using this sample buffer, and the dilutions adjusted to
233 find the suitable range for individual samples. Here, MOMP-tNLP samples are diluted 500-fold in
234 sample buffer and 25µl are loaded into each well of an Endosafe PTS cartridge with 0.05EU/ml
235 sensitivity (Thermo Fisher Scientific). The endotoxin levels of MOMP-tNLP and empty tNLP used
236 in the mouse studies described below are between 0.4 to 12 EU/µg protein depending on the
237 sample.

238 **9. Lyophilization**

239 9.1. The MOMP-tNLP nanoparticles can be lyophilized and stored long-term (up to years) at -
240 20°C. To prepare tNLP and MOMP-tNLP solutions for lyophilization, trehalose is added to serve
241 as a protectant during the freezing and lyophilization process. This process has been extensively
242 validated for a variety of tNLP formulations[6,7].

243 9.2. The current volume of the MOMP-tNLP solution is divided by 9 to obtain the volume of 1M
244 trehalose in sterile DI water that must be added to reach a final concentration of 0.1M trehalose.
245 Make note of the final volume.

246 9.3. The mixed solution is then frozen at on dry ice and lyophilized overnight using a Labconco
247 lyophilizer. The dried formulations can then be stored at -20°C until needed.

248 9.4. Lyophilized tNLPs are reconstituted using DI water. Gently roll until lyophilized cake is fully
249 dissolved and rehydrated. To remove trehalose, the solution may be dialyzed against PBS using
250 a 3.5 kDa cutoff dialysis membrane.

251 **10. Adjuvant addition**

252 Our vaccine formulations incorporate two adjuvants: CpG-ODN1826 and FSL-1. CpG-ODN1826
253 (Biosearch Technologies) is a modified Class B CpG oligonucleotide (5'-tccatgacgttcctgacgtt-3')
254 with a full phosphorothioate backbone featuring a 5' cholesterol moiety (5'-chol-C6). The
255 conjugation of CpG-ODN1826 to tNLPs is mediated by the hydrophobic interactions between the
256 cholesterol moiety and the phospholipid bilayer of the tNLP, and has been demonstrated and
257 well-characterized as previously reported[8,9].
258
259 10.1. Prior to incorporation into our formulations, the cholesterol-modified CpG is purified by
260 reversed phase chromatography to remove contaminating endotoxin as well as any unmodified
261 CpG molecules.
262
263 10.1.1. Upon receipt from the vendor, the lyophilized CpG material is rehydrated in endotoxin-
264 free water, and subsequently purified on a preparative C4 RP-HPLC column using separation
265 gradient consisting of 10mM triethylammonium acetate (TEAA) (mobile phase A) and acetonitrile
266 (mobile phase B).
267
268 10.1.2. Fractions containing cholesterol-modified CpG are pooled and lyophilized. To ensure
269 complete removal of residual TEAA, CpG is reconstituted with 15ml endotoxin-free water and re-
270 lyophilized three times.
271
272 10.1.3. After the final lyophilization, CpG is reconstituted in endotoxin-free water (>20mg/ml final
273 CpG concentration), aliquoted, and stored frozen at -80°C until needed. For addition to
274 formulations, CpG is first diluted to a concentration of 1-2.5 mg/ml.
275
276 10.2. FSL-1 is available as a vaccine-grade, lyophilized powder from InvivoGen (San Diego, CA).
277 This is reconstituted using sterile and endotoxin-free water at a concentration of 1 mg/ml.

278 10.3. Our vaccine is administered intramuscularly (i.m.), with each dose containing 10 µg of
279 MOMP in a total volume of 50 µl. To achieve the desired formulation dose, the nanoparticles
280 typically need to be concentrated following dialysis into PBS using a centrifugal vacuum
281 concentrator (e.g. Eppendorf Vacufuge) before adjuvant addition. Care should be taken when

282 doing this to prevent the formulation from completely drying the sample—check the sample
283 volume every 20-30 minutes during centrifugation.

284 10.4. Adjuvant addition is carried out under sterile conditions in a biosafety cabinet. To assess
285 successful incorporation, the final formulations, as well as their individual components, can be
286 analyzed by analytical size-exclusion chromatography (SEC). For our preparations, we use a
287 Superdex 200, 5/150 GL column, (GE Healthcare) in PBS buffer (0.5 ml/m in flow rate) and detect
288 elution using a UV-vis diode array detector (Shimadzu).

289 10.5. The adjuvanted MOMP-tNLP and empty tNLP can be stored at 4°C prior to animal use for a
290 period of up to 14 days, but stability will vary from formulation to formulation. To fully assess the
291 stability of a new tNLP formulation, the stored tNLPs need to be periodically analyzed by SEC.

292 **11. Serum testing**

293 11.1. All animal studies were performed at the Lawrence Livermore National Laboratory in Public
294 Health Service (PHS)-assured facilities in accordance with guidelines set by the Institutional
295 Animal Care and Use Committee. Female 3-week-old mice (BALB/c) are purchased from The
296 Jackson Laboratory (Bar Harbor, ME).

297 11.2. Mice (n=6) are vaccinated intramuscularly (i.m.) with 10 µg of MOMP in the form of MOMP-
298 tNLP adjuvanted with 5 µg of CpG and 1 µg FSL1.

299 11.3. Four weeks after the initial vaccination (prime), animals are vaccinated a second time
300 (boost).

301 11.4. Whole blood is drawn on day 56 for analysis.

302 11.5. Serum antibodies specific for MOMP are tested using Western blotting techniques as
303 described above. Mouse sera from all immunized mice is pooled and this pooled serum is used
304 in place of a primary antibody at 1:5,000 dilution.

305
306
307 **REPRESENTATIVE RESULTS:**
308 The SDS-PAGE profile of the Ni affinity purification of MOMP-tNLP from a 1 ml cell-free reaction
309 is shown in Figure 1b. The reaction resulted in high levels of expression for both the MOMP and
310 the Δ49ApoA1 protein. Our previous results showed that the cell-free expression of Δ49ApoA1
311 in the presence of DMPC and telodendrimer resulted in the formation of telodendrimer
312 nanolipoprotein particles (tNLPs). The co-elution of MOMP with Δ49ApoA1 indicated that MOMP
313 is associated with tNLPs, as the His-tag is only present on the tNLP scaffold Δ49ApoA1 and not
314 on MOMP. We also know that MOMP is a highly insoluble protein that can only be eluted through
315 complexing with tNLPs, which have been shown to facilitate solubilization of membrane proteins.

316 The elution fractions containing MOMP-tNLPs were pooled and the total protein concentration
317 were determined using Qubit® 3.0 Fluorometer (Life Technologies) (or other protein
318 quantification techniques, such as Nanodrop). To allow for precise dosing of the MOMP vaccine,
319 it is also important to determine the concentration of MOMP in the purified complexes. We
320 developed a method to quantify MOMP based on gel densitometry (Figure 2). We used a purified
321 recombinant MOMP with known concentration to be the standard. By establishing the standard
322 curve and comparing it to our MOMP-tNLP sample, we can accurately quantify the MOMP
323 concentration. The determination of MOMP concentration in the purified sample enabled us to
324 estimate the yield of MOMP in cell-free reactions at various scales, which is important for the
325 planning of reaction setup appropriate to the downstream studies (Table 1).

326 MOMP needs to form oligomers to elicit a robust immune response[10]. To test the oligomeric state
327 of MOMP, we analyzed MOMP-tNLP in the presence and absence of both heat and the reducing
328 agent DTT (NuPAGE™ Sample Reducing Agent (10X), Life Technologies) (Figure 3a). Higher-order
329 oligomers of MOMP were identified through SDS-PAGE when samples are not treated with heat
330 and DTT. In comparison, samples that are treated with heat in the presence of DTT showed
331 primarily two distinct bands on the gel, corresponding to MOMP and Δ49ApoA1 (approximately
332 40 kD and 22 kD, respectively). These results closely resemble the gel banding pattern attributed
333 to oligomer formation of MOMP, which is critical to its effectiveness. Further western-blot
334 analysis using MAb40, an antibody against the linear epitope on the variable domain of MOMP
335 protein, showed similar banding pattern, confirming the oligomer formation by MOMP protein
336 in its non-denatured state (Figure 3b). An important factor that can impact MOMP oligomer
337 formation is the ratio between MOMP plasmid and the Δ49ApoA1 plasmid during the cell-free
338 reaction setup. Table 2 lists the ratio of plasmids and the resulting insertion rate of MOMP into
339 tNLPs. Previous studies indicated that chlamydial MOMP and other outer membrane proteins
340 may exist primarily as trimers[11]. To maximize the trimer formation in the cell-free reaction, it is
341 desirable to have the insertion rate close to three MOMP proteins per NLP, which corresponds
342 to ~25:1 MOMP-to-Δ49ApoA1 plasmid ratio.

343 We also used dot blots assay as a more streamlined method to detect the presence of MOMP
344 and tNLP. The MAb40 antibody was used to detect total MOMP. The MAbHIS antibody which is
345 targeted to the His-tag on the Δ49ApoA1 scaffold of the tNLP was used to assess the presence of

346 tNLP. The co-signaling of MAb40 and MAbHIS antibodies indicated MOMP-tNLP formation. The
347 control reaction produced empty tNLP which only showed positive signal from MAbHIS (Figure
348 3c).

349 To test the immunogenicity of MOMP-tNLPs produced the in cell-free reaction, we adjuvanted
350 MOMP-tNLP with CpG + FSL1 and injected intramuscularly (i.m.) into mice in a prime-boost
351 regimen as described above. Sera were collected from the immunized mice and MOMP-specific
352 IgG antibody was measured using a western blot assay (Figure 4). The sera from mice injected
353 with adjuvanted MOMP-tNLP showed strong MOMP binding, indicating MOMP-tNLP could elicit
354 an immune response *in vivo*.

355 **FIGURES AND TABLES:**

356

357 **Materials:**

358

1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) as powder (Avanti, catalog No. 850345)
1.5 ml endotoxin-free centrifuge tubes (Eppendorf, catalog No. 002600028)
1M Trizma hydrochloride solution (Sigma, catalog No. T2194)
Acetic acid, glacial, ACS reagent, ≥99.7% (Sigma-Aldrich, catalog No. 695092)
Bio-Dot apparatus (Bio-Rad, catalog No. 1706545)
Buffer Dam for XCell SureLock™ (Life Technologies, catalog No. EI0012)
C24 Incubator shaker (New Brunswick Scientific)
Cell-Free Expression System: RTS 500 ProteoMaster E. coli HY Kit (BiotechRabbit, catalog No. BR1400201).
cOmplete His-Tag Purification Resin (Roche Molecular Diagnostics, catalog No. 5893682001)
cOmplete, EDTA-free Protease Inhibitor Cocktail (Roche Molecular Diagnostics, catalog No. 4693132001)
CpG-ODN1826 (Biosearch Technologies)
D-(+)-Trehalose dihydrate (Sigma-Aldrich, catalog No. T9449)
Dialysis tubes D-Tube™ Dialyzer Maxi (Sigma-Aldrich, catalog No. 71509)
Disposable, polypropylene fritted columns 10 ml capacity (Bio-Rad, catalog No. 7311550EDU).
Dulbecco's Phosphate Buffered Saline (PBS) (Sigma-Aldrich, catalog No. D8537)
Electrophoresis Power Supply
Endosafe PTS™ cartridge (Thermo Fisher Scientific, catalog No. NC9594798)
Endosafe-PTS™ Testing System (Charles River, Charleston, SC)
Gel wash solution: 10% methanol, 7% acetic acid
HCl and NaOH solutions for pH adjustment
HPLC with UV-vis diode array detector (Shimadzu)
HyClone HyPure culture-grade water (VWR, catalog No. 82007-328)

iBlot™ 2 Dry Blotting System (Life Technologies)
iBlot™ 2 Transfer Stacks, PVDF (Life Technologies, catalog No. IB24001)
Image Studio V2.0 software (LI-COR Biosciences)
Imidazole (Sigma-Aldrich, catalog No. I5513)
Immun-Blot PVDF Membrane (Bio-Rad, catalog No. 1620177)
LI-COR Odyssey Fc imager (LI-COR Biosciences)
Lyophilizer (Labconco)
Methanol (≥99.9%)(Sigma-Aldrich, catalog No. 34860)
Microcentrifuge
Micropipettes
Microwave oven
NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, catalog No. ND-ONE-W)
NuPAGE™ 4 to 12%, Bis-Tris, 1.0 mm (Life Technologies, catalog No. NP0321)
NuPAGE™ LDS Sample Buffer (4X) (Life Technologies, catalog No. NP0007)
NuPAGE™ MES SDS Running Buffer (20X) (Life Technologies, catalog No. NP000202)
NuPAGE™ Sample Reducing Agent (10X) (Life Technologies, catalog No. NP0009)
Odyssey™ Blocking Buffer in TBS (LI-COR Biosciences, catalog No. 927-50000) containing 0.2% Tween 20
Orbital Shaker
PBS-T (1xPBS, 0.2% Tween 20, pH 7.4)
PEG5K-CA8 Telodendrimer (custom synthesis product)3
pIVEX2.4d vector (Roche Molecular Diagnostics)
Plasmid Maxi Kit (Qiagen, catalog No. 12162)
Primary antibody: MAb40 (monoclonal antibody to the variable domain 1 (VD1) of C. muridarum MOMP, de la Maza laboratory)4
Primary antibody: MAbHIS, Penta-His antibody (Qiagen, catalog No. 34660)
Probe sonicator
Qubit™ 3.0 Fluorometer (Life Technologies, catalog No. Q33216)
Qubit™ Protein Assay Kit (Life Technologies, catalog No. Q33212)
Secondary antibody: IRDye 800CW goat (polyclonal) anti-mouse IgG (heavy and light) (LI-COR Biosciences, catalog No. 926-32210)
SeeBlue™ Plus2 Pre-stained Protein Standard (Life Technologies, catalog No. LC5925)
Sodium chloride NaCl (Sigma-Aldrich, catalog No. S7653)
Sodium phosphate monobasic NaH2PO4 (Sigma-Aldrich, catalog No. S0751)
Superdex 200, 5/150 GL column, (GE Healthcare, Piscataway, NJ)
Synthetic diacylated lipoprotein-TLR2/6 FSL-1 (Invivogen, catalog No. tlrl-fsl)
SYPRO™ Ruby Protein Gel Stain (Life Technologies, catalog No. S12001)
TWEEN™ 20 (Sigma-Aldrich, catalog No. P1379)
UV light source

Vacufuge Bench Top Centrifuge (Eppendorf)
Vortexer
XCell SureLock™ Mini-Cell (Life Technologies, catalog No. EI0001)

359

360 **Table 1.** The quantity of lipids, telodendrimer and plasmids used for differently scaled cell-free
361 reactions and the corresponding yields.

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

362 **Table 2**. The plasmid ratios in a cell-free reaction and the resulting MOMP insertion rates.

Ratios of plasmid input, MOMP : Δ49ApoA1	1:1	5:1	10:1	25:1	50:1	100:1
Ratios of the amount of protein produced, MOMP : Δ49ApoA1	0.02	0.32	0.64	3.46	6.55	20.04
Estimated number of MOMP insertion per tNLP	0.03	0.37	0.75	4.04	7.65	23.39

363
364
365 **FIGURE AND TABLE LEGENDS:**

366 **Table 1.** The quantity of lipids, telodendrimer and plasmids used for differently scaled cell-free
367 reactions and the corresponding yields.

368 **Table 2.** The plasmid ratios in a cell-free reaction and the resulting MOMP insertion rates.

369 **Figure 1. Expression and purification of MOMP-tNLP.** (a) Image of tubes containing small aliquot
370 of cell-free reaction that successfully expressed GFP controls luminating under UV light source.
371 (b) SYPRO Ruby Protein Gel stained SDS-PAGE of the purification profile of MOMP-tNLP.
372 Molecular weight marker (MW), total cell-free lysate (T), flow through (FT), Washes 1 and 6 (W1,
373 W6), and Elutions 1 through (E1-E7). MOMP migrates at 40 kDa and the Δ49ApoA1 migrates at
374 22 kDa.

375 **Figure 2. Quantification of MOMP in MOMP-tNLP samples.** (a) SYPRO Ruby Protein Gel stained
376 SDS-PAGE of the quantification of MOMP. Recombinant MOMP with known concentration was

377 loaded onto the gel to form the standard curve. Each lane contained 0.1µg, 0.2µg, 0.5µg, 1µg and
378 2µg of MOMP. MOMP-tNLP samples that were being quantified were loaded on the same gel.
379 (b) The MOMP concentration standard curve was generated using densitometry. An equation
380 relating normalized band density and the amount MOMP was established. We then used the
381 equation to calculate the MOMP content in the unknown samples.

382 **Figure 3. Cell-free produced MOMP-tNLP allow MOMP to form higher order structures.** (a)
383 SYPRO Ruby Protein Gel stained SDS-PAGE of MOMP-tNLP with and without treatment of heat
384 and reducing agent DTT. With heat and DTT, MOMP primarily appeared as a monomer band at
385 ~40 kDa, as heat and reducing agent broke down the majority of higher order MOMP structure.
386 In the absence of heat and DTT, the higher order bands were present, indicating MOMP oligomer
387 conformation. (b) Western blot of MOMP-tNLP and MOMP alone, untreated and treated with
388 heat and DTT. After transfer, the membrane was probed with MAb40 (1:1000 dilution). A banding
389 pattern similar to the SYPRO Ruby stained gel was observed, confirming that the higher molecular
390 weight bands were indeed MOMP oligomers. (c) Dot blot of MOMP-tNLP and empty tNLP
391 samples (in duplicate) probed with MAb40 and MAbHIS.

392 **Figure 4. Cell-free produced MOMP-tNLP is highly immunogenic.** Serum from immunized mice
393 showed strong anti-MOMP specific IgG signal. MOMP-tNLP adjuvanted with CpG + FSL1 was used
394 to immunize mice. Serum from six immunized mice was collected, pooled, and used to probe
395 MOMP-tNLP. The serum was able to bind to MOMP in a western blot assay and showed strong
396 IgG signal. The western blot using MAb40 as primary antibody showed similar bands, indicating
397 that the serum contained MOMP specific IgG.

398

399 **DISCUSSION:**

400 Chlamydia is the most common sexually transmitted infection that affects both men and women.
401 Vaccine research on Chlamydia spans decades, yet a safe and effective vaccine that can be scaled
402 to mass production has remained elusive[12]. The chlamydial MOMP is considered the lead
403 candidate as a protective vaccine antigen, however MOMP is highly hydrophobic and prone to
404 incorrect folding[13,14]. Further study has revealed that MOMP exists in oligomeric states that are
405 essential for its immunogenicity[10]. Here we validated our cell-free co-expression method in
406 producing oligomeric MOMP formed within tNLP nanoparticle as a vaccine.

407 We have previously published on using cell-free expression to produce membrane proteins
408 embedded within NLPs. In the method reported here, we describe how this procedure can be
409 adapted to scale-up expression of bacterial membrane proteins that are promising candidates as
410 antigens for use in subunit vaccines. Not only does this procedure produce solubilized MOMP,
411 but the overall nanoparticle structure is amenable to further modification using a variety of
412 lipophilic vaccine adjuvants including, but not limited to, CpG conjugated to a cholesterol moiety
413 or FSL-1. This procedure can be further scaled for industrial production, increasing its prospects
414 as a useful approach for generating vaccines.

415

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421

422 **DISCLOSURES:**

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

425

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427

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467 trachomatis major outer membrane protein. *Vaccine.* **27** (36), 5020-5025,
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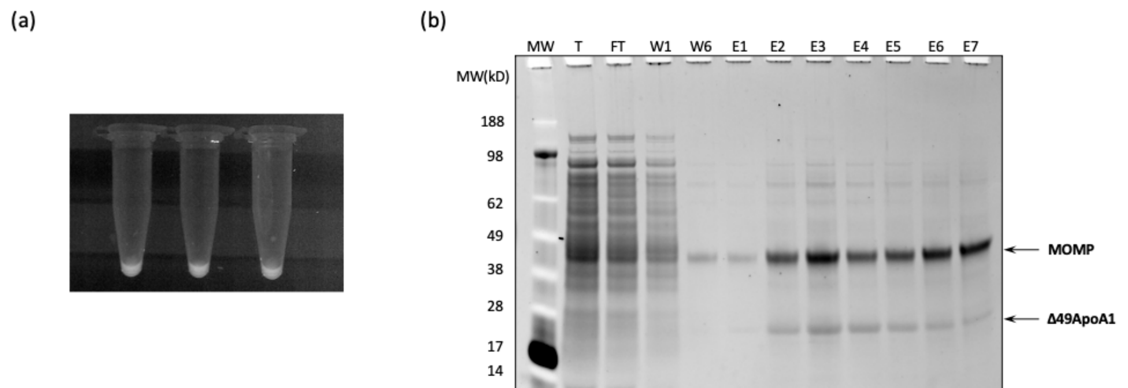
469

470

Figure 1



471

Figure 2

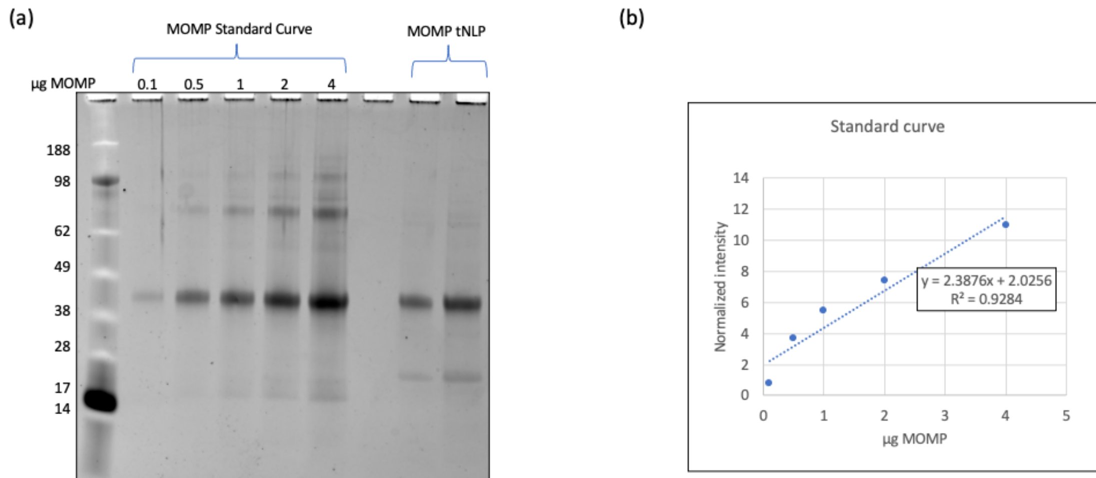


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Figure 3

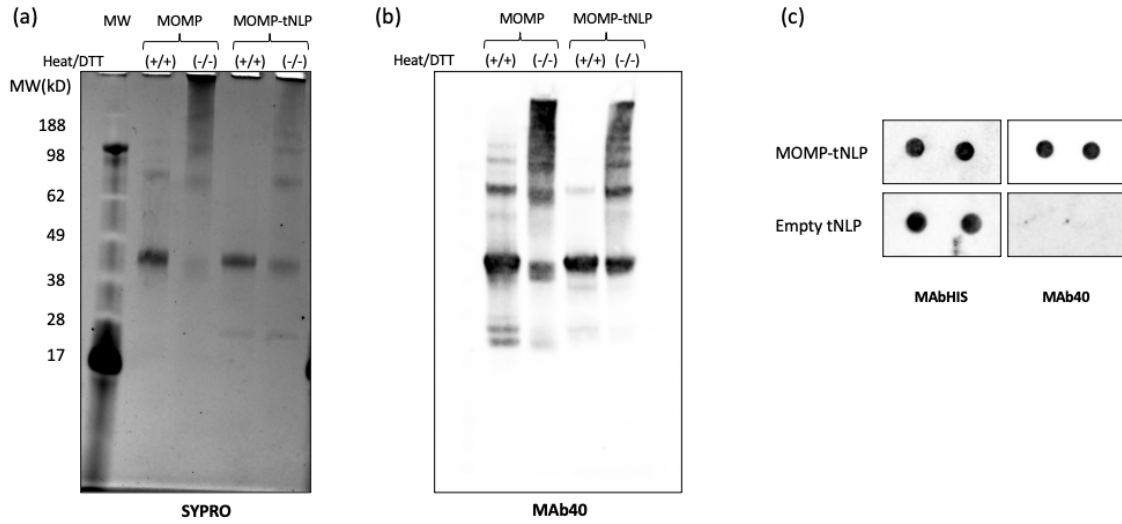

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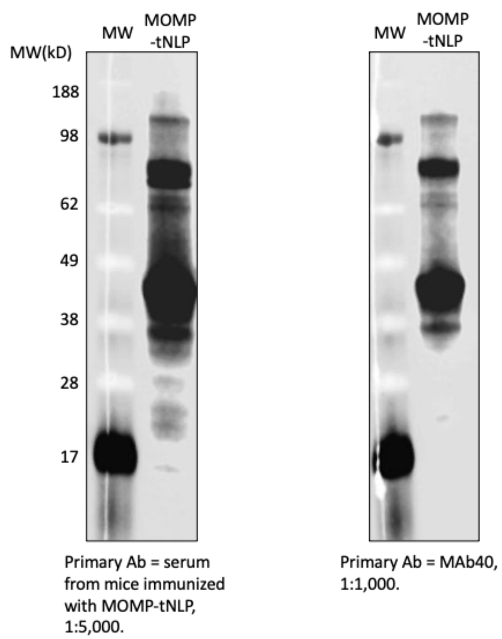
Figure 4



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

Primary Ab = MAb40, 1:1,000.

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