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Tracing Endotoxin Levels Throughout HPV16 L1 Vaccine Purification and Particle Production

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Tracing Endotoxin Levels Throughout HPV16 L1 Vaccine Purification and Particle Production

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Abstract

Cervical cancer is the second most common cancer among women worldwide, and the most common cancer among women in developing countries\(^1\). The causal role of human papillomavirus (HPV) infections in invasive cervical cancers is acknowledged beyond reasonable doubt, and the ability to vaccinate against HPV can drastically reduce the incidence of cervical cancer worldwide. There are currently marketed HPV vaccines that make use of HPV L1 virus-like particles (VLPs), and while these VLP vaccines confer immunogenicity, they are expensive, thermosensitive, and require multiple doses\(^2\). Expression of the HPV L1 protein in *Escherichia coli* (*E. coli*) bacteria has the potential to reduce production costs, while lyophilization creates a thermostable powdered vaccine formulation, and atomic layer deposition (ALD) generates a platform for a single-administration prime-boost vaccination.

By using *E. coli* cells for protein expression, endotoxin is therefore present and must be removed prior to vaccine injection. This is done through protein purification and further development into a powderized vaccine particle. The research project described in this thesis details the tracing of endotoxin levels throughout HPV16 L1 vaccine purification and particle production. Endotoxin is removed by the multi-step purification and it is hypothesized that it is removed in varying extents during the different steps. It is also hypothesized that endotoxin is not reintroduced during particle production. In order to test these hypotheses, and determine which stages yield the most removal or reintroduction of endotoxin, an endotoxin assay is run with samples at each stage of the protein purification, spray drying, and ALD coating processes. The endotoxin assay is crucial in determining where, if anywhere, endotoxin is reintroduced after purification. The results of the endotoxin assay are used to effectively fine-tune the vaccine development process in order to create a safe and effective second generation HPV vaccine.
**Introduction**

Human Papillomavirus (HPV) is an etiological agent for cervical, oropharyngeal, anal, vulvar, and penile cancers\(^3,4\). Out of hundreds of types of HPV, 15 types are called oncogenic or high-risk, and are significantly associated with invasive cervical cancer\(^5\). 99% of cervical cancers are HPV-attributable, which is the highest worldwide attributable fraction for a specific cause of any major human cancer\(^6\). Of these high-risk, oncogenic HPV types, most are phylogenetically related to either HPV 16 or HPV 18\(^7\). Therefore, the ability to produce a prophylactic vaccine against HPV16 and HPV18 has the potential to prevent more than two-thirds of worldwide invasive cervical cancers\(^8\).

HPV is a non-enveloped, double-stranded DNA virus that infects squamous epithelium\(^9\). The 8kb genome encodes 6 early proteins responsible for viral replication, and 2 structural proteins, L1 and L2. The T=7 icosahedral virion shell is made up of 72 pentameric subunits, called capsomeres, with each subunit comprising of five interconnected L1 proteins\(^10\). The interaction of L1 with heparin sulfate carbohydrates displayed on host proteoglycans initiates viral infection. After attachment to host cells, L1 must become pliable enough to ultimately allow release of the viral genome into the target cell\(^11\). Consequently, the L1 major capsid protein has been the focus of HPV vaccines. Recombinant HPV L1 capsomeres self-assemble into L1 Virus-Like Particles (VLPs) that resemble the HPV capsid and produce type-specific neutralizing antibodies in inoculated animals\(^12\). HPV L1 VLPs are antigenically almost identical to native virions, and this technology has been exploited to produce HPV L1 VLP subunit vaccines, notably Cervarix\(^\text{®}\) and Gardasil\(^\text{®}\), that prevent HPV infection\(^13,14\).

Cervarix\(^\text{®}\) (GlaxoSmithKline), is a bivalent L1 VLP vaccine protecting against HPV types 16 and 18, produced using baculovirus-infected insect cell lines\(^15\). Gardasil 9\(^\text{®}\) (Merck)
Protects against HPV types 6, 11, 16, 18, 31, 33, 45, 52, and 58, and is produced in yeast cell lines expressing the type-specific HPV L1 VLP\textsuperscript{15}. These current VLP vaccines are expensive, require multiple doses, and are temperature sensitive, necessitating cold-chain storage and handling, during which the vaccines must be kept at 35\textdegree C at all times\textsuperscript{2}. HPV L1 proteins can be expressed in \textit{E. coli}, potentially offering a lower cost expression system\textsuperscript{16,17}. Expression of HPV L1 in \textit{E. coli} has shown to produce self-assembling capsomers that produce neutralizing antibodies in viral challenge in animal models\textsuperscript{16}. In addition, the HPV L1 capsomers have been lyophilized into a thermostable formulation, one that requires no refrigeration, and this formulation has the ability to elicit neutralizing antibodies after incubation at 50\textdegree C for 12 weeks\textsuperscript{18}. This shows potential to eliminate the need for the cold-chain, especially during shipping and long-term storage in developing countries\textsuperscript{19}.

Developing countries have higher incidences of cervical cancer, due to a lack of resources for screening programs, and vaccine distribution\textsuperscript{20,21}. Issues such as reliable refrigeration and patient communication affect the ability to vaccinate the population with multiple booster doses of the HPV temperature sensitive vaccines, leading to the prevalence of cervical cancer as the most common female cancer in developing countries, where 80\% of new cases arise\textsuperscript{1}. 87\% of cervical cancer deaths occur in less developed regions of the world, and the possibility of a thermostable, single-administration prime-boost HPV vaccine could minimize the incidence of cervical cancer in low-resource areas\textsuperscript{22,23}.

Development of a thermostable single-administration vaccine is collaborative effort between the Randolph and Garcea groups at the University of Colorado. They have previously developed a thermostable\textbackslash second-generation HPV vaccine, expressed in \textit{E. coli}, that remains stable at 50\textdegree C, even after three months\textsuperscript{18}. In this lyophilized formulation, the HPV L1 protein is
embedded in a glassy trehalose matrix\textsuperscript{24}. In addition, a single-administration vaccine, one that contains the prime and booster doses in a single injection, bypasses the need for a booster dose. This concept is achieved through the use of a newly developed atomic layer deposition process, in which the spray dried vaccine particles are uniformly coated with adjuvants and antigens to create a temporally-separated primer and booster dose\textsuperscript{25}.

One obstacle with this vaccine production platform is the inherent endotoxin presence because of the use of \textit{E. coli} cells, and the potential introduction of endotoxin during particle production. Endotoxin is innate to the vaccine development process due to the use of \textit{E. coli} cells for protein expression, and can also be introduced through glassware, plasticware, water, and human handling\textsuperscript{26}. Endotoxin is a lipopolysaccharide membrane component of gram negative bacteria and can cause a pyrogenic response within the human body, ranging from fever and chills to fatal septic shock. According to the FDA, endotoxin must fall below a certain limit for vaccine administration, depending on the dose volume, potency and maximum endotoxin exposure.

It is hypothesized that endotoxin is removed in varying amounts during L1 protein purification process. It is also hypothesized that endotoxin is NOT reintroduced during particle production. In order to test these hypotheses, samples were taken at each step in the process of purification and further production into a spray dried vaccine particle. In order to test the endotoxin presence, the FDA-approved Limulus Amebocyte Lysate assay was used. This assay uses the aqueous extract of blood cells from the pericardium of the horseshoe crab. By measuring endotoxin levels during all stages of protein purification and particle production, procedures can be assessed for adequate sterility. This is important for creating a safe, and effective single-administration, prime-boost vaccination platform that will eliminate cold chain requirements,
reduce the number of required doses, and help vaccinate populations in low-resource areas of the world.
Materials and Methods

Materials

HMS174 *E. coli* competent cells were purchased from Novagen (San Diego, CA). Isopropyl β-D-1-thiogalactoside (IPTG) for protein induction was purchased from Research Products International (RPI) (Mount Prospect, IL). Centrifugal filters used for concentrating protein were purchased from Merck Millipore (County Cork, Ireland). Ion exchange columns, Q-resins, HiTrap desalting columns, AKTA FPLC and AKTA purifier were purchased from GE Healthcare (Piscataway, NJ). The Panda homogenizer for cell lysis and homogenization was purchased from GEA Niro Soavi (Bedford, NH). The MiniSprayDryer B-290 for spray drying the protein formulation was purchased from Buchi (Fawil, Switzerland). The LyoStar Freeze-Dryer, used for further drying of the spray dried powder, was purchased from FTS Systems Inc. (Stoneridge, NY). Limulus Amebocyte Lysate QCL-1000 kits, used to quantify endotoxin presence, was purchased from Lonza (Basel, Switzerland). Nunc 96-well plates used for the ELISA and sterile Costar 96-well plates for endotoxin assays were purchased from Costar (Corning, NY). The ELx808 plate reader was purchased from BioTek Instruments (Winooski, Vermont). An Eppendorf BioPhotometer Plus spectrophotometer was used for Bradford assays and was purchased from Eppendorf (Hamburg, Germany). A Hitachi SU-3500 Scanning Electron Microscope was used to render images of particles (Krefeld, Germany). Alkaline phosphatase and HRP conjugated secondary IgG antibodies were purchased from Promega (Madison, WI) and used for Western blotting. The Hoefer IE70 Semi-dry Transfer Unit for Western blotting was purchased from Amersham Biosciences, (Madison, WI). All chemical reagents were purchased from Sigma Aldrich (Darmstadt, Germany) unless stated otherwise. Protocols were previously developed for effective HPV16 L1 expression, and purification18.
Transformation and Protein Expression

HPV16 L1 DNA was transformed in HMS174 (DE3) E. coli competent cells, containing a kanamycin resistance gene, by heat shock. 1µg DNA was added to 50µL thawed E. coli cells. This mixture was held at 42°C for 45 seconds and then cooled on ice for 2 minutes. 450µL of S.O.C. media was added, after which the cells were incubated at 37°C for 2 hours with gentle mixing every 15 minutes. The cells were spread on Luria Broth (LB) agar plates containing 50µg/mL kanamycin (KAN) and incubated overnight at 37°C.

3mL of Terrific Broth (1.2% Tryptone, 2.4% Yeast extract, .94% K2HPO4, .22% KH2PO4) with 3µL of 50µg/mL KAN was inoculated with a single colony from the transformation plate and incubated for 6 hours at 30°C with shaking. After 6 hours, 200µL of the 3mL culture was added to 50mL of TB containing 50µL of 50µg/mL KAN and incubated overnight at 30°C with shaking. The following day, 500µL of 50µg/mL KAN was added to each of six 500mL warmed baffle flasks of TB. Each 500mL flask was inoculated with 2mL of the 50mL overnight culture and incubated at 37°C at 275 rpm until the OD₆₀₀=4.0. The cultures were then chilled to 25°C in a cold water bath. 200µL of 0.5M IPTG was added to each 500mL flask. These flasks were then incubated at 25°C until OD₆₀₀=8.0. Each 500mL culture was centrifuged at 5858 x g for 15minutes at 4°C. A sample of the supernatant was discarded and the pellets containing E. coli cells were stored at -20°C until further use.

Lysis of E. coli cells

Each 500 mL culture pellet was resuspended with 50mL of a 200mM NaCl buffer (200 mM NaCl, 50 mm Tris, 10% glycerol, 5 mM DTT, 0.01% Tween 80, 1 mM EDTA, pH 8.1) and lysed by two to three passes through the Panda homogenizer at 800-1000 bar. After the initial passes through the homogenizer, the lysate was cooled back down to 4°C before a second set of
passes through the homogenizer at 800-1000 bar. This whole cell lysate (WCL) was centrifuged at 22,000 x g for 30 minutes at 4°C. The supernatant/soluble fraction (S1) contained the HPV16 L1 protein and was then purified, while the pellet (P1) containing the L1 protein in inclusion bodies and cell debris, was sampled and discarded.

**L1 Purification: Q Sepharose Fast Flow Column Chromatography (QFF)**

A XK 50/20 GE column was packed with 300 mL QFF resin and equilibrated with the 200mM NaCl buffer using the AKTA FPLC. The S1 was loaded onto the column at 7 mL/min and the flow through (QFF FT) containing the L1 protein was collected and saved for the subsequent NH₄SO₄ precipitation.

**L1 purification: Ammonium Sulfate Precipitation**

Solid NH₄SO₄ was slowly added with stirring to the QFF FT to reach 30% saturation. This suspension was incubated for 2 hours at 4°C, and then centrifuged at 13,000 x g for 30 minutes. The L1 protein was present as a precipitated pellet (AS Pel), while unwanted proteins were contained in the supernatant (AS Sup).

**L1 purification: Q Sepharose High Performance Column Chromatography (QHP)**

The ammonium sulfate precipitate was resolubilized in 10 mL of a 25 mM NaCl buffer (25 mM NaCl, 50 mM Tris, 10% glycerol, 5 mM DTT, 0.01% Tween 80, pH 8.5) and passed through the Panda homogenizer at 500 bar to re-homogenize the protein. The homogenate was centrifuged at 13,000 x g for 30 minutes and the supernatant was retained. The conductivity of the resolubilized L1 protein was adjusted to match that of the 25 mM NaCl buffer using a 0 NaCl buffer (50 mM Tris, 10% glycerol, 5 mM DTT, 0.01% Tween 80, pH 8.5). A XK 26/20 GE column was packed with 70mL of QHP resin. The column was equilibrated by alternating the 25 mM NaCl buffer (loaded in A lines of the AKTA purifier; Buffer A) with a 1 M NaCl buffer.
(1M NaCl, 50 mM Tris, 10% glycerol, 5 mM DTT, 0.01% Tween 80, pH 8.5; Buffer B), then Buffer A again. The protein sample was loaded onto the column and a sample of the flow through (QHP FT) was collected. Residual protein was washed off the column by running Buffer A until the UV, as read by the AKTA detector, equilibrated back to baseline. The L1 protein was eluted using an elution gradient that increased to 50% Buffer B from Buffer A over 12 column volumes. The L1 protein contained in the fractions was then quantified using a Bradford assay. Fractions containing protein were combined (QHP Pool). The QHP chromatography was repeated a second time (QHP Polish) to yield cleaner, more concentrated L1 protein. As with the initial QHP, fractions were quantified using a Bradford assay and combined (QHP Polish Pool). Protein was flash frozen in liquid nitrogen and stored at -80°C until further use.

**Buffer exchange**

In order to continue with particle production, the L1 protein needed to be exchanged from an approximately 200 mM NaCl buffer as eluted from the QHP Polish, into a 54 mM histidine buffer, pH 7.1, for thermostability. Two 5 mL HiTrap desalting columns were linked in series and equilibrated with 54 mM histidine buffer. The QHP polish pool/ L1 protein was loaded onto the linked columns 2 mL/run and collected in 0.5mL fractions. In between each 2 mL run, the column was washed with 5CV of the histidine buffer in order to remove the NaCl from the column. This process was repeated to exchange the remaining L1 protein. Fractions were analyzed for protein content by Bradford assay, pooled, and stored at 4°C for immediate use, or otherwise flash frozen in liquid nitrogen and stored at -80°C until further use.

**Spray Drying**

A 10 mL protein formulation for spray drying was made containing 15% trehalose, 0.01% HPV16 L1 protein, 2.5% hydroxyethyl starch, 54 mM histidine, 3.6 mM Tween 80, pH
6.0. Inlet temperature on the spray dryer was set to 90°C, the aspirator was set to 20 m³/hr, and the drying gas (ultrapure nitrogen) pressure was set to 55 mm Hg. Once the temperatures stabilized, water was run through the apparatus with the pump set at 10% (2 mL/min). The protein formulation was then run through the spray dryer. The spray dried particles containing the HPV16 L1 protein were aliquoted into vials containing approximately 1mg of particles each, then further dried at 30°C overnight under vacuum in the LyoStar Freeze-Dryer. The next morning, the vials were backfilled with nitrogen, sealed, and kept at room temperature until further use.

**Atomic Layer Deposition (ALD)**

Atomic Layer Deposition was used to form a protective alumina barrier coating the spray dried particles. This process was performed by Milly Dong, a graduate student in Ted Randolph’s laboratory, following the procedure developed by Al Weimer for the use of a fluidized bed reactor for ALD. This procedure included repetitive, alternating cycles subjecting the protein particles to 100 layers of “ABC” chemistry, which involves trimethyl aluminum, ethanolamie, and maleic anhydride, purging the reactor bed in between each chemistry (Fig. 1). Subsequently, the particles were coated with 500 layers of “AB” chemistry, which involved a cycle of trimethyl aluminum followed by water.

![Fig. 1: Self-limiting surface ABC-type reactions to deposit molecular layers of alucone. A) Hydroxyl groups on substrate (e.g., trehalose microparticles) react with trimethyl aluminum. B) Ethanolamine reacts, leaving terminal amine groups on the surface; C) Maleic anhydride reacts with terminal amine groups, regenerating surface with hydroxyl groups. ABC steps may then be repeated to add additional layers as desired.](image-url)
Analysis

Various assays were used to analyze HPV16 L1 concentration, purification, immunogenic conformation, and endotoxicity.

Bradford assays were used to measure protein concentration using 1X Bradford reagent with all samples in a 1:100 protein to reagent ratio, measured in an Eppendorf BioPhotometer at 595nm. A BSA standard curve was generated and protein concentrations were calculated accordingly.

HPV16 L1 was monitored throughout the purification process on 10% SDS PAGE gels. Each sample consisted of the protein sample, 3X SDS-PAGE sample buffer, and β-mercaptoethanol (BME). Samples were boiled in a 95°C sand bath for 5 minutes, then quickly spun down. 10% SDS-PAGE gels were loaded with samples and separated at 150 V, 150 mA for 70 minutes. The stacking gels were removed and the resolving gels were placed in a container with Coomassie stain, and placed on a rocker for 1 hour. Gels were destained in a 40% methanol, 10% glacial acetic acid solution overnight.

Western blots were used to confirm the identity of the protein being visualized on the SDS PAGE as being HPV16 L1. A duplicate 10% SDS-PAGE was made for Western blotting. The gel was washed for 10 minutes in 1X Transfer Buffer (250 mM Tris, 2 M glycine), and transferred to a nitrocellulose membrane using a Hoefer IE70 Semi-dry Transfer Unit at 15 V, 500 mA for 45 minutes. Once the protein had transferred to the membrane, the membrane was blocked overnight at 4°C in Block Buffer (5% milk in 1X Tris-Buffered Saline solution with 0.1% Tween20 (TBST)). The following day, the block buffer was removed and the membrane was rinsed 3 times with 1XTBST. The 1° antibody, mouse sera antibodies generated from mice injected with Gardasil®, was diluted 1:1000 in 1XTBST and was added to the membrane. The
membrane was incubated with rocking at room temperature for 1 hour. After an hour, the 1° antibody was removed and the membrane was washed three times for 5 minutes each with 1XTBST. A 2° antibody solution, an anti-mouse alkaline phosphatase conjugated IgG antibody diluted 1:5000 in 1XTBST, was added and the membrane was incubated with rocking for 1 hour at room temperature. After an hour, the membrane was washed three times for 5 minutes each with 1XTBST. 20 mL of the developer solution consisting of 66µL of 50mg/ml BCIP (5-bromo-4-chloro-3′-inolylphosphate-p-toluidine) and 88µL of 50mg/mL NBT (nitro-blue tetrazolium chloride) in a 1X alkaline phosphatase developing solution (0.4 M Tris, 1 M NaCl, 0.2 M MgCl₂) was added to the membrane and was allowed to react until visible banding appeared. The reaction was stopped with dH₂O and the membrane was allowed to air dry.

An enzyme-linked immunosorbent assay (ELISA) was used to test correct protein conformation for immunogenic response. Serial dilutions of HPV16 L1 samples in 1X Phosphate-buffered saline (PBS) were added to a Nunc 96-well plate, so that the final concentrations were 0.25 µg, 0.13 µg, and 0.06 µg in the wells, with each concentration run in duplicate for both V5 and L1 reactivity. A fourth well for each sample contained no protein in order to serve as a negative control. After adding protein, the plate was covered and incubated at 4°C overnight to allow the protein to bind to the wells. The next day, the plate was washed three times using 50 µL/well Wash Buffer (1X PBS, 0.05% Tween20) in order to remove any non-bound L1 protein. 100 µL/well of Block Buffer (1X PBS, 0.05% Tween20, 5% milk) was added and the plate was incubated for 1 hour at 37°C. Block Buffer was removed and 50 µl/well of a primary antibody solution consisting of either V5 monoclonal, or L1 polyclonal, antibody diluted 1:1000 in Block Buffer was added to their respective wells and incubated for 1 hour at 37°C. Positive controls were primary anti-L1 polyclonal antibodies and anti-V5 monoclonal antibodies.
against L1 protein and conformationally correct HPV16, respectively. Negative controls were primary anti-V5 antibodies against plated HPV45 L1 protein and a set of wells using primary anti-V5 antibodies against murine polyomavirus structural protein VP1 (MPyV VP1). The plate was incubated for 1 hour at 37°C and then washed three times with 50 µL/well Wash Buffer. 50 µL/well of anti-mouse horseradish peroxidase (HRP)-conjugated IgG secondary antibodies diluted 1:500 in Wash Buffer was added to the wells testing for V5 reactivity, while 50 µl/well of anti-rabbit HRP-conjugated IgG secondary antibodies diluted 1:5000 in Wash Buffer was added to wells testing for L1 reactivity. The plate was incubated for 1 hour at 37°C. After incubation, the plate was washed three times with 50 µL/well Wash Buffer and 50 µL/well of TURBO 3,3’,5’5 Tetramethylbenzidine (TMB) substrate was added. After a blue color satisfactorily developed, the reaction was stopped with 50 µL/well of 1 M sulfuric acid. Absorbance values were read using an ELx808 absorbance reader at a wavelength of 450 nm.

In order to quantify endotoxin presence throughout the HPV16 L1 protein purification process and particle production, a Lonza Limulus Amebocyte Lysate (QCL-1000) Endotoxin Kit was used. Each sample taken throughout the purification process as well as particle formation was run in triplicate. An E. coli endotoxin control, present in the kit, was reconstituted in an appropriate volume of endotoxin-free (EF) water, according to vial-specific concentrations stated in the certificate of analysis included in the kit. Once reconstituted, the endotoxin was vigorously vortexed for 15 minutes at room temperature. From this reconstituted endotoxin solutions, a 1.0 EU/mL endotoxin standard was prepared by diluting 100µL of the endotoxin solution with an appropriate volume of EF water, dependent on the solution concentration. From the 1.0 EU/mL standard, four other standards were created (0.5 EU/mL, 0.4 EU/mL, 0.25 EU/mL, 0.1 EU/mL). For the test proteins, several serial dilutions were made ranging from undiluted to
Samples taken from the initial steps of purification (S1 1, S1 2, QFF FT, AS Pel Post 1, AS Pel Post 2) were tested at 1:10,000,000, 1:100,000,000, and 1:1,000,000,000 dilutions in EF water. Samples taken at the end of purification (QHP FT 1, QHP FT 2, QHP Polish Load), were tested at 1:1000, 1:10,000 and 1:100,000 dilutions in EF water. Samples taken during after purification (Polish Pool, Post His, Post SD, Post FD, ALD) were tested in undiluted, 1:10 and 1:100 dilutions in EF water. A Costar pyrogen-free 96-well plate and the chromogenic substrate were pre-equilibrated in 37°C, as the entirety of the assay was performed at 37°C. 50 µL of each endotoxin standard was dispensed into appropriate wells, in triplicate. For the negative control, 50 µL of EF water was added as a 0 EU/mL standard. 50 µL of each test protein dilution was dispensed into appropriate wells, in triplicate. A timer was started and at T=0 minutes, 50 µL of the Limulus Amebocyte Lysate (LAL) reagent was added to all wells. At T=10 minutes, 100 µL of pre-warmed chromogenic substrate was added to all wells. The amebocytes contain coagulogen which reacted with gram negative bacterial endotoxin and turned into a semi-solid mass. This clotting cascade was then used as a qualitative test for endotoxin presence in the sample. In order to quantify results, the chromogenic substrate was added, and as the lysate cleaved the substrate, the solution became yellow, given that the time required for the change was inversely proportional to the amount of endotoxin present. At T=16 minutes, 100 µL of 10% SDS was added to all wells to stop the reaction. Any bubbles formed from the addition of the stop solution were carefully aspirated. Absorbance values were read using an ELx808 absorbance reader at a wavelength of 405nm.
Results

Verifying Protein Expression and Purification

The “pETAC 16Δ” lab plasmid containing the HPV16 L1 DNA as well as a kanamycin resistance gene was successfully transformed into HMS 174 DE3 competent *E. coli* cells. After the transformation was plated and incubated overnight, a single starter colony was selected and used for the culture expansion technique that resulted in 6 L of HPV16 L1 culture, separated into two 3 L purification processes. In order to verify L1 protein expression, samples were taken pre and post induction with IPTG. The results were visualized with 10% SDS Page gels and Western blotting (Fig. 2 A-D). The molecular weight of our unique HPV16 L1 protein is 52 kDa. Based on the results of the SDS Page gels and Western Blotting, the HPV16 L1 protein was correctly expressed in the *E. coli* cells.

![Fig. 2: A,C: 10% SDS-PAGE and B,D: Western blots used to identify and assure accurate protein expression as HPV16 L1. Lane 1: MW ladder, lanes 2& 3: 5µL pre-IPTG induction, lanes 4&5: post-IPTG induction. Desired protein is seen at 52kDa](image)

Samples of the L1 protein solution were taken at multiple steps in the purification process, including the whole cell lysate before homogenization (WCL), the soluble L1 fraction after homogenization (S1), the pellet after homogenization (P1), the flow through of the QFF column chromatography (QFF FT), the ammonium sulfate supernatant (AS Sup), the ammonium sulfate precipitation pellet pre-centrifugation (AS Pel Pre) and post-centrifugation (AS Pel Post),
the flow through of the QHP column chromatography (QHP FT), the elution fractions of the QHP column (A9-B9), the load and flow through of the QHP polish (QHP Polish Load, QHP Polish FT), and the elution fractions of the QHP Polish (B12-B9). In addition to the samples taken throughout the protein purification process, samples were also taken from the histidine buffer exchange (QHP Pool/His load), the fractions resulting from the histidine buffer exchange (A-F), the post histidine buffer exchange pool (Post His), post spray drying (Post SD), and post lyophilization (Post FD). All samples were analyzed with 10% SDS PAGE and Western blotting (Fig 3 A-N).

Fig. 3 A-D, First 3 L purification process 10% SDS-PAGE and corresponding Western blot to confirm presence of HPV16 L1 and analyze purification. A,B: Lane 1-3: 5 µL WCL, S1, P1, respectively. Lane 4: 5µL MW marker. Lane 5-7: 10µL AS Sup, AS Pel Pre, AS Pel Post, respectively. Lane 8: 30µL QHP FT. C,D: Lane 1: 5µL MW marker. Lanes 2-9: 30µL QHP elution fractions. Desired protein is seen at 52kDa.

Fig. 3 E-H, Second 3 L purification process 10% SDS-PAGE and corresponding Western blots to confirm presence of HPV16 L1 and analyze purification. E,F: Lane 1-3: 5 µL WCL, S1, P1, respectively. Lane 4: 5µL MW marker. Lane 5-8: 10µL QFF FT, AS Sup, AS Pel Pre, AS Pel Post, respectively. Lane 8: 30µL QHP FT. G,H: Lane 1: 5µL MW marker. Lanes 2-9: 30µL QHP elution fractions. Desired protein is seen at 52kDa.
An unforeseen obstacle arose in the first 3L protein purification process. The QFF was intended to be used as the first column for chromatography; however, the resin in the column had solidified causing back pressure with the potential to damage the column and FPLC. In response to this problem, the supernatant of the whole cell lysate, which contained the solubilized L1 protein, was subjected to ammonium sulfate precipitation, foregoiing the QFF column chromatography (see in Fig 3 A, C). This change in protocol was eventually shown to in no way detract from protein purification and the comparison of the two 3L processes showed that the QFF was not inherently necessary and has since been removed from the purification procedure.

**Imaging the Powered L1 Protein Particles**

After protein purification and histidine buffer exchange, the protein solution was subjected to spray drying, in which the HPV16 L1 was stabilized in a glassy matrix as thermostable powder.
“glassy” trehalose matrix in particle form. This L1 protein particle was transferred to glass vials (~1 mg/vial) and further dried in a lyophilizer overnight at 30°C under vacuum at 60 mTorr, backfilled with nitrogen and stored in vials at room temperature (Fig 4). Qualitatively this powder was very electrostatic and individual particles tended to agglomerate. In order to view them on a microscopic level, a sample of the spray-dried particles was visualized using scanning electron microscopy (SEM) by Natalie Meinerz (Fig 5 A, B).

![Fig. 5 A,B:](image)

HPV16 L1 spray dried and lyophilized particles visualized using scanning electron microscopy. The particles are seen to have a large size distribution and significant agglomeration. 5B showing a magnified image of 5A.

There was a large size distribution among the particles, as well as significant necking, which is the annealing of two particles’ surfaces to each other. Yield was calculated by assuming the 1.5g of trehalose in the protein formulation would all solidify, thus 1.5g was the theoretical yield. 0.9936g of the spray-dried powder was collected, for 66% yield, and using a Bradford assay, the HPV16 L1 protein concentration was found to be 2.99µg/1mg of spray-dried powder. After the protein solution had been spray-dried into powdered particles, the particles were
subjected to atomic layer deposition (ALD) using a fluidized bed reactor, courtesy of Dr. Weimer’s lab. The ALD was performed by Milly Dong. These HPV16 L1 particles were first coated with 100 layers of “ABC chemistry” followed by 500 layers of “AB chemistry” at 100°C. The particles again appeared to agglomerate, and were then visualized with scanning electron microscopy. The layers introduced by atomic layer deposition were visible, as well as other interesting characteristics: the particles displayed a large size distribution and many particles had depressions on their surface, potentially from smaller particles adhering to the surface and then falling off. Some particles had broken in half, leaving a shell of layered coatings, while others had holes in the surface that led to the formation of hollowed particles (Fig. 6 A-C).

Fig. 6 A-C: HPV16 L1 particles after ALD with 100 layers of ABC chemistry and 500 layers of AB chemistry. The particles are seen to agglomerate, break apart, and appear as hollow, empty shells. 5B is a magnified image of 5A showing broken shells of particles. 5C is a magnified image of 5A showing a hollow particle, probably due to necking and subsequent dissociation of a smaller particle.
ELISA: L1 and V5

An enzyme-linked immunosorbent assay was used to determine the presence of L1 and V5 capsomere epitopes in samples after purification. L1 measurement is used to verify the capsomere specificity, while V5 binds a neutralizing epitope specific to conformationally intact capsomeres. Samples after the desalting column used for histidine buffer exchange, as well as after spray drying, and after lyophilization (FD) were tested. All three steps in the particle production procedure were shown to not affect HPV16 L1 capsomere structure (Fig. 7). A previously processed HPV16 L1 sample was used as the positive control, and HPV45 L1 and murine polyomavirus capsid protein, VP1, were used as a negative controls.

![HPV16 L1 Reactivity](image)

**Fig. 7:** ELISA absorbance data for protein condition after purification. Each sample was run with three concentrations of HPV16 L1 and absorbance was read at 450nm. The MuPyV VP1 negative control shows cross some reactivity with L1, and the HPV16 L1 positive control show an error in V5 reactivity to sample degradation.
LAL Endotoxin Assay

Samples taken at each step in the purification process were tested for endotoxin using a Lonza LAL endotoxin assay. The results were presented as endotoxin units per mL (EU/mL), with one EU equal to approximately 0.1 to 0.2 ng endotoxin/mL of solution\textsuperscript{26}. The first samples tested were the supernatant samples containing the HPV16 L1 protein before any column chromatography. The first sample (S1 1) was taken from the first 3L purification process and as expected, the endotoxin level was very high at 3.3E+06 EU/mL. This process skipped the QFF column chromatography as previously discussed, and thus the next sample was from the ammonium sulfate precipitate post-centrifugation. The endotoxin level significantly dropped to 8.7E+05 EU/mL, showing that the ammonium sulfate precipitation itself drastically reduces endotoxin presence in the sample. In contrast, the second 3L process followed the standard procedure, incorporating both the QFF column chromatography and ammonium sulfate precipitation. The supernatant sample (S1 2) endotoxin level was again very high at 5.9E+06 EU/mL, and after the QFF column chromatography the level was 5.2E+06 EU/mL. This small change in overall endotoxin level indicates that the QFF column chromatography is not a significant purification step. This conclusion is further supported by the data from the sample taken after ammonium sulfate precipitation, in which the endotoxin level was 1.6E+06 EU/mL. The comparison between the two 3L processes supports the conclusion that QFF column chromatography can be removed from the procedure without significantly effecting protein purification (Fig. 8).
Following the ammonium sulfate precipitation, the protein sample was subjected to the QHP column chromatography for further purification. Samples were taken of the QHP flow-through, which did not contain the HPV16 L1 protein, as well as the pooled elution fractions that were subsequently run through a second QHP Polish column. The flow-through of the first 3L process through the QHP column showed undetectable levels of endotoxin. This follows previously seen data from the SDS Page gels and Western blots, which did not detect any protein.

Fig. 8: LAL endotoxin assay on purification samples taken during HPV16 L1 protein purification. Absorbance read at 405nm.
presence in the QHP flow-through of the first process. In contrast, the SDS Page gels and Western blots for the second process’ QHP flow-through showed HPV16 L1 protein in the flow-through and this corresponds to the 5.7E+04 EU/mL measurement for QHP FT 2. This loss of protein in inherent to the purification process, potentially due to column overload. After the two separate 3L purification processes, the elution fractions from each QHP column were combined and loaded onto the second QHP column for final purification. The endotoxin present in the QHP Polish load was measured to be 6.9E+04 EU/mL. This is a significant reduction in endotoxin due to QHP column (Fig. 9).

![Endotoxin Levels During Later Steps of Purification](image)

The last step in protein purification was running the HPV16 L1 protein solution through a second QHP column, known as the QHP Polish. Both the flow-through from this column chromatography, as well as the combined elution fractions were sampled for endotoxin levels. Endotoxin levels in the flow-through were measured to be 0.93 EU/mL while endotoxin levels measured in the pooled elution fractions (QHP polish pool) were measured to be 6 EU/mL. This
level of endotoxin is predicted to be within FDA regulations, contingent on the finalized vaccine dose volume. After the second QHP column chromatography, the protein solution was run through a desalting, buffer exchange column, and a sample of this solution had an endotoxin measurement of 4.5 EU/mL. Samples were also taken after spray drying the HPV16 L1 protein solution into a powdered particle resulting in an endotoxin level of 2 EU/mL. Assuming a final dose of 50 µg of HPV16 L1 per injection, and based on the protein concentration, the final endotoxin level would be 1.1 EU/50µg dose. The spray-dried particles were then coated using ALD and two measurements for endotoxin presence were taken. The first measurement, ALD Sup, was taken by subjecting the coated particles to PBS to test their dissolution. The supernatant was sampled and endotoxin levels were undetectable. In theory, the coated protein particles could have not dissolved and thus any endotoxin could have been sequestered to the interior of the particle. In order to test this, the particles were digested with exposure to heat and mechanical degradation in hopes of breaking apart any intact particles. A sample was then taken, and endotoxin levels were still undetectable. From the samples taken after the QHP Polish, it appears that endotoxin is not reintroduced during particle formation or ALD coating (Fig. 10).

![Endotoxin Levels During Particle Production](image)

**Fig. 10**
LAL endotoxin assay on samples taken during the HPV16 L1 protein purification process, spray drying, and ALD. After purification, endotoxin levels measured at 6 EU/mL and no endotoxin is reintroduced during drying or coating processes. Absorbance was read at 405nm.
Discussion

HPV16 is a major etiological agent for cervical cancer as it accounts for 50% of HPV-attributable cervical cancers. HPV16 is thus a valid target for vaccine development and although there are effective HPV vaccines commercially available that protect against HPV16, the high cost and cold-chain requirements make these vaccines difficult to use in resource poor areas of the world. In addition, the booster requirements present another hindrance in places without reliable transportation and patient communication. Current HPV VLP vaccines include Gardasil® and Cervarix®, which are produced in yeast and baculovirus-infected insect cells, respectively. Alternatively, HPV L1 capsomere-based vaccines expressed in E. coli cells may provide a lower cost alternative. It has been shown that these adjuvanted HPV L1 capsomere-based vaccines are effective and thermostable when lyophilized at 50°C for 12 weeks, but the use of E. coli cells for protein expression introduces a potential obstacle.

E. coli is a gram negative bacteria that contains endotoxin, a lipopolysaccharide membrane component, which produces a pyrogenic response in humans ranging from fever and chills to fatal septic shock. Endotoxin is also potentially present due to glassware, plasticware, water, and human handling. The FDA requires that endotoxin must fall below a certain threshold that is determined by the maximum allowable endotoxin exposure, maximum human dose volume, and drug potency. Future experiments will confirm the dose volume, but an estimated limit is around 5EU/mL.

In order to effectively measure endotoxin levels throughout protein particle production, samples were taken at each step in the expression, purification, and particle production procedures for HPV 16 L1. Samples taken early in the process show endotoxin levels upwards of 6.0E+06 EU/mL using the LAL Endotoxin Assay, while SDS Page gels and Western blots show...
the presence of other cellular debris. After a purification process that includes anion exchange
column chromatography and ammonium sulfate precipitation, the endotoxin levels drop
significantly to 6 EU/mL. This million-fold reduction in endotoxin reflects a robust and efficient
purification process. This data supports the hypothesis that endotoxin was removed in varying
amount during the HPV16 L1 protein purification process.

A second-hand conclusion shown during this experiment was the unimportance of the
initial QFF column chromatography. Due to danger of column damage, QFF column
chromatography was not used on the first 3L purification process. The issue was resolved and the
second 3L purification process proceeded as usual with the initial QFF ion exchange
chromatography. The SDS Page gels, Western blots, and endotoxin data of each process were
compared to determine the efficacy of the primary QFF ion exchange chromatography. It was
determined that this step in the purification process was unnecessary and has resulted in adapted
protocol for HPV16 L1 purification going forward.

With endotoxin levels within an acceptable range after L1 purification, samples were
taken throughout the particle production process to determine if endotoxin was reintroduced due
to glassware, plasticware, water, or human handling. The HPV16 L1 protein was purified in a
NaCl buffer, and was exchanged into a histidine buffer through gel filtration chromatography.
This desalting process did not intro any endotoxin into the sample, as the endotoxin
measurement was 4.5 EU/mL.

HPV16 L1 particle formation started with spray drying the protein in combination with
trehalose, starch, and Tween 80. Trehalose served as the glassy matrix that thermostabilzed the
L1 protein. Hydroxyethyl starch was used to increase the viscosity of the solution and add
texture to the particle in hopes of improving the particles ability to be fluidized in a fluidized bed
reactor during atomic layer deposition. Tween 80 was used to keep the antigen away from the air/surface antigen and also increase the ability to be fluidized. The spray-dried HPV16 L1 particles were tested for endotoxin presence and were measured to contain 2 EU/mL.

After the protein solution was spray-dried into particles, the particles were subjected to atomic layer deposition in a fluidized bed reactor. Endotoxin levels were measured to determine whether or not endotoxin was reintroduced due to materials or human handling. Endotoxin levels were undetectable in the samples taken after ALD. The endotoxin levels measured from samples taken during HPV16 L1 particle production process support the hypothesis that endotoxin is not reintroduced during the single administration prime-boost vaccine production process.

This vaccine manufacturing process was effectively used for HPV16, and has the ability to be applicable to a variety of other types of HPV, as well as other antigens. Endotoxin levels were undetectable in the finalized HPV16 L1 layered particle, showing that the current procedure for purification, spray drying and coating, is effective and no other purification techniques need to be added to the established protocol.

Current and future directions of this project include optimization of the spray drying step to produce particles with a narrower size distribution. Any major change in protocol would render the need for repeated endotoxin analysis. In addition, experiments to determine the optimal number of layers required for delivering an effective prime-boost interval are needed, again with repeated endotoxin analysis with any major protocol changes. Overall, with acceptable endotoxin levels, this second-generation HPV vaccine development technique has the potential to produce effective, lower cost vaccine alternatives that will help decrease HPV infections worldwide.
References


