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TITLE:

Production of *E. coli*-expressed Self-Assembling Protein Nanoparticles for Vaccines Requiring Trimeric Epitope Presentation

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SUMMARY:

A detailed method is provided here describing the purification, refolding, and characterization of self-assembling protein nanoparticles (SAPNs) for use in vaccine development.

ABSTRACT:

Self-assembling protein nanoparticles (SAPNs) function as repetitive antigen displays and can be used to develop a wide range of vaccines for different infectious diseases. In this article we demonstrate a method to produce a SAPN core containing a six-helix bundle (SHB) assembly that is capable of presenting antigens in a trimeric conformation. We describe the expression of the SHB-SAPN in an *E. coli* system, as well as the necessary protein purification steps. We included an isopropanol wash step to reduce the residual bacterial lipopolysaccharide. As an indication of the protein identity and purity, the protein reacted with known monoclonal antibodies in Western blot analyses. After refolding, the size of the particles fell in the expected range (20 to 100 nm), which was confirmed by dynamic light scattering, nanoparticle tracking analysis, and transmission electron microscopy. The methodology described here is optimized for the SHB-SAPN, however, with only slight modifications it can be applied to other SAPN constructs. This method is also easily transferable to large scale production for GMP manufacturing for human vaccines.

INTRODUCTION:

While traditional vaccine development has focused on the inactivated or attenuated pathogens, the focus of modern vaccines has shifted toward subunit vaccines[1]. This approach can lead to a more targeted response, and potentially more efficacious vaccine candidates. However, one of the main drawbacks is that subunit vaccines are not particulates like whole organisms which can result in reduced immunogenicity[2]. A nanoparticle as a repetitive antigen display system can have the benefits of both the targeted subunit vaccine approach as well as the particulate nature of the whole organism[1,3].

Among the existing types of nanovaccines, rationally designed protein assemblies allow for the design and development of vaccine candidates that can present multiple copies of the antigen potentially in a native-like conformation[1,4-6]. One example of these protein assemblies are the self-assembling protein nanoparticles (SAPNs)[7]. SAPNs are based on coiled-coil domains and are traditionally expressed in *Escherichia coli*[8]. SAPN vaccine candidates have been developed for a variety of diseases such as malaria, SARS, influenza, toxoplasmosis, and HIV-1[9-19]. The design of each SAPN candidate is specific to the pathogen of interest, however, the production, purification, and refolding techniques are generally broadly applicable.

One of our current interests is an effective HIV-1 vaccine. In RV144—the only Phase III clinical trial of an HIV-1 vaccine that demonstrated modest efficacy—the reduced risk of infection was correlated with IgG antibodies to the V1V2 loop of the envelope protein[20,21]. The native-like trimeric presentation of this region is thought to be important for protective immunogenicity[22]. To present the V1V2 loop in as close to native-like conformation as possible, we developed a proof of principle SAPN vaccine candidate that contained the HIV-1 envelope post-fusion six-helix bundle (SHB) to present the V1V2 loop into the correct conformation[9]. This candidate was recognized by known monoclonal antibodies to HIV-1 envelope protein. Mice immunized with V1V2-SHB-SAPN raised V1V2 specific antibodies, that, most importantly, bound to gp70 V1V2, the correct conformational epitopes[9]. The SHB-SAPN core could have other functions beyond the role as a carrier for the HIV-1 V1V2 loop. Here we describe a detailed methodology for the expression, purification, refolding, and validation of the SHB-SAPN core. The sequence selection, nanoparticle design, the molecular cloning, and transformation of *E. coli* have been previously described[9].

PROTOCOL:

1. Expression of the SHB-SAPN Protein in *E. coli* BL21(DE3)

1.1. Mix 95 mL of component A and 5 mL of component B of the media in a 2 L sterile glass Erlenmeyer flask as per the manufacturer's instructions (see the **Table of Materials**). Add ampicillin to a final concentration of 100 µg/mL.

1.2. Inoculate the media with *E. coli* from a previously established glycerol stock culture. Incubate culture at 30 °C with shaking at 200 rotations per minute (rpm) for 48 h.

NOTE: The used *E. coli* BL21 (DE3) stock contained the ampicillin resistant expression vector[23] with the SHB-SAPN gene. Although the general protocol of the media recommends 24 h of incubation at 37 °C, 48 h of incubation at 30 °C gave higher yield for SHB-SAPN.

1.3. Transfer the culture to two 50 mL conical tubes. Centrifuge the tubes at 4,000 x *g* for 10 min with a fixed angle rotor at 4 °C. Remove the supernatant and save the pellet to harvest cells.

NOTE: The cell pellet can be either processed immediately or frozen at -80 °C until use.

2. Lysis of *E. coli* BL21(DE3) by sonication

NOTE: Use nonpyrogenic plasticware and glassware baked at 250 °C for at least 30 min. Tris(2-carboxyethyl)phosphine (TCEP) as a reducing agent breaks the disulfide bonds within and between proteins. TCEP is necessary in the buffers during this protocol if the displayed antigen contains S-S bonds. For SHB-SAPN core only, the presence of TCEP in the buffers is not essential.

2.1. Prepare imidazole-free buffer (8 M Urea, 50 mM sodium phosphate monobasic, 20 mM Tris base, 5 mM TCEP) pH 8.0 (adjusted with 5 N NaOH) and filter it using a 0.22 µm vacuum bottle filtration unit.

2.2. Resuspend the pelleted cells (from step 1.3) with 40 mL of imidazole-free buffer in one 50 mL conical tube. Sonicate the resuspended cells with a probe on ice for 5 min (4 s of sonication, 6 s of rest) with a sonication output of 150 W.

2.3. Centrifuge the cellular lysate (40 mL) at 29,000 x *g* at 4 °C for 25 min in a fixed angle rotor to generate clarified supernatant. Transfer the supernatant to a 150 mL sterile flask and discard the pellet. Dilute the supernatant to 100 mL using the imidazole-free buffer (later in the protocol referred to as “sample”).

NOTE: This dilution step is needed to prevent the FPLC system pressure from becoming too high during lysate loading on the column.

3. Protein purification using a His-column

NOTE: This protocol was performed using an FPLC instrument, but it can be adapted to gravity flow.

3.1. Prepare the following buffers and filter them using a 0.22 µm vacuum bottle filtration unit: (i) imidazole-free buffer “Buffer A” (8 M Urea, 50 mM sodium phosphate monobasic, 20 mM Tris base, 5 mM TCEP) pH 8.0; (ii) 500 mM imidazole buffer “Buffer B” (8 M Urea, 50 mM sodium phosphate monobasic, 20 mM Tris base, 5 mM TCEP, 500 mM Imidazole) pH 8.0; and (iii) isopropanol wash (20 mM Tris, 60% isopropanol) pH 8.0.

NOTE: pH for each buffer was adjusted with 5 N NaOH.

3.2. Equilibrate the His-column.

NOTE: For lab scale production, this protocol uses a 5 mL prepacked His-column, but any larger sized column can be used.

3.2.1. Open the FPLC software and click on the **New method** option. It will immediately open to the **Method settings** menu. Under the drop-down menu for column position choose **C1 port 3**.

3.2.2. On the **Shown by technique** drop down menu choose **affinity**. On the **Column type** drop-down menu choose **others, Histrap HP, 5 mL**. The column volume and the pressure boxes will be automatically set to the appropriate values.

3.2.3. Click on the **Method outline** button. Drag the following buttons from the **Phase library** popup menu: **equilibration**, **sample application**, **column wash**, and **elution** next to the arrow in that exact order. Close the **Phase library** menu.

3.2.4. Click on the **equilibration** button. The values listed in the table should be “initial buffer B” (4%), “final buffer B” (4%), and “volume (CV)” 5.

3.2.5. Click on the **Sample application** box. In the sample loading box click the radio button for **Inject sample on column with sample pump**. Make sure the box next to the **Use flow rate from method settings** is checked in the sample injection with system pump box. Next to the volume box on the right side of the screen change the value to **20 mL**.

3.2.6. Click on the **Column wash** button. The values listed in the table should be “initial buffer B” (4%), “final buffer B” (4%), and “volume (CV)” 5. Next to the fraction collection scheme unclick the **Enable box**.

3.2.7. Click on the **elution** button. The values listed in the table should be “initial buffer B” 0%, “final buffer B” 100%, and “volume (CV)” 5. Next to the fraction collection scheme click on the **Enable** box. Unclick the **Use fraction size from method settings** and adjust fraction size to 4 mL in the fill-in box below.

3.2.8. Click the **save as** button on the top of the of the software. Name the file “equilibration”.

3.2.9. Connect a 5 mL prepacked His-column to the corresponding column port 3 on the FPLC. Both pump A and pump B tubing as well as the sample pump tubing should be placed into 0.22 µm filtered deionized water. Run the equilibration program.

3.2.10 Place both pump A and pump B as well as the sample pump tubing into the imidazole-free buffer (Buffer A) and run the equilibration protocol again.

3.3. Bind the sample to the column and purify the protein.

3.3.1. Open the FPLC software and click on the **New method** option. It will immediately open to the **Method settings** menu. Under the drop-down menu for **Column position** choose **C1 port 3**. On the **Shown by technique** drop down menu choose **affinity**. On the column type drop-down menu choose **others, Histrap HP, 5 mL**. The column volume and the pressure boxes will automatically be set to the appropriate values.

3.3.2. Click on the **Method outline** button. Drag the buttons from the **Phase library** popup menu: **equilibration**, **sample application**, **column wash** (Wash 1), **column wash** (Wash 2), **column wash** (Wash 3), and **Elution** next to the arrow in that exact order. Close the **Phase library** menu.

3.3.3. Click on the **Equilibration** button. The values listed in the table should be "initial buffer B" 4%, "final buffer B" 4%, and "volume (CV)" 5.

3.3.4 Click on the **Sample application** box. In the sample loading box click the radio button for I**nject sample on column with sample pump**. Make sure the box next to the **Use flow rate from method settings** is checked in the sample injection with system pump box.

3.3.5. Next to the volume box on the right side of the screen change the value to 100 mL. Next to the fraction collection scheme click the **Enable** button. Unclick the **Use fraction size from method settings** box and then change the fraction size to 4 mL.

3.3.6. Click on the first column wash button (Wash 1). The values listed in the table should be "initial buffer B" 4%, "final buffer B" 4%, and "volume (CV)" 10. Next to the fraction collection scheme click the **Enable** box. Unclick the **Use fraction size from method settings** and then change the fraction size to 4 mL.

3.3.7. Click on the second column wash button (Wash 2). The values listed in the table should be "initial buffer B" 0%, "final buffer B" 0%, and "volume (CV)" 5. Next to the fraction collection scheme click the **Enable** box. Unclick the **Use fraction size from method settings** and then change the fraction size to 4 mL.

3.3.8. Click on the third column wash button (Wash 3). The values listed in the table should be "initial buffer B" 0%, "final buffer B" 0%, and "volume (CV)" 5. Next to the fraction collection scheme click the **Enable**. Unclick the **Use fraction size from method settings** box and then change the fraction size to 4 mL.

3.3.9. Click on the **Elution** button. In the table right click the information listed and, on the menu that comes up, click **Delete step**. Drag the isocratic gradient button onto the table twice, so that there are two entries.

3.3.10. The value for the first entry should read "initial buffer B" 30%, "final buffer B" 30%, and "Volume (CV)" 10. The value for the second entry should read "initial buffer B" 100%, "final buffer

B" 100%, and "volume (CV)" 10. Next to the fraction collection scheme click the **Enable** button. Click the box next to **Use fraction size from method settings**.

3.3.11. Click the **Save as** button on the top of the of the software. Name the file "purification". Pump A tubing of the FPLC should be placed into the imidazole-free wash buffer while pump B tubing should be placed into the 500 mM imidazole buffer. The sample pump tubing should be placed into the 100 mL sample.

3.3.12. Run the "purification" program and wait for the time when the 60% isopropanol is needed (Wash 2). Pause the program, move pump A tubing from the imidazole-free wash into the 60% isopropanol wash. Restart the program.

3.3.13. Once the isopropanol step is completed, pause the program again and move pump A tubing back into the imidazole-free wash buffer. Restart the purification program (the rest of the run is automated).

4. Purity assessment and protein identification by SDS-PAGE

4.1. Combine all the fractions corresponding to (i) flow-through (the cell lysate that did not bind to the His column), (ii) Wash 1, (iii) Wash 3 (60% isopropanol wash), and (iv) Wash 3 in separate 50 mL conical tubes. Do not combine the 2 mL fractions from the elution steps.

4.2. Mix 15 µL from each of the pooled fractions and all fractions from elution steps with 2x Laemmli sample buffer in a 0.5 mL microcentrifuge tube and denature them at 95 °C for 10 min.

4.3. While the protein denatures, set up the gel running apparatus with 3 stain-free 4–20% precast polyacrylamide gels in 1x Tris-glycine SDS-PAGE running buffer.

4.4. Load 8 µL of molecular weight marker to the first well and 30 µL of denatured sample to the other wells of the gel. Run the gels at 200 V until the dye front hits the bottom of the gel (about 30 min). Remove the gels from the apparatus and briefly rinse with deionized water. Image the gel immediately using the stain-free imaging system.

4.5. Identify the fractions that contain protein bands with the correct size (18.07 kDa). Pool all these fractions.

5. Protein identification by western blot

5.1. Run a western blot using an His-specific antibody (anti-6x HisTag) and a SHB-specific antibody (167-D-IV) to identity the purified full-length protein. The anti-6x HisTag antibody recognizes the N terminus of the protein and the 167-D-IV antibody recognizes the C terminus demonstrating the presence of the full-length protein.

5.2. Determine the protein concentration of the (i) flow-through, (ii) Wash 1, (iii) Wash 2 (60% isopropanol wash), (iv) Wash 3, and all fractions from the elution steps with the spectrometer instrument at an absorbance of 280 nm. Generate dilutions that contain 100 ng of protein in 15 µL of imidazole-free buffer for each of these groups.

5.3. Add 15 µL of 2x Laemmli sample buffer to each 15 µL of the samples and denature them as in step 4.1. Once the denaturing is completed spin down the tubes to ensure all the protein can be transferred.

5.4. Load samples and the pre-stained marker into a stain-free 4–20% precast polyacrylamide gel. Run the electrophoresis at 200 V until the dye front reaches the bottom of the gel.

5.4. While the gel runs, make 1 L of TBS-T (20 mM Tris, 150 mM NaCl, and 0.1% Tween 20) and 200 mL of 5% non-fat milk in TBS-T.

5.5. Use a western blot transfer system to transfer protein onto a nitrocellulose membrane. Use a pre-assembled transfer stack and place the gel on it. Set up the system to run at 25 V for 7 min. Check for the presence of the pre-stained marker on the nitrocellulose membrane indicating a complete transfer.

NOTE: All the subsequent steps are performed on an orbital shaker set at 100 rpm at room temperature (RT).

5.6. Once the transfer is completed, wash blots two times with TBS-T for 10 min each.

5.7. Block the nitrocellulose membranes (blot) with 5% non-fat milk in TBS-T for at least 1 h. Wash blots two times with TBS-T for 10 min each.

5.8. Dilute the primary 167-D-IV and anti-6x HisTag antibodies to 1 mg/mL in TBS-T (stock Ab). Dilute the stock Abs of the 167-D-IV 10,000-fold and the anti-6x HisTag 5,000-fold by adding 2 µL of the stock 167-D-IV and 4 µL of the stock anti-6x HisTag antibodies to two different tubes containing 20 mL of TBS-T. Add the total 20 mL volume of primary antibodies, one to each blot, and incubate blots for 1 h. Wash blots two times with TBS-T for 10 min.

5.9. Dilute 4 µL of 1 mg/mL of the mouse anti-human secondary antibody conjugated with alkaline phosphatase in 20 mL of TBS-T (1:5,000 dilution). Dilute 4 µL of 1 mg/mL of the goat anti-mouse secondary antibody conjugated with alkaline phosphatase in 20 mL of TBS-T (1:5,000 dilution). Add secondary antibodies to corresponding blots.

NOTE: The anti-human secondary antibody binds to 167-D and the anti-mouse binds to the anti-6x HisTag. Wash blots two times with TBS-T for 10 min.

5.10. Add enough BCIP/NBT alkaline phosphatase substrate to cover the blots. Develop the blots for about 10 min until bands appear. Rinse blots with cold tap water and let them dry before scanning with a flatbed scanner.

6. Refolding the SHB-SAPN

6.1. Add the pooled protein (10–20 mL total) to a 10 kDa molecular weight cut off dialysis cassette and dialyze it into 8 M urea, 20 mM Tris, 5% glycerol, 5 mM TCEP pH 8.5 at RT (18–26 °C) overnight.

6.2. Slowly dialyze the urea off the sample by decreasing the urea concentration in the dialysis buffer stepwise by 2 M every 2 h. At a urea concentration of 2 M, move the dialysis apparatus to 4 °C (do not use TCEP in the dialysis buffer from this step). Finish the refolding by dialyzing the sample into 120 mM urea, 20 mM Tris, 5% glycerol, pH 8.5 at 4 °C overnight.

6.3. Remove the refolded protein (SHB-SAPN) from the dialysis cassette. Filter the SHB-SAPN using a 0.22 µm polyvinylidene fluoride (PVDF) syringe filer. Aliquot SHB-SAPN into sterile tubes and freeze them at -80 °C, leaving at least 100 µL at RT for subsequent analyses.

7. Validation of particles by size and appearance

7.1. Dynamic light scattering (DLS)

7.1.1. Measure the mean particle size of the SHB-SAPN by the following parameters: select protein as the material, create a complex buffer for 120 mM urea, 20 mM Tris, 5% glycerol 25 °C for temperature, select disposable cuvettes for the analysis, select automatic measurement, set for 5 runs.

7.1.2. Add 45 µL of SHB-SAPN to a disposable cuvette and run the software by clicking on the green arrow. Select the percentage volume for the readout.

7.2. Nanoparticle tracking analysis (NTA)

7.2.1. Dilute the sample by 1:20 in the refolding buffer. Make 10 mL of diluted sample.

7.2.2. Using 3 mL syringes, flush the NTA instrument with the refolding buffer and load about 1.5 mL of sample to equilibrate the instrument. Use rest of the sample for the analysis.

7.2.3. Create a new SOP in the NTA software by clicking on the **SOP** tab. Under the tab change the number of captures to 3 and change the capture time to 30 s. Press the **autofocus** button on the left side of the screen to bring the sample into focus. Use the manual focus knob on the side of the machine to fine tune the focus.

7.2.4. Run the created SOP, when the system prompts load a small volume of sample with the syringe. After the system has taken all the captures it will automatically bring up the analysis screen. Slide the detection limit bar so that all the real particles are marked with red crosses. Press the run analysis button and the analysis will automatically begin.

7.3. Transmission electron microscopy (TEM)

7.3.1. Glow discharge formvar/carbon 400 mesh copper TEM support films.

7.3.2. Add 3 µL of sample at a 0.075 mg/mL concentration to the grid for 30 s. Wick off the liquid using a filter paper.

7.3.3. Wash the grid with 3 µL of deionized water three times, each time wicking off the water with filter paper.

7.3.4. Add 3 µL of 0.5% uranyl acetate to the support film and allow it to sit for 30 s. Wick off most of the uranyl acetate but leave a thin film on the surface. Allow the samples to dry before imaging them on the transmission electron microscope.

7.3.4. Image samples at 80 kV on a TEM.

8. Determination of endotoxin levels in the samples using a kinetic limulus amoebocyte lysate (LAL) assay

8.1. Remove the kit and samples from the refrigerator and allow to equilibrate to RT.

8.2. To perform this assay, a plate reader with a heat block and the ability to read the samples for 40 reads at a wavelength of 405 nm at 37 °C is required. Write a program template so that wells are read every 150 s to identify the onset time point (the OD increased by 0.2 in comparison with the first read).

8.2. Dilute the sample to the immunization dose concentration in PBS.

NOTE: The pH of the refolding buffer is outside the range of the LAL assay. The dilution of the sample in PBS will set the pH to the acceptable range.

8.3. Resuspend the control endotoxin in the appropriate volume of endotoxin-free LAL water as determined by the certificate of analysis to generate 50 EU/mL. Vigorously vortex the vial for 15 min to ensure complete resuspension of the endotoxin.

8.4. Generate the endotoxin standard curve by preforming a 10-fold serial dilution in glass vials in the range of 50 EU to 0.005 EU/mL. For each dilution, add 0.1 mL of the previous dilution to 0.9 mL of LAL water. Vortex vigorously after combination for 1 min.

8.5. Add standard curve dilutions and SHB-SAPN samples in duplicate to a 96-well plate. Use LAL water as a negative control in duplicate as well. Preincubate the plate at 37 °C for 15 min.

8.6. Towards the end of the incubation, resuspend the assay reagent vial with 2.6 mL of LAL water. Gently mix content with a serological pipette.

8.7. Add 100 µL of the assay reagent to each well of the 96-well plate. Quickly move the plate to the plate reader and run the program template written in step 8.2.

8.8. Once the program is completed, generate a standard curve using the log value of the controls versus the log value of the onset time. Use the formula generated from this curve to calculate the endotoxin concentration in the samples.

REPRESENTATIVE RESULTS:

The fully assembled SHB-SAPN shown here is built upon protein sequences (**Figure 1A**) that are predicted to fold into a particle that contains 60 copies of the monomer (**Figure 1B**). **Figure 2** provides an outline of the method for the production, purification, and identification of the SHB-SAPN core. *E. coli* from a glycerol stock that contained a pPep-T expression vector with gene sequence of the SHB-SAPN core were induced in BL21 (DE3) *E. coli.* Bacterial cells were successfully grown and lysed under denaturing and reducing conditions.

Total cell lysate was used to purify SHB-SAPN monomers by FPLC using a Ni^{2+} column (**Figure 3A**). The FPLC chromatograph demonstrates that protein eluted both at 150 mM and 500 mM imidazole (**Figure 3A**). The chromatogram also shows two other peaks at 185 mL and 210 mL total volume corresponding to the isopropanol wash and the imidazole-free wash, respectively. The fractions and the purity of the recombinant protein were identified by gradient SDS-PAGE gels (**Figure 3B**). The protein of interest was primarily located in fractions 68–79 (278–300 mL total volume). These fractions were combined for further analyses. Western blot with anti-His antibody (N-terminal) and 167-D-IV antibody (C-terminus) indicated that the pooled fractions were indeed the protein of interest (**Figure 4A,B**). These blots also demonstrated the presence of the SHB-SAPN multimers. Earlier washes and elution fractions tended to contain a higher concentration of multimerized protein and were therefore excluded.

The samples that contained the protein monomers of interest were folded into the fully assembled SHB-SAPN by dialysis. Particle size distribution was determined by DLS and nanoparticle tracking analysis (**Figure 5A,B**). The DLS identified particles with a Z-average hydrodynamic diameter of 67 nm while the NTA system measured a mean size of 81 nm. The slight size differences were due to the particle sizing techniques, however the size from both analyses were in the expected range of 20–100 nm[8,24,25]. SHB-SAPNs were visualized by TEM and the images showed well-formed individual particles with the size distribution obtained from the two particle sizing techniques (**Figure 5C**).

During the purification of the protein, the column was washed with isopropanol to decrease the LPS contamination in the final SHB-SAPN product. To verify if the endotoxin level was acceptable

for immunization, the concentration of LPS in SHB-SAPN samples purified with or without the isopropanol wash step was determined by a kinetic LAL assay. The results indicated that the isopropanol wash decreased the endotoxin levels from >0.25 EU/μg to 0.010 EU/μg of SHB-SAPN protein (**Table 1**).

FIGURE AND TABLE LEGENDS:

Figure 1: SHB-SAPN protein sequence and structure. (**A**) The amino acid sequence of the SHB-SAPN monomer. (**B**) Computer model of the structure of the fully assembled SHB-SAPN core consisting of 60 protein monomers. Color scheme for amino acid sequences: Grey = HisTag; Green = pentamer; Dark blue = de novo designed trimer; Light blue = six-helix bundle.

Figure 2: Flowchart of the protocol for SHB-SAPN production. Color scheme: Black = expression of the protein monomer in *E. coli*; Dark grey = monomer purification; Medium grey = monomer identification; Light grey = refolding and characterization; White = fully assembled SHB-SAPN product. In steps labeled with dark and medium grey, the protein is under denaturing and reducing conditions.

Figure 3: Protein purification of the SHB-SAPN monomers. (**A**) Chromatograph from the FPLC purification. Green line above the chromatogram indicates the purification steps. Blue line in the chromatogram represents the optical density of the fractions at 280 nm wavelength. The black line shows what percent of buffer B (8 M urea, 20 mM Tris, 50 mM sodium phosphate monobasic, 5 mM TCEP, 500 mM Imidazole, pH 8.5) that was used in each stage of the purification. (**B**) SDS-PAGE gels of the pooled fractions from the lysate (L), flow through (FT), first wash (W1), isopropanol wash (W2), third wash (W3), and individual fractions from the 150 mM imidazole (E150) and 500 mM imidazole (E500) elution steps of the purification. Molecular markers in the first lane (M) identify bands between 10 and 250 kDa. Target protein is indicated by a black arrow.

Figure 4: Identification of the protein by western blot. All lanes are loaded with 100 ng of protein. (**A**) Results of a western blot with anti-6x HisTag. (**B**) Results of a western blot with a 167-D-IV HIV-1 monoclonal antibody. Lanes are labeled as: M = molecular weight marker; 1 = lysate; 2 = Flow through; 3 = first wash; 4 = isopropanol wash (second wash); 5 = third wash; 6 = pooled volume fractions 56–61 (first elution peak), 7 = pooled volume factions 62–67 (between the two peaks), 8 = pooled volume fractions 68–78 (second elution peak). Target protein with the expected band size of 18.07 kDa as the monomeric SHB-SAPN band is indicated by a black arrow. Extra banding in lanes 7 and 8 are dimers, trimers, and multimers of the SHB-SAPN (red arrow).

Figure 5: Characterization of the refolded SHB-SAPN. (**A**). Particle size distribution as determined by DLS. (**B**) Particle size as determined by nanoparticle tracking (system). (**C**) Visualization of the SHB-SAPN particles by TEM.

Table 1: Endotoxin levels of refolded SHB-SAPNs. Endotoxin levels in SHB-SAPN samples purified with or without an isopropanol wash presented both as endotoxin units/mL and endotoxin units/μg of SHB-SAPN protein.

DISCUSSION:

Nanotechnology provides many advantages and solutions for subunit vaccine development. Nanovaccines can repeatedly present antigens as particulates to the host immune system increasing immunogenicity[26]. While there are many different types of nanovaccines, we believe that ones composed of de novo designed protein seem to be the strongest approach for vaccine development[1]. They can be engineered without any sequence homology to the host proteins and present the antigen of interest in close to native-like conformation while providing low production cost and high product yields. A prime example of this approach is the SAPN technology, which we have applied to vaccines against multiple infectious diseases[7]. Addressing the difficulties in HIV-1 vaccine development, we have engineered a unique SHB-SAPN core to effectively present the V1V2 antigen in a native-like trimeric conformation[9]. Many vaccine targets, particularly for viral diseases, are present as trimers[27]. This phenomenon indicates that our SAPN design has wide implications for the development of subunit vaccines.

In this method, we demonstrate how to produce SHB-SAPNs in an *E. coli* expression system. We expressed high yields of protein (about 6 mg/100 mL of culture). The protein contained 10 histidines and was easily purified using an immobilized metal affinity chromatography with Ni^{2+} column. This length of the His-Tag was found to be the optimal for the highest protein yield. The purified protein contained the full-length of the designed protein as indicated by the presence of both the N-terminal HisTag and the C terminal heptad repeat. We utilized widely accepted techniques and optimized them for the expression, production, and characterization of the SHB-SAPN core. Lack of the production of the full-length protein during the development of a SAPN containing a new protein epitope could indicate an expression problem of the gene in the host cell. If it happens, the gene and the expression system must be redesigned and adapted to the described protocol. Modification of sonication time or intensity may also increase the concentration of the predicted full-length protein.

Refolded particles were in the expected size range (20 to 100 nm)[8,24,25] as determined by DLS and nanoparticle tracking analysis. These results were further confirmed by using TEM. If there are problems in this step, it is normally due to a problem with the pH or ionic strength of the refolding buffer. When large size particles are detected on the particle sizing techniques, it indicates aggregation, which can be avoided by increasing the pH of the refolding buffer. If the particles are not detected by DLS, verify the concentration of the protein and check the pH of the buffer. The final protein concentration for DLS should be at least 100 µg/mL. If the concentration is not the problem, it indicates the abundance of small, incompletely formed particles, whose concentration can be reduced by decreasing the pH. Alternatively, the sodium chloride concentration can be adjusted to the optimum range to minimize the presence of particles with unwanted size.

Finally, by using an isopropanol wash step during purification we were able to reduce contaminating LPS from the host *E. coli* to 0.01 EU/µg of SAPN which is below the Food and Drug Administration (FDA) limit of 5 EU/kg of body weight for injectable products[28]. This level can be further reduced by using an anion exchange column also known as Q column. If high levels of

endotoxin are still present, check all materials that were used for buffer preparation. Remember to use only depyrogenated glassware and endotoxin free plasticware in this method.

These results indicate that we have successfully developed a method to produce the SHB-SAPN core that can be used for pre-clinical immunization studies. This method with only slight modifications, if any, can be applied to the purification of SHB-SAPNs when an antigen of interest is added. Using this method as a starting point one of the major changes is in the elution step. Different proteins elute at different imidazole concentrations that must be determined experimentally. The other major difference might be the composition of the refolding buffer. Optimization would require testing different pH conditions as well as ionic strengths.

In consideration of future work, only two slight modifications are needed to allow human application of the SHB-SAPN. The first is that the expression vector needs to be changed to a kanamycin resistance selectable marker due to the ampicillin allergy in humans[29]. The other major requirement of the protein manufacturing for human use is to produce the SHB-SAPN in animal product-free media. A small-scale study already indicated a reasonable yield of protein in a plant-based media. The work presented here is easily scalable for ultimate GMP production as demonstrated with a malaria vaccine candidate, FMP014[16]. This large scale FMP014-SAPN production included both the anion exchange and the cation exchange steps to further reduce LPS and Ni^{2+} content from the final product. This bacterial-expressed SAPN has been already scaled up for an upcoming Phase 1/2a clinical trial.

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DISCLOSURES:

The views expressed are those of the authors and should not be construed to represent the positions of the US Army or the Department of Defense. Peter Burkhard has an interest in the company called Alpha-O Peptides AG and has patents on the technology. The other authors have no affiliation or financial involvement with any company with a financial interest on the subject matter presented in this paper.

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A

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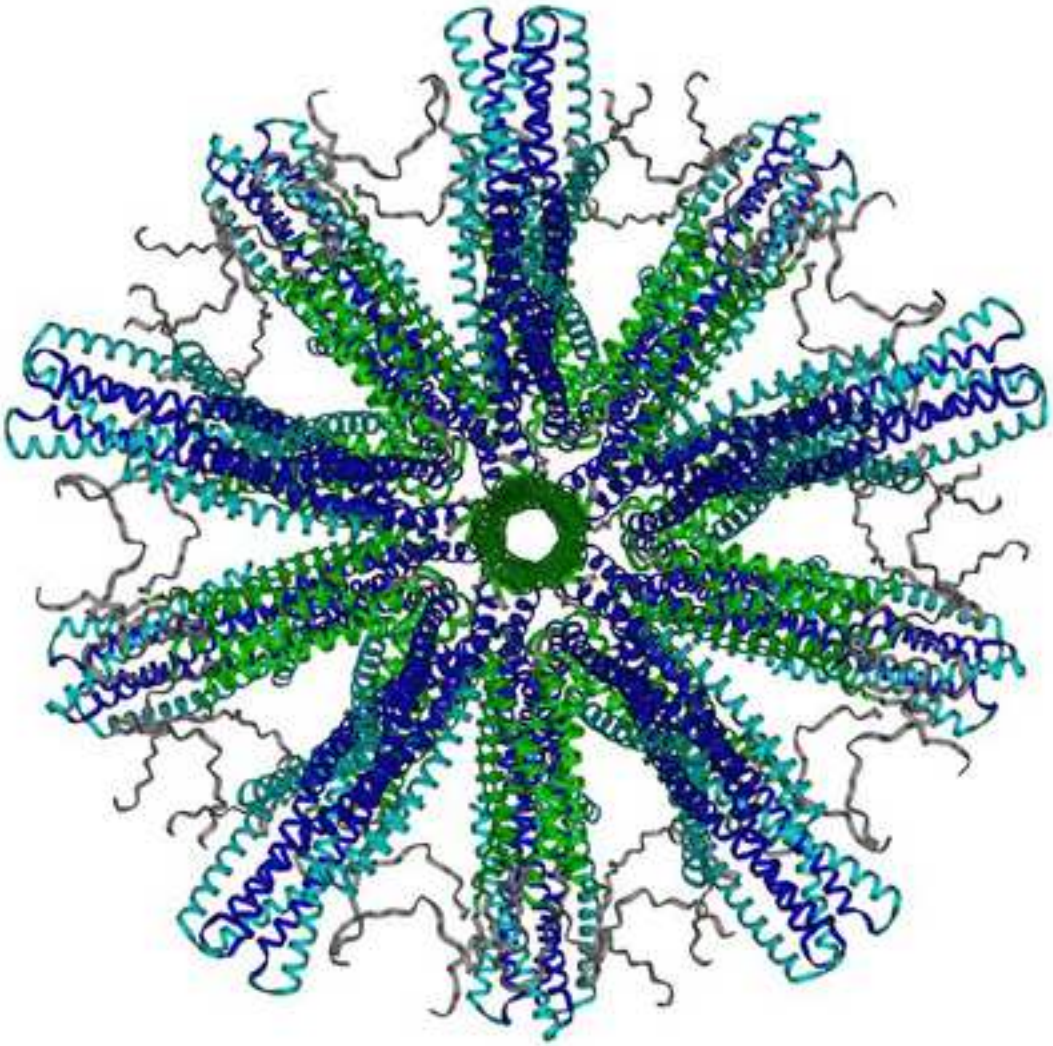

Figure2

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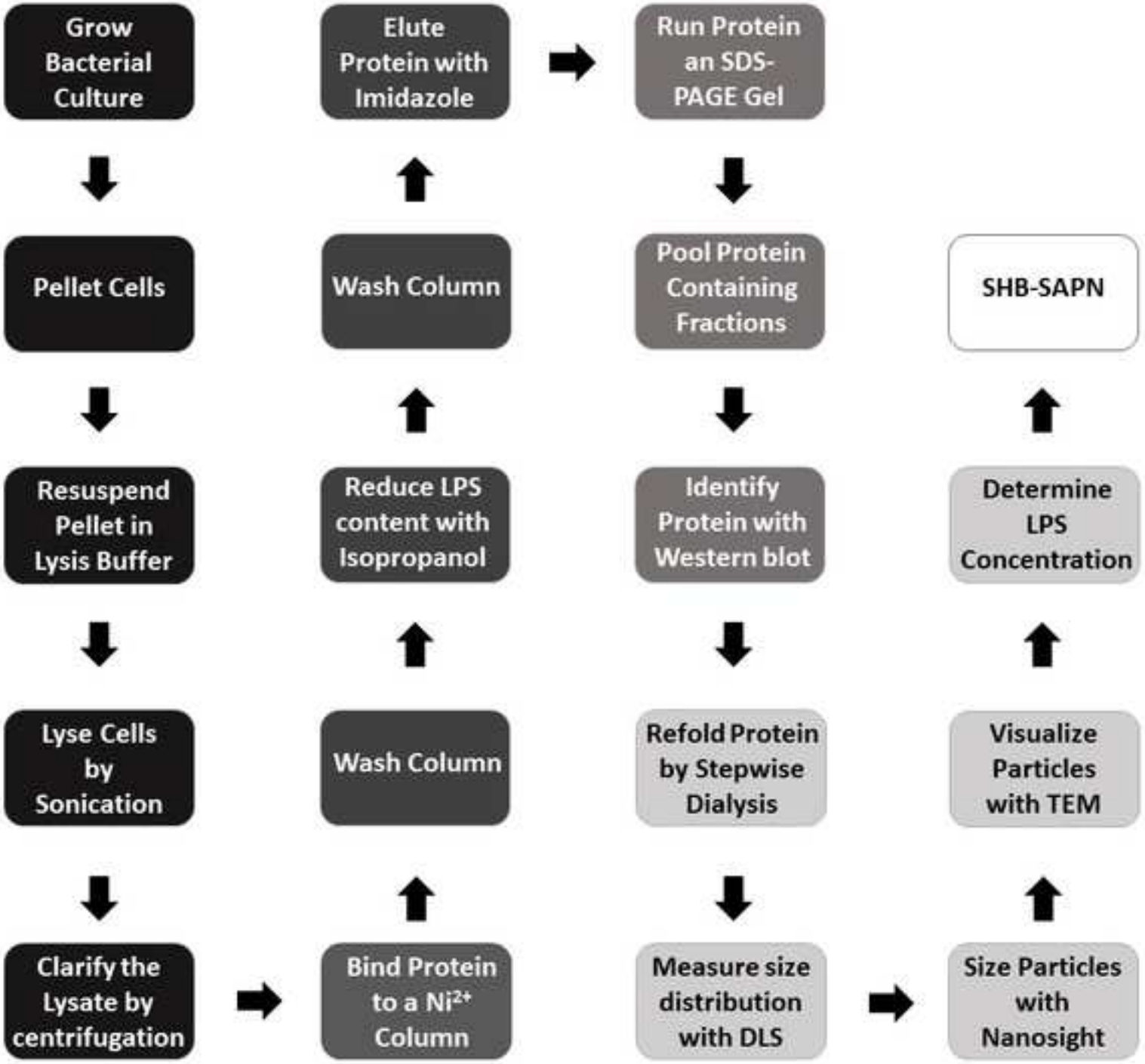

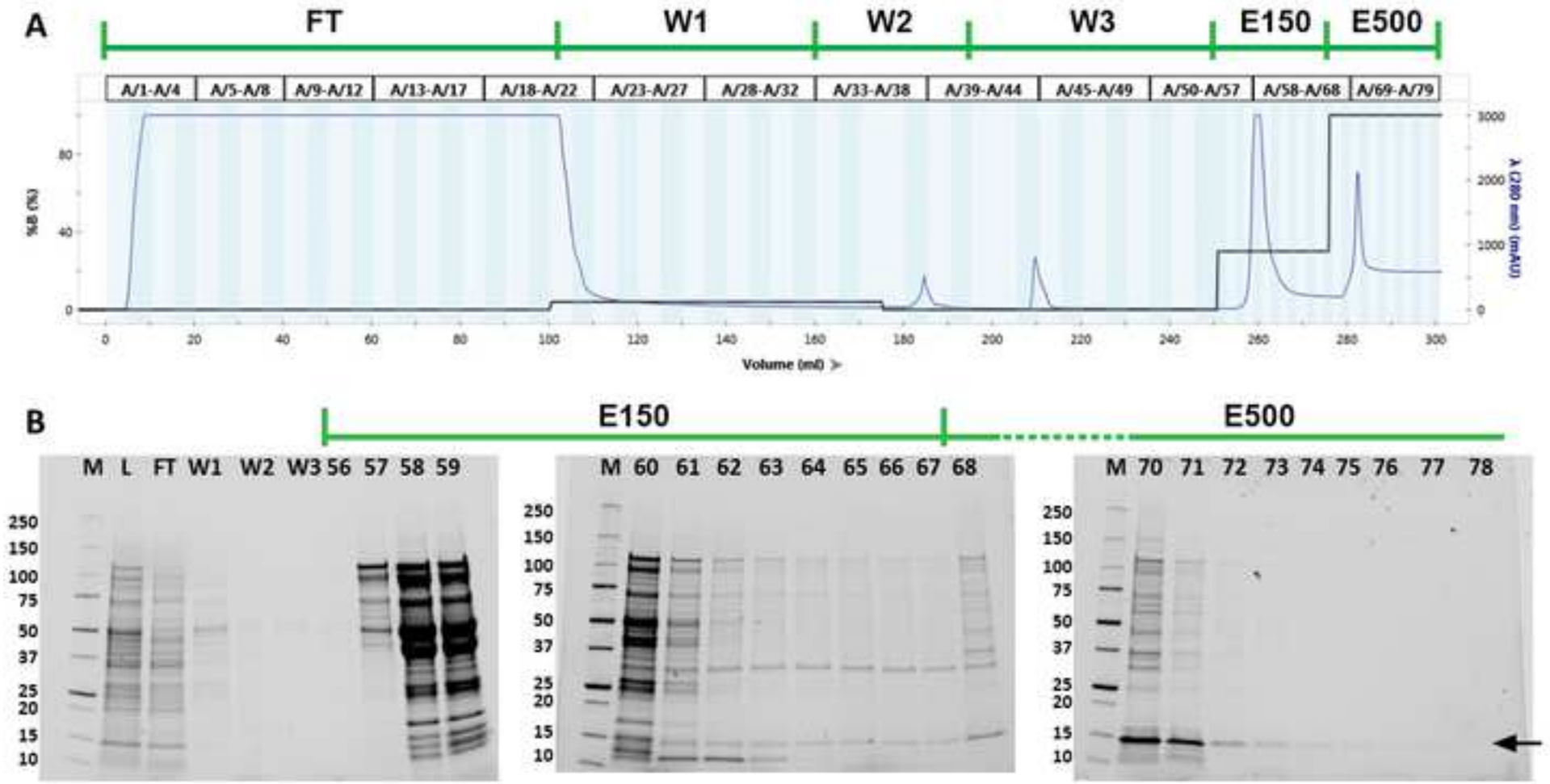

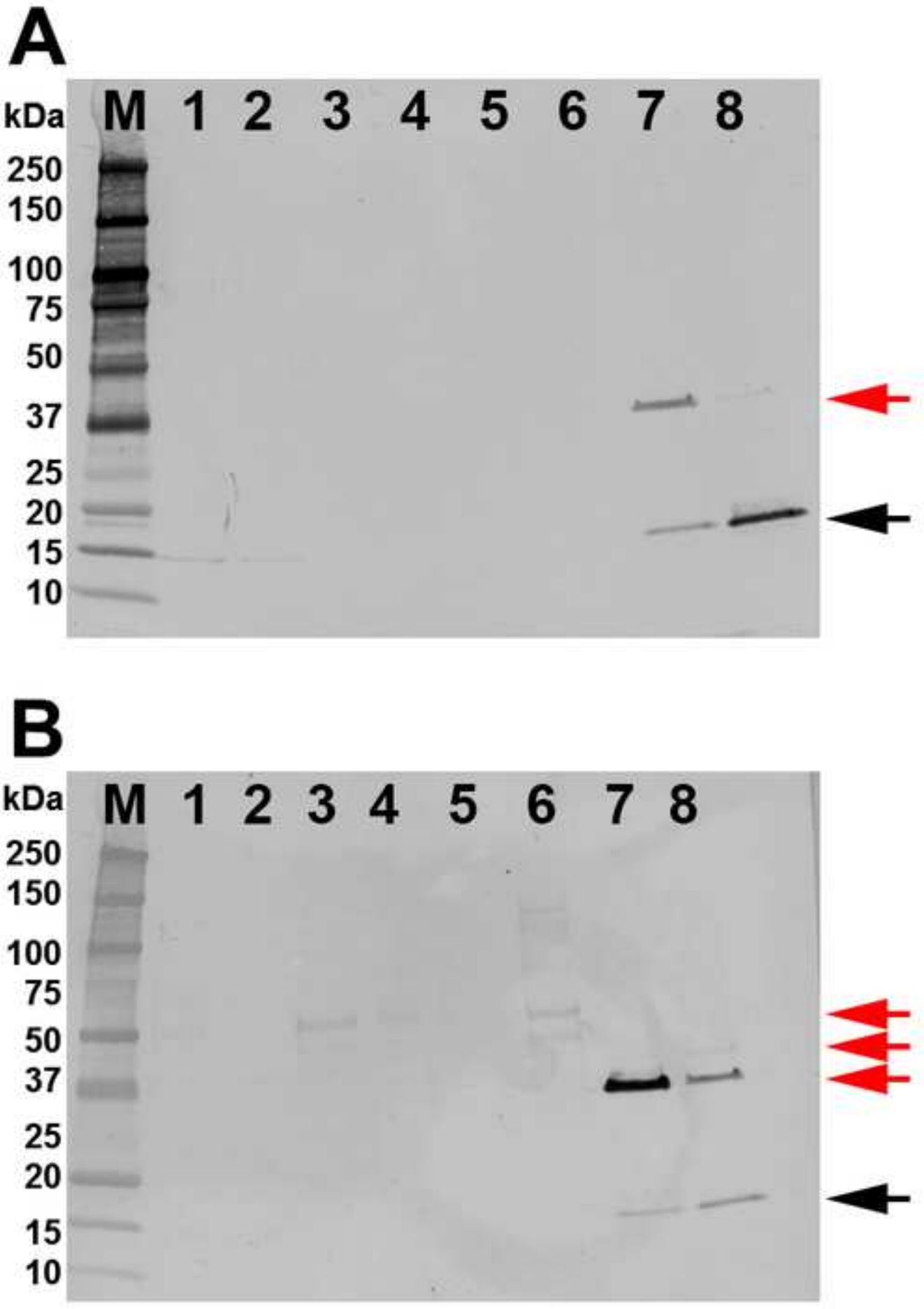

Figure5

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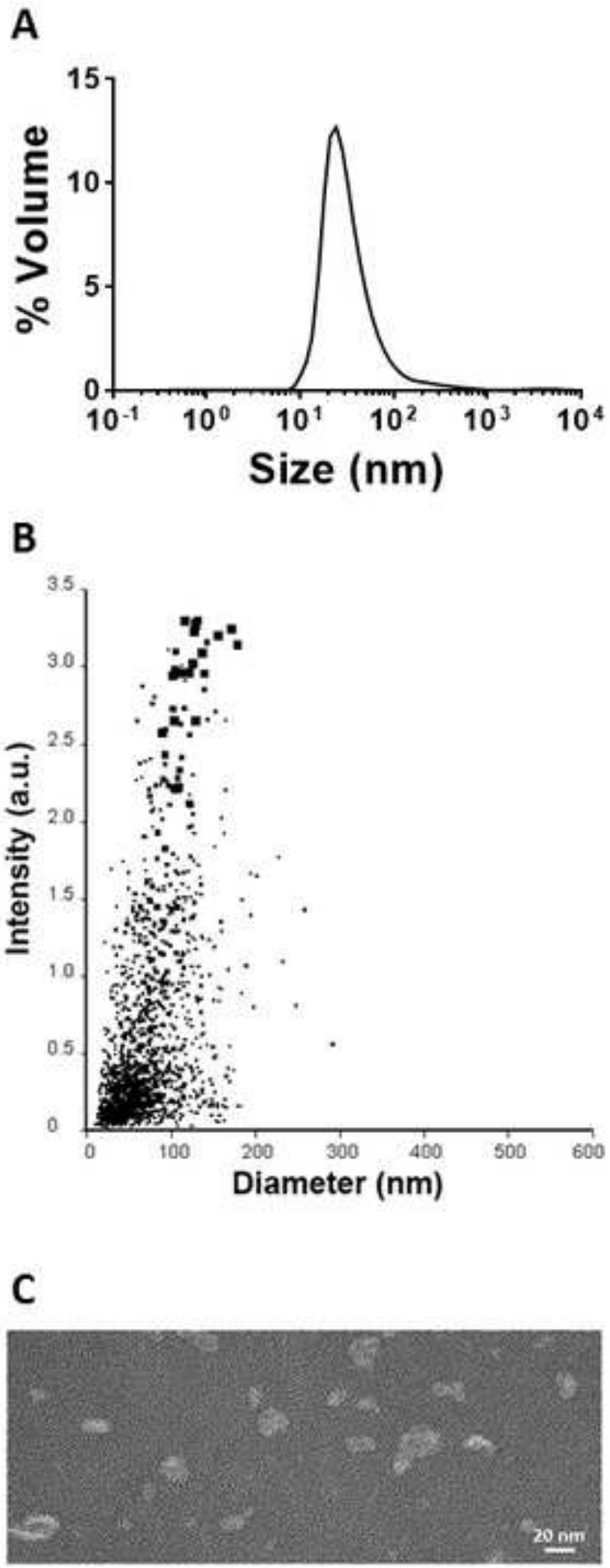

Sample	Endotoxin (EU/mL)	Endotoxin (EU/µg of Protein)
SAPN with Isopropanol Wash	2.02	0.01
SAPN without Isopropanol Wash	>50	>0.25
Negative Control	Below detection level	N/A

Table of Materials

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Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
10x Tris/Glycine/SDS	BioRad	1610732	1 L
2-Mercaptoethanol	BioRad	1610710	25 mL
2-propanol	Fisher	BP26181	4 L
2x Laemmli Sample Buffer	BioRad	1610737	30 mL
40ul Cuvette Pack of 100 with Stoppers	Malvern Panalytical	ZEN0040	100 pack
4–20% Mini-PROTEAN TGX Precast Protein Gels, 10-well, 30 µl	BioRad	4561093	10 pack
Ampicillin	Fisher	BP1760-25	25 g
Anti-6X His tag antibody [HIS.H8]	AbCam	ab18184	100 µg
Anti-HIV-1 gp41 Monoclonal (167-D IV)	AIDS Reagent Repository	11681	100 µg
BCIP/NBT Substrate, Solution	Southern Biotech	0302-01	100 mL
Corning Disposable Vacuum Filter/Storage Systems	Fisher	09-761-108	A variety of sizes
Formvar/Carbon 400 mesh, Copper approx. grid hole size: 42µm	Ted Pella, Inc	01754-F	25 pack
GE Healthcare 5 mL HisTrap HP Prepacked Columns	GE HealthCare	45-000-325	5 pack
Glycerol	Fisher	BP229-4	4 L
Goat Anti-Mouse IgG H&L (Alkaline Phosphatase)	ABCam	ab97020	1 mg
Imidazole	Fisher	O3196-500	500 g
Instant NonFat Dry Milk	Quality Biological	A614-1003	10 pack
Kinetic-QCL Kinetic Chormogenic LAL Assay	Lonza Walkersville	50650U	192 Test Kit

LAL Reagent Grade Multi-well Plates	Lonza Walkersville	25-340	1 plate
Magic Media *E. coli* Expression Medium	ThermoFisher	K6803	1 L
MilliporeSigma Millex Sterile Syringe 0.22 μm Filters	Millipore	SLGV033RB	250 pack
Mouse Anti-Human IgG Fc-AP	Southern Biotech	9040-04	1.0 mL
One Shot BL21 Star (DE3) Chemically Competent *E. coli*	ThermoFisher	C601003	20 vials
Precision Plus Protein Unstained Protein Standards, Strep-tagged recombinant,	BioRad	1610363	1 mL
Slide-A-Lyzer Dialysis Cassettes, 10K MWCO, 12 mL	ThermoFisher	66810	8 pack
Sodium Chloride	Fisher	BP358-212	2.5 kg
Sodium Phosphate Monobasic	Fisher	BP329-500	500 g
Tris Base	Fisher	BP152-1	1 kg
Tris-(2-carboxyethyl)phosphine hydrochloride	Biosynth International	C-1818	100 g
Uranyl Acetate, Reagent, A.C.S	Electron Micoscopy Services	541-09-3	25 g
Urea	Fisher	BP169-500	2.5 kg
Whatman qualitative filter paper	Sigma Aldrich	WHA10010155	pack of 500

Equipment	**Company**	**Catalog Number**	**Comments/Description**
ChromLab Software ver 4	BioRad	12009390	Software
Epoch 2 Microplate Spectrophotometer	BioTek	EPOCH2	Plate Reader

Fiberlite F14-14 x 50cy Fixed-Angle Rotor	ThermoFisher	096-145075	Rotor
Gel Doc EZ Gel Documentation System	BioRad	1708270	Gel Imager for Stain free Gels
JEOL TEM	JEOL	1400	Transmission Electron Microscope
Mini-PROTEAN Tetra Vertical Electrophoresis Cell for Mini Precast Gels	BioRad	1658004	To run gels
NanoDrop One Microvolume UV-Vis Spectrophotometer	ThermoFisher	ND-ONE-W	For Protein Concentration
NanoSight NS300	Malvern Panalytical		Particle Sizing
NanoSight NTA software NTA	Malvern Panalytical		Particle Sizing
New Brunswick Innova 44/44R	Eppendorf	M1282-0000	Incubator/Shaker
NGC Quest 10 Chromatography System	BioRad	7880001	FPLC to aid in protein purification
PELCO easiGlow Glow Discharge Cleaning System	Ted Pella, INC	91000S	To clean grids
PowerPac Universal Power Supply	BioRad	1645070	To run gels
Rocker Shaker	Daigger	EF5536A	For Western
Sonifer 450	Branson	also known as 096-145075	Sonicator
Thermo Scientific Sorvall LYNX 4000 Superspeed Centrifuge	ThermoFisher	75-006-580	Centrifuge
Trans-Blot Turbo Mini Nitrocellulose Transfer Packs	BioRad	1704158	For Western
Trans-Blot Turbo Transfer System	BioRad	1704150	For Western
Vortex-Genie 2	Daigger	EF3030A	Vortex

Zetasizer Nano ZS	Malvern Panalytical		Particle Sizing
Zetasizer Software	Malvern Panalytical		Particle Sizing

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Signature:  Date: 04/02/2019

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Dear Dr. Singh,

Thank you very much for the editorial and peer-review of our manuscript, entitled "Production of *E. coli*-expressed Self-Assembling Protein Nanoparticles for Vaccines Requiring Trimeric Epitope Presentation" for publication in JoVE Bioengineering as an open access video article. We addressed all the questions that were raised by the reviewers and highlighted our answers and the changes according to the reviewers' comments with red color in the manuscript. We thank the reviewers for their comments that contributed to the improvement of our manuscript. We submitted each figure in 300 dpi resolution.

Best regards,

Zoltan Beck, Ph.D.
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Editorial Comments:

- Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

  We have proofread the manuscript.

- Protocol Detail: 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 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.

  To avoid branding we had moved the instrument and software identifications to the materials and reagent table. Although the main point of the video will not be the use of software of FPLC, we added more specific details (e.g. "button click" actions and numerical settings) to protocol.

- Protocol Numbering: There must be a one-line space between each protocol step.

  We have inserted one-line between steps.

- Protocol Highlight: After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer

than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

We have highlighted about 2.5 pages in yellow to identify the steps for visualization

- Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

  The discussion covers points 1-5

- Commercial Language: JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are magic media, magic media, ChromLab, HisTrap HP, Trans-Blot Turbo Transfer, Trans-Blot Turbo Mini Nitrocellulose Transfer Packs, Zetasizer software 7.12, NanoSight, Kinetic-QCL, etc.

  We have located and replaced the commercial sounding language in the manuscript with generic names where it was possible. All commercial products are referenced in the table of materials. We also have removed the registered trademark symbols from the table of materials.

- If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher.

  All figures and tables are original and have not been published previously

Reviewer #1:

Major Concerns:

1. In Protocol 3.3.3., the authors suggest the use of 60% isopropanol to wash the HisTrap column after capturing the protein target. The authors explain that this step helps removing endotoxins from the protein target, however, 60% isopropanol treatment seems extremely harsh for proteins. Even for the very stable proteins (e.g. monoclonal antibodies) isopropanol of greater than 15% causes irreversible denaturation (Bobaly et al., J. Pharm. Biomed. Anal. 2016). There are many other ways to remove endotoxins from protein solutions with much less harmful conditions by using surfactant detergents and/or cationic

polymers (Petsch et al., J. Bacteriol. 2000). I am wondering if the authors tried other conditions instead of 60% isopropanol?

In the manuscript we discussed the use of a Q-column as an alternative method or a further purification step to reduce the endotoxin. Previously, we have attempted multiple techniques for SAPNs, but the isopropanol wash worked the best. The reviewer is right that 60% isopropanol could be harsh for some protein, but we could not see any evidence for irreversible denaturation. Our protein was capable of refolding after the 60 % isopropanol wash as the DLS and electron microscopy images demonstrated. We have been successfully using this approach for many years (Karch *et al., J Nanobiotechnology*, 2017; Li *et al., PLoS One.* 2018; Seth *et al, Vaccine* 2017, Kaba *et al., Vaccine 2018*, El Bissati *et al., NPJ Vaccines*, 2017, and Karch *et al., Nanomedicine* 2017).

2. In Discussion, the authors mention that the sonication may lead to the protein degradation and expression problem of the gene in the host cell. What exactly is the problem in the host gene expression? Did the author mean fragmented host genomic contamination? Also have the authors tried other methods for lysing E. coli cells (e.g. French press and/or chemical lysis)?

   For lab scale production sonication is the most convenient technique due to the size of the culture and the availability of equipment. We have previously used a microfluidizer to lyse *E. coli* in the large-scale production of SAPNs (Seth *et al, Vaccine* 2017), which resulted in no difference in biophysics or immunogenicity when the cells were sonicated (Kaba *et al.*, Vaccine 2018. Sonication has no relation to the gene expression. We believe that this protocol will be used with various proteins displayed on the SAPNs. The comment about the problem of gene expression relates to the troubleshooting steps. During the optimization of the production of the new protein, if no protein is obtained, one of the potential problems could be the gene expression. The text of the manuscript has been revised accordingly.

Minor Concerns:

1. In Protocol 4.3., 200 mV should be 200 Volts instead.

   Thank you for calling our attention to this typo. The protocol has been updated to reflect this change.

2. For Figure 3A, the image looks a little blurry and it might be hard for readers to understand what is shown. I would suggest adding label on each step (e.g. sample loading, wash with xx mM imidazole, elution with xx mM imidazole, column regeneration, etc). It would also be helpful to indicate the peak that contains the product.

   We tried to increase the image quality of Figure 3A, but please note that the image is generated by the software of the FPLC. We will work with the journal's production team

together to make the highest resolution possible. We added additional labels on Figure 3A. We also indicated the peak that contains most of the product.

3. For Figure 3B, place arrow to indicate the target protein.

   The arrows have been now added indicating the protein of interest.

4. For Figure 4A, it appears that the lane 7 and 8 are positive from the image. This may just be the incorrect gel cropping.

   The reviewer is right, the lane markers were slightly off, which probably occurred during saving the file. Lanes have been adjusted correctly in the new version of figure 4A.

5. For Figure 4A and B, place arrow to indicate the target protein.

   The arrows have been now added indicating the protein of interest.

Reviewer #2:

Major Concerns:

Lack of a method to detect correct formation of trimeric antigen epitope(s).

The reviewer is right about the lack of a method to detect trimeric formation. However, it was not the scope of this manuscript. The purpose of this protocol was to describe the production and purification method of the core of the self-assembling protein nanoparticles that can be used for various antigens. We did not use any specific antigen in this work. The trimeric presentation is strongly dependent on the antigen and design of the construct. In our previous study (Karch *et al.*, *Nanomedicine*, 2019), using an HIV-1 Env antigen, we provided methods to detect the correct formation of the trimeric V1V2 epitope.

Minor Concerns:

Overall, numerous details missing.

Reviewer is right. We have included more details in the revised manuscript

Specific reviewer comments:

Protocol section, part 1.1, should specify the origin/manufacturer of Magic Media.

This information is included in the reagents/materials table according to the journal's instructions.

1.2 Specify origin of pPep-T vector.

We have added a reference to specify the origin of the used expression vector (Babapoor *et al. Influenza Research and Treatment*, 2011). We removed the name of the pPep-T vector, because other expression vectors can be also used.

And, 200 rotations per minute is not informative if we don't know the size of incubator rotation radius.

The information on the incubator is in the reagents/materials table.

1.3. 4000g in fixed angle or horizonal centrifuge? Temperature?

The centrifugation was performed with a fixed angle rotor at 4^{o}C. We have added this change to the text.

2 Note: 'bonds' not 'bounds'

Thanks for calling our attention to this typo. “Bounds” has been fixed in the text.

2.1 - use Tris buffer, is that Tris-HCl or Trizma base? How is the pH adjusted? Same query applies to parts 3.1 etc. (I see Tris base specified in the table page 20-22, perhaps also mention in main text).

Tris base has been used and the pH was adjusted for “buffers” with 5 N NaOH. Text has been updated.

2.2 - for a 40mL volume, what sort/size of sonicator probe should be used?

We used the probe that comes with the instrument. (Cat: 101-147-040). It’s referred to in the manual as the standard solid disruptor horn. It has also been listed as a separate item in the reagents/materials table now.

2.2 and 2.3. Please specify how many tubes are being processed. i.e. in step 1, two pellets were generated, each from approx. 50mL, from the 100mL total. Now in step 2.2 and 2.3, how many tubes are being processed. Is it necessary to use 1 x 150mL sterile flask for each '50mL-pellet' being processed? E.g. 'Transfer the supernatant (approx. 45mL) to a 150mL….' ?

Once the cells were resuspended, they were in one tube. These details have been added to the manuscript now. 150 mL flask is needed because the 40 mL of cellular lysate was diluted to 100 mL.

2.3 - horizontal or fixed angle rotor?

It is a fixed angle rotor; this information has been added to the text.

2.3 - discard the pellet? Why not keep the pellet for analysis later to see if it contains target protein, as a 'control' that the sonication step worked efficiently?

We agree with the review that the pellet can be analyzed for the efficiency of the lysis. We have done it for the method development, but it is a well-established method that does not necessarily require this step.

2.3 - refers to 'this step'. Question: which step? Part 2.3 contains multiple steps.

Thank you for this comment, it was not clear which step we referred to. The referred step has been now clarified.

General:

3.2. Provide an example of a His-column e.g. source, size, manufacturer, how the size might change if processing pellets of different volume/expected yield, which may influence purity.

The details about the His-column is included in the reagents/materials table. Traditionally for lab scale production we use 5 mL prepacked GE Healthcare HisTrapHP columns (please see references: Kaba *et al., Vaccine 2018*, El Bissati *et al., NPJ Vaccines*, 2017, Karch *et al., Nanomedicine* 2017). The reviewer is right that the pellet size and yield can vary from construct to construct. We generally modify the culture media and other growth conditions to generate protein that purifies with few impurities and at high enough yield for downstream animal experiments with this specific column size. Reference 13 (Seth *et al, Vaccine* 2017) covers the method development with a larger column. We could not see a difference in purity by changing the size of the column.

3.2.1 Choose, not chose. (same corrections in 3.3.1)

Thanks for calling our attention to this misspelling. It has been fixed in the text.

3.2.1 Buffer B has not been defined. What is the composition? Is it necessarily the buffer into which the tubing of pump B is placed? If so, should part 3.3.2 appear earlier in the method?

The composition of Buffer B has been defined in 3.1 (8M urea, 50 mM Sodium phosphate, 20 mM Tris-base, 5 mM TCEP, 500 mM Imidazole). The tubing of pump B is placed in Buffer B during the purification method.

3.3.1 Isopropanol second and third washes are done as two separate washes of 5 and 10 CVs each? Why not simplify and just have one step of 15 CVs?

Only the second wash is the 60% isopropanol wash. The third wash is to remove the remaining isopropanol before the elution steps.

3.3.3 Worth adding a cautionary note to avoid dripping/carryover transfer of isopropanol back into imidazole-free buffer?

It is a good question/suggestion. However, we did not detect any carryover of the buffers, because the closed pump does not let the buffer drip. Therefore, we believe that there is no need for a cautionary note.

Titles to sections 4 and 5: so far, most titles are descriptive of the aim (e.g. protein expression, protein purification etc). However, title to section 5 is 'Western Blot' which is a method, not an aim. What is the purpose of section 5 (WB)? Perhaps section 4 could be

'recovery and overall purity assessment' while section 5 could become 'protein identification by WB'? Or similar…

These are very good suggestions that increase the consistency of the protocol. We have changed the titles of section 5 to reflect this change.

4.1 the term 'flow through' has not been defined

We added this description to define flow through, "…the cell lysate that did not bind to the His column."

4.2 - manufacturer of gel, example?

This information is included in the reagents/materials table.

4.3 - manufacturer of MW marker, example?

This information is included in the reagents/materials table.

4.4 - correct size of what? Of the target antigen? Specify.

We agree that is was not clear. The sentence has been clarified by including "protein" and the "expected size of the band".

5.1 - 'run a Western blot' is insufficient information. Please provide full experimental details. Provide details of where to obtain the detection antibodies mentioned.

This is just introducing the idea; the full experimental details are explained in the subsequent steps (5.2-5.10). We have slightly changed the text to be more descriptive. Antibody sources are included in the reagents/materials table.

5.2 - 'determine the concentration' - of what? E.g. determine the total protein concentration by measuring Absorbance at A280nm….

The sentence was modified to include the phrase the "protein concentration at an absorbance of 280 nm".

5.3 'as before' is vague, please refer to specific part numbering that contains the aforementioned method.

Text has been updated to reference the specific step that describes the denaturing step.

5.4. Please specify gel type and running buffer type. If this is an SDS-PAGE system, it should be mentioned (reasonable doubt exists, given that the Abstract claims that WB was used to detect conformational epitopes whereas it is generally understood that conformational epitopes are not maintained under the denaturing conditions of SDS-PAGE but could feasibly be maintained were this a native PAGE).

The gel information is included in the reagents/materials table. Yes, conformation of a protein is not maintained under denaturing conditions of SDS-PAGE. The purpose of the Western blot in steps 5.1-5.10 was purely to demonstrate that protein of interest was the

correct size, and to verify that it was not truncated on either the N or C terminus. Thanks for this comment, the sentence in the abstract has been modified to clarify the role of the Western blot in the protocol.

5.5 Whereas much of the methods are not dependent on a specific manufacturer, here a very specific system is named. Is this necessary or sufficient?

In this protocol we used a transfer system from a specific manufacturer that requires only 7 minutes for the transfer. Text has been updated and the name of the manufacturer has been moved to the reagent/materials table.

6.1 Specify expected range of RT (e.g. 18 to 26 deg C?)

18 to 26 C as room temperature has been defined in the text now.

7.1.1 - any comments about data analysis? This seems to be a method to determine mean particle size. What happens if there are multiple populations of distinct particles of diverse sizes?

We added sentences that address this potential problem to the discussion section. If there are multiple populations of the particles, the quality of the product is not acceptable and further optimization of the method is required. The methodology for the "empty" SHB-SAPN core described in this manuscript is well-established. However, when a new antigen sequence is added, the methodology must be fine-tuned for the new construct.

7.2.3 Knob, not nob.

Thank you. Text has been updated to include the correct spelling

Figure 1a: quick check: are all colors used in the sequence correctly identified?

Yes, the colors are correct.

Figure 2: box 14 in the flow diagram mentions 'refold protein….'. For clarity, should box 3 (or later) mention the denaturing/unfolding step?....to mark the point at which the protein is unfolded or solubilized in denaturing conditions?

We agree with the reviewer that clarifying the steps with the protein monomers, denatured monomers, and fully assembled SAPN construct would help to understand the flowchart of the protocol. Therefore, the legend of Figure 2 has been updated now.

Figures 3 & 4: What is the expected/theoretical MW of a single chain? Can this be indicated somewhere.

The theoretical size is 18.07 kDa and it has been added to step 4.4 and to the figure legend.

Add an arrow to highlight he monomeric band on the gel/WB? Based on the MW of single chain, where would the dimer appear on the gel? The text says '…blots demonstrated the presence of the SHB-SAPN multimers'. The WB only shows two bands in lanes 6,7,8,

perhaps monomer and dimer, or monomer and trimer…but only two species, not a ladder of multimers. Please comment/clarify. Are these due to disulphide bond formation?

We added arrows to highlight the monomeric band. We agree with the reviewer that the strong bands are the monomers, dimers and perhaps trimers. There are some faint bands that are weakly detected by the image capture system. The dimers, trimers, and multimers, if any, formed by the nature of coiled-coils, which can stay associated in a denaturing SDS-PAGE gel. Although the SHB-SAPN core construct itself does not have any cysteines, we included the use of a TCEP in this protocol, because most of the antigens that will be used with this core will contain cysteine, and therefore, require a reducing agent.

After figures 3 and 4 - the reader cannot know how pure the sample is, with respect to contaminating E coli proteins. The gels in fig 3b are promising, but the later fractions that contain the protein are so dilute that one cannot tell whether other contaminants are present. The WBs are Ab-dependent, and might not reveal contaminants. The authors should present a gel of a concentrated sample, after the refolding step. Fig 5 suggests nanoparticles are formed, but does not inform about how many other contaminants may also be present.

The SAPN proteins have very little of contaminating *E. coli*. proteins as Figure 3b shows. We fully agree with the reviewer that the WBs may not reveal any bacterial contaminants since the antibodies used were specific for the SAPN construct. Although, theoretically, a gel with concentrated sample after the refolding step may increase the signal of the contamination, due to nature of the SAPN with the tendency for multimerization, it is not a doable approach.

In the representative results' section. Sizes are mentioned in nm. Please add clarity. Is this diameter (or radius)?

It is the hydrodynamic diameter. The text has been updated.

The text mentions that measured sizes were 'within the expected range'. What was the 'expected range' and how was it determined?

We added the expected size range of 20-100 nm to the sentence. The size of SAPNs has been reviewed in our previous publications that were referenced here, specifically in (Raman *et al.*, *Nanomedicine* 2006, Indelicato *et al.*, *Biophys J.* 2016 and Indelicato *et al.*, *R Soc Open Sci* 2017). Some SAPN are not completely formed and there are some aggregates, however, the majority of the constructs fall into a narrow, 68-81 nm size range, as measured by DLS.

Figure 5 panel C shows particles with quite high variability in size. Please indicate which are closely matching the 68-81nm sizes determined by DLS/Nanosight. If the scale bar is really 20nm, where is a particle 3-4 times broader than the scale bar?

There is a limitation of the use of DLS on polydisperse samples, because the larger size particles mask the smaller ones and the technique highly overestimates the frequency of

the big size particles. DLS measures the fluctuations in intensity of scattered light from all particles undergoing Brownian motion. (Pecora, 1985). Intensity is then statistically autocorrelated to the diffusion coefficient, from which the hydrodynamic diameter is determined. Electron microscopy identifies the individual particles and the detected size of the particles is precise. However, due to the lack of the real-time detection and the limitation of the size of the field of detection, this technique does not provide size distribution data. The two technique measure similar size of homogenous particles, however, the higher the polydispersity of the samples the higher the detected size by the two technique.

Discussion section:

Histidine, not histadine

Spelling has been corrected

"This length of the His-Tag found to be the optimal for the highest protein yield." - poor English

The missing word, “was”, was added to the sentence to now read as, “This length of the His-Tag was found to be the optimal for the highest protein yield.”

Discussion mentions expected size range of 20-100nM. That seems like a large range. As queried above, how was this expected/theoretical range calculated?

Particles follow a Gaussian distribution. The expected size range was assumed from previous works with different SAPN constructs that are cited in this manuscript. The actual size and size distribution of the SHB-SAPN particles, as discussed above, was measured by EM and DLS.

"This method without or only slight modifications can be applied when an antigen of interest is added to the SHB-SAPN core" - poor English

The reviewer is right about the sentence. It has been rewritten as “This method with only slight modifications, if any, can be applied to the purification of SHB-SAPNs when a new antigen of interest is added”.

Other comments:

In this method, the authors have included SDS-PAGE and WB as methods to show purification and identity of sample, and have included methods to show nanoparticle assembly, but have not included a method to demonstrate/check correct folding of a trimeric antigen/epitope. It is conceivable that the designed sequences self-assemble into trimers and beyond into larger nanoparticles, without necessarily 'faithfully recreating' the correctly folded 'target trimer' of the antigen of interest; in my opinion. i.e. it is conceivable that 60 copies of a peptide fragment of a target antigen are being presented but not necessarily in the right trimeric conformation. Can the authors comment on and address

this query? In this context, the authors merely cite reference 6, "…we have engineered a unique SHB-SAPN core to effectively present the V1V2 antigen in a native-like trimeric conformation". The Abstract here says "As an indication of the correct conformation, the purified protein reacted with known monoclonal antibodies in

Western blot analyses.", but the WB technique is not able to indicate correct conformation, only the presence of a target sequence under denaturing conditions, irrespective of whether this is known as an Ab targeting a conformational epitope (it may bind to a conformational epitope with high affinity, but with low-but-detectable affinity to a sub-fragment of that epitope under 'denaturing' conditions of the WB procedure)

We agreed with the reviewer and explained why we did not include a method to demonstrate/check correct folding of a trimeric antigen/epitope (see above answers). We agree with the reviewer's statement that "It is conceivable that the designed sequences self-assemble into trimers and beyond into larger nanoparticles, without necessarily 'faithfully recreating' the correctly folded 'target trimer' of the antigen of interest; in my opinion. i.e. it is conceivable that 60 copies of a peptide fragment of a target antigen are being presented but not necessarily in the right trimeric conformation.". Although, generally speaking it is a true statement, we are still very confident that because of the strong interactions in the coiled-coil motifs in particular in combination with the SHB, forces the designed trimeric antigen into the native-like trimeric conformation (Karch et al, *Nanomedicine*, 2019, Raman *et al., Nanomedicine* 2006, Indelicato *et al., Biophys J.* 2016 and Indelicato *et al., R Soc Open Sci* 2017). Thanks for the reviewer's other comment, it is true that WB technique is not able to indicate correct conformation. We have updated the abstract and removed the reference to the conformation and focused on protein purity instead.

Reviewer #3:

Comments:

1. The statement that a repetitive antigen display system can have the same benefits as a sub-unit vaccine is not necessarily an obvious conclusion - this sentence could be rewritten for clarity.

We have discussed the benefits of a repetitive antigen display system in two reviews (Karch *et al., Biochem Pharmacology* 2016, Kelly *et al., Expert Rev Vaccines* 2019).

2. SAPNs are an example of designed protein assemblies but are no more leading than the work of others including the groups of Woolfson, Yeates and Baker. The work of these groups as it relates to vaccines should be included for full acknowledgment of the literature.

Their work has been now fully acknowledged by citing three references.

3. 1.2 - are E.coli really grown for 48 hours? Is there a reason for this? ThermoFisher's protocols for expression in Magic Media recommend overnight growth only. Is cell viability still good after this time?

The reviewer is right, the general protocol from ThermoFisher recommends 24 hours incubation at 37ºC. During the method optimization with this SHB-SAPN core, we have tested different media and multiple growth conditions. We gained the best protein yield when we grew the culture at 30ºC for 48 hours.

4. 2.2 - During lysis are protease inhibitors, nucleases etc added to the cells?

There were neither protease inhibitors nor nucleases added to the cells during the lysis. The cells were lysed under denaturing and reducing conditions, which would inactivate any proteases and nucleases present.

5. 3.2 - the description of using ChromLab could be clearer. Hopefully the video will make this more understandable than the written section on its own.

Since the journal avoids commercialization, we removed all of the branding, such as the BioRad HPLC with the ChromLab software, from the text and moved the specific information to the reagents/materials table. However, based on the reviewer's suggestions for better understanding, we have now included the detailed steps for the setup of the ChromLab software that will be further clarified by the video file.

6. 5.3 - add "in step 4.1" to the sentence ending "denature them as before"

The text has been updated to reflect this suggestion. Thank you.

7. 5.5 - the instructions for transferring the gel onto the nitrocellulose membrane could be expanded on. The level of detail is lacking in comparison to other sections

We would like to emphasize again that the journal tries to avoid commercialization, and therefore, we have moved all the branding, such as the Trans-Blot® Turbo™ Transfer System, from the text to the reagents/materials table. However, the reviewer is right, and for better understanding and consistency, we have now included more details of the transfer to the nitrocellulose membrane.

8. 5.8 - it would be useful to note the total final dilution of your antibodies (it appears to be 1:1000 and 1:2000?)

Thanks for this suggestion. Although we usually calculate with the actual concentration of the given antibody, we agree that most of the time the dilution factor has been provided. We have added this information to the text

9. 5.9 - as point 8. The dilution ratio of antibodies is possibly more useful than just how many uL are being added

We have added the dilution factor to the text.

10. 6.1 - what are the relative volumes for dialysis (eg 25 mL pooled protein was dialyzed against 4L, with several changes of buffer, for 20 total L)

Based on the upstream application (e.g. characterization only or animal immunization studies) the volume of the pooled protein varies between 10 and 20 mL. The volume of the refolding buffers is 1 L. As described in the refolding section the buffer is changed 4 times every two hours decreasing the urea content by 2 M every 2 hour. Then it is dialyzed overnight into the final buffer condition.

11. 6.2 - is urea still necessary in the refolded protein?

It is a very valid question, the presence of the urea is not essential, but we have found that this concentration helps the protein folding.

12. 7.1.1 - line break required between 7.1.1/7.1.2

Missing line break has been added to corresponding lines.

13. In the discussion you state that these proteins can be expressed at high yield and are easy to purify using IMAC. For clinical products the presence of a his-tag is not ideal. Are alternate methods for purification without his-tags being considered? This may make the process more difficult.

The reviewer is right, for a clinical product the presence of the His-Tag is not ideal. In previous work, we have used ion exchange and size exclusion chromatography to purify the protein, in current animal studies the SAPNs have contained a His-Tag. Also, a malaria SAPN based vaccine that contains a HisTag has been manufactured and will soon be used in a clinical phase I/IIa trial (Seth *et al*, *Vaccine* 2017).

14. In the discussion the sentence "This method without or only slight modifications can be applied..." does not make sense as currently written.

We agree with the reviewer and the sentence has been updated to read: “This method with only slight modifications, if any, can be applied to the purification of SHB-SAPNs when a new antigen of interest is added”.

15. Does the selection marker of the expression vector matter? (regarding ampicillin allergy in humans) In a final protein-based product there should be no left-over antibiotic after a thorough purification. This does not seem like a sentence necessary to the discussion of the work.

It is a good question. The FDA does not recommend using β-lactam antibiotics because they are used in humans and there may be hypersensitivity or allergic reactions. This recommendation is outlined in the FDA guideline, "Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals."

16. The resolution of Fig 3a could be improved

We tried to improve the quality of Figure 3a. The chromatogram was exported from the HPLC software in the highest as possible resolution. The Figure has been saved in a Tiff

format with the requested 300 dpi resolution. However, we will work with the journal's production team to further improve the resolution if it is required.

17. Why are multiple bands observed in lanes 6&7 of Fig 4. No reference to these bands are made in the figure caption

These bands are dimers of the protein which tend to happen with coiled-coils structures. These bands have been now referenced in the figure caption.

18. Is there a reason for presenting the DLS data in Fig 5a in terms of %volume rather than the more analytically correct %intensity?

The reviewer is right. DLS measures the intensity of scattered light. Volume is then mathematically calculated. Since the size range of the SHB-SAPN particles is relatively wide, the % volume presentation of the size distribution correlates better with the results of the EM.