



APPLICATION NOTE

Optimization of Chromatographic Processes for Antigen Purification of an Influenza Vaccine Candidate During Early Discovery

Abera develops a pipeline of vaccine candidates based on its proprietary technology, where Outer Membrane Vehicles (OMVs) are decorated with antigens to elicit an immune response. This pipeline includes a nasal influenza vaccine. Bio-Works manufactures and markets chromatographic agarose-based resins, integral to the purification processes in biopharmaceutical applications. In this collaborative study, we focus on optimizing the vaccine manufacturing process during early discovery, the so-called proof-of-concept, phase, with particular emphasis on the downstream chromatographic purification steps. These steps include immobilized metal affinity chromatography and buffer exchange of the influenza antigen, followed by antigen-delivery vesicle conjugation.

Introduction

Vaccination plays a critical role in maintaining a healthy human population, particularly in regions where the prevalence of disease is high, and treatments are becoming less effective, for example due to antimicrobial resistance. Vaccines come in different forms, including, mRNA vaccines, vector-based vaccines, and virus-like particles. Key factors determining the success of a vaccine candidate reaching the market include safety, efficacy, delivery and stability.

Vaccine platform – Outer Membrane Vehicles decorated with antigens

Abera's vaccine delivery platform – BERA – is based on Outer Membrane Vesicles (OMVs) derived from genetically optimized Gram-negative bacteria. The technology allows for high-density decoration of the OMVs with recombinant antigens (Figure 1) and other functional proteins to develop effective vaccines and

potent immunotherapeutics. This technology gives the option to decorate the OMVs with one unique antigen or several different antigens, to provide the optimal immunity for as many subtypes of viruses as needed.

Another important aspect of vaccination is its potential not only to provide the individual with immunity, but ideally also to prevent the spread of the disease. Vaccines administered nasally and orally can induce the local mucosal responses in addition to systemic immunity. Pathogen elimination at the mucosal surface effectively blocks transmission between individuals.

OMVs are nanosized spherical buds from the outer membrane of gram-negative bacteria that cannot replicate, and thus have an improved safety profile compared to whole cell based bacterial vaccines. Moreover, the OMV production hosts have been engineered to circumvent the natural reactogenicity of bacteria. This results in a potent, yet safe, vaccine delivery platform, as demonstrated in several toxicology studies.

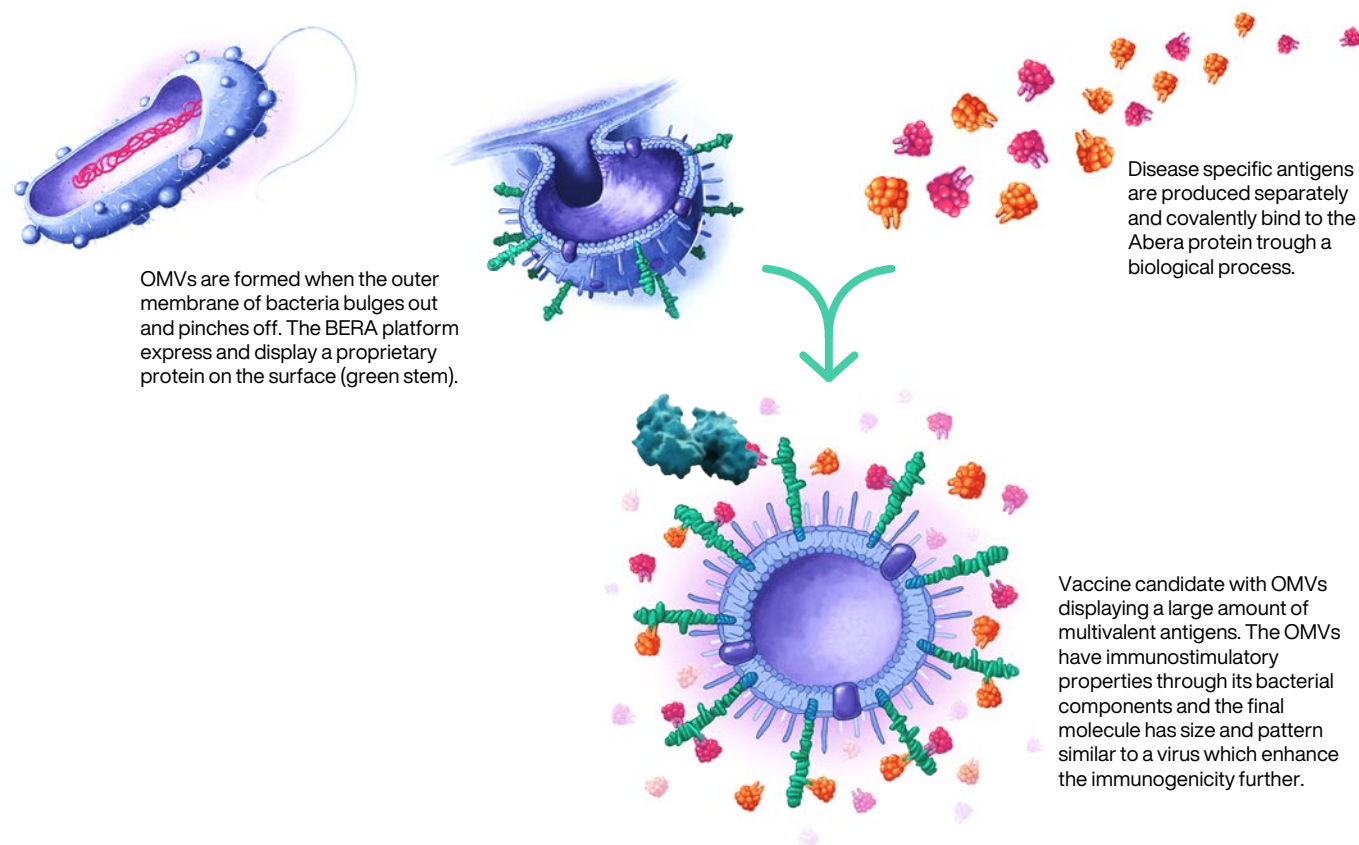


Figure 1. A schematic illustration of the Abera platform, BERA.

The development route for new vaccines

The development of all new vaccines and pharmaceuticals follows a standard pathway, consisting of Research and Development (R&D), development of the manufacturing process, Proof-of-Concept (PoC) studies and clinical testing (phase I-III) before final approval of the vaccine or drug. The PoC phase is an early discovery stage where the vaccine must show ability to elicit an immune response in small animal models, commonly mice. At this stage, adjustments can be made to the vaccine to enhance its efficacy. Vaccine effectiveness is a critical metric as it measures how well vaccination protects people against outcomes such as infection, symptomatic illness, hospitalization, and death. If the vaccine demonstrates promising results, it progresses to clinical trials for testing in people. In the PoC phase, **speed is of essence** to ensure timely advancement to subsequent stages.

Development phases for vaccines

1. Research and Discovery
2. Proof-of-Concept
3. Testing the vaccine
4. The manufacturing process
5. Approval

Abera's influenza vaccine candidate prevents spread of infection

The intranasal influenza vaccine candidate aims to create strong immunological protection directly in the respiratory tract, which is the main infection pathway for influenza viruses, thereby preventing their spread.

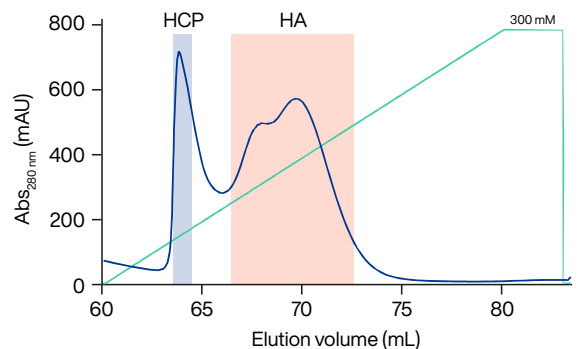
The aim of this study is to optimize chromatographic processes for the influenza antigen hemagglutinin (HA), to obtain sufficient quantities of pure material to ensure efficient coupling to pre-purified OMVs. The resulting OMV-HA complex (the vaccine candidate) will undergo additional purification steps prior to animal testing. In these tests the OMV-HA vaccine will be intranasally administered in mice, which will later be challenged with an influenza strain to evaluate the immune response.

PoC study

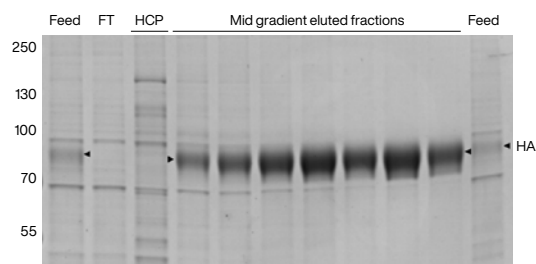
HA was expressed in mammalian cells and filtered through a 0.22 μm filter post harvest. To facilitate the purification, the antigen was fused to a histidine tag (HA-His₆) at the C-terminus. WorkBeads™ Ni-NTA was the resin of choice since it commonly yields high recoveries of His-tagged proteins, even for proteins expressed at low levels. To initially evaluate the binding strength between target and ligands compared to impurities such as the host cell proteins (HCP), 45 mL of supernatant was loaded onto a GoBio™ Mini Ni-NTA 1 mL column, and elution was conducted using a gradient of increasing imidazole concentration. See Figure 2A.

Column: GoBio Mini Ni-NTA 1 mL
 Load: 45 mL mammalian supernatant expressing HA-His₆
 Binding buffer: 300 mM NaCl, 50 mM sodium phosphate, pH 8
 Elution buffer: 300 mM NaCl, 50 mM sodium phosphate, 300 mM imidazole, pH 8
 Gradients: (A) linear, 0-100% for 20 CV, (B) steps à 3 CV as indicated in chromatogram
 Flow rate: (A) 0.25 mL/min; 4 min residence time, (B) 1 mL/min, 4 min residence time

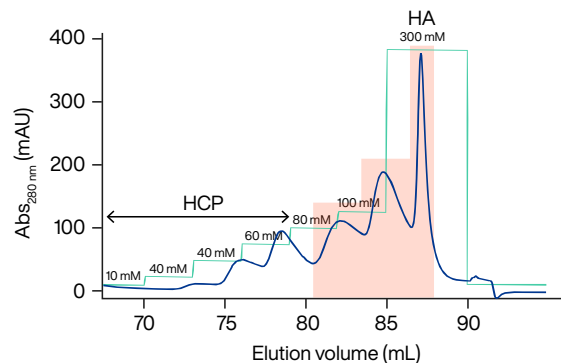
(A) 45 mL feed – 1 mL GoBio Ni-NTA, gradient elution



Analysis of eluted fractions



(B) 45 mL feed – 1 mL GoBio Ni-NTA, step elution



Analysis of eluted fractions

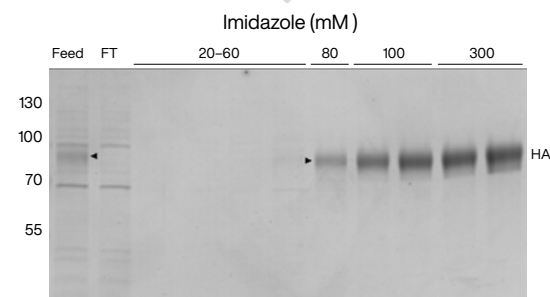


Figure 2. A linear elution gradient (A) and a step elution gradient (B) run for purification of HA-His₆. In the chromatograms, red highlighted areas are collected fractions containing the target and the blue highlighted area is the collected HCP fraction. The analyses of these are shown below in the corresponding SDS-PAGE, as indicated.

The chromatogram from the linear gradient elution purification (Figure 2A) indicates that the HCPs are eluted earlier in the gradient compared to HA-His₆, which is confirmed by SDS-PAGE analysis of collected fractions. No HA-His₆ was visible in the flow-through (FT) compared to the feed (as indicated by arrows in the SDS-PAGE image).

Based on these findings, a step gradient was conducted to establish the minimal imidazole concentration required to remove the HCP from the eluted target protein (Figure 2B). Since the target protein started to elute at approximately 80-100 mM imidazole, 10 mM imidazole was included in the binding buffer to further enhance purity. Additionally, the flow rate was increased to a residence time of 1 minute (1 mL/min) to shorten the cycle time.

As seen in the step gradient study, the HA-His₆ starts to elute significantly at a concentration of imidazole of 80 mM, whereas the majority of HCP eluted at lower concentrations of imidazole. No HA-His₆ was visible in the flow-through (FT in SDS-PAGE) compared to the feed (see arrow in the SDS-PAGE image).

Based on the gradient runs, an optimized process was set up for a larger load purification, 1.3 L supernatant was

loaded onto a GoBio Mini Ni-NTA 5 mL column with a wash step of 50 mM imidazole to eliminate residual HCP with a final elution step at 300 mM imidazole (Figure 3).

Column: GoBio Mini Ni-NTA 5 mL
 Load: 1.3 L mammalian supernatant expressing HA-His₆
 Binding buffer: 300 mM NaCl, 50 mM sodium phosphate, 10 mM imidazole, pH 8
 Elution buffer: 300 mM NaCl, 50 mM sodium phosphate, 300 mM imidazole, pH 8
 Wash: 50 mM imidazole for 10 CV
 Elution: 300 mM imidazole for 10 CV
 Flow rate: 5 mL/min, 1 min residence time

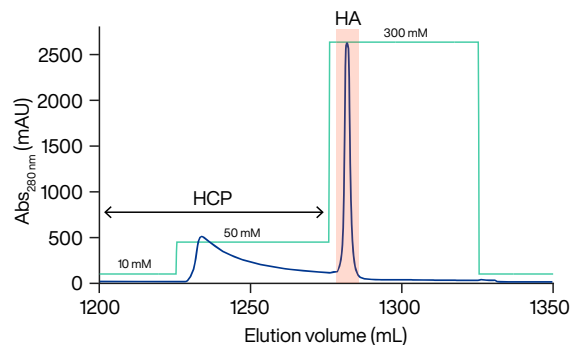
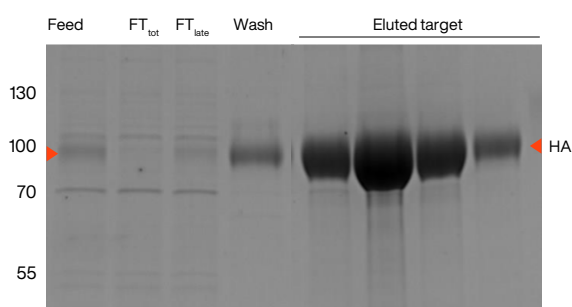


Figure 3. A large loading purification of HA-His₆. In the chromatogram, red highlighted area is collected fraction containing the target.

A pure eluate consisting of HA-His₆ was obtained, however, the yield was lower than expected. Analysis on SDS-PAGE revealed that some target protein was eluted in the wash step (see Figure 4A). Further investigation concluded that components present in the supernatant or media negatively impacted the nickel-ligand interaction. This caused partial stripping of nickel ions, thus reducing the resin's target-metal binding capacity and resulting in target protein loss. As the yield of the target collected was still high enough to do coupling experiments, approximately 10 mg HA-His₆ per run (from 2 batches processed in series), was further processed for the PoC study in order to save time. In parallel, another larger load was purified using a tolerant nickel-IMAC resin, WorkBeads NiMAC, to investigate if a more robust method could be obtained. The aim was to optimize the process for future vaccine candidates expressed in similar growth media without delaying the timeline for entering the next phase. The WorkBeads NiMAC, with tightly bound nickel ions, significantly reduces nickel ion leakage. A load of 750 mL supernatant was applied to a GoBio Mini NiMAC 5 mL column with no significant target loss observed in the flow-through or the wash step (see Figure 4B), indicating a more robust IMAC method for future processes.

(A) GoBio Ni-NTA



(B) GoBio NiMAC

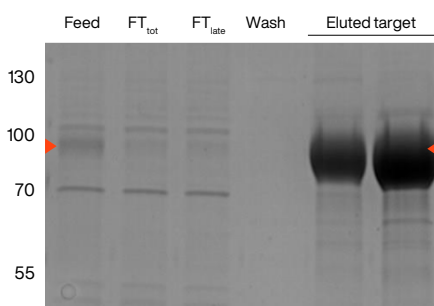


Figure 4. SDS-PAGE analysis of fractions collected from purifications of HA-His₆ using GoBio Ni-NTA vs. GoBio NiMAC. The red arrows indicate the position of the target protein. FT refers to the flow-through fraction, both the complete flow-through (tot) and its final portion (late).

Following the larger loading runs, sufficient material was collected for subsequent coupling to pre-purified OMVs. However, a buffer exchange was required first. 5 mL of Ni-NTA eluate was loaded onto a GoBio Prep Dsalt column (20 mL) and isocratically eluted with PBS at a flow

rate of 5 mL/min, 120 cm/h (Figure 5). The imidazole was effectively removed (as indicated by the conductivity peak), along with other small impurities such as potential leaked nickel residues. The target eluate protein was successfully collected. This desalting step was crucial to ensure complete removal of imidazole from the targets, which may interfere with the coupling reaction to the OMVs.

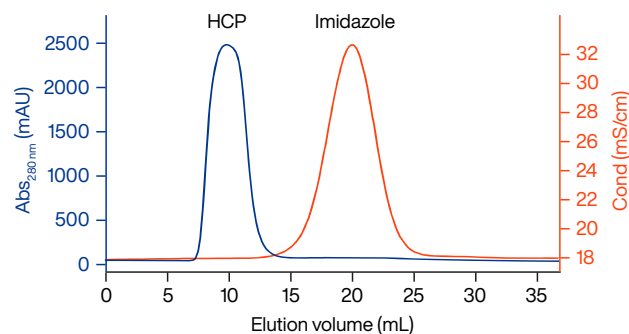


Figure 5. Buffer exchange using WorkBeads DSalt. Target peak shown by absorbance at 280 nm (blue) is base line separated from conductivity/imidazole peak (red).

At this stage the antigen, HA-His₆, is ready for coupling to the OMVs. An anchor protein on OMVs was targeted for the coupling reaction. Figure 6 provides a visual representation of the coupling procedure and how the coupling efficiency was detected using Abera's proprietary technology.

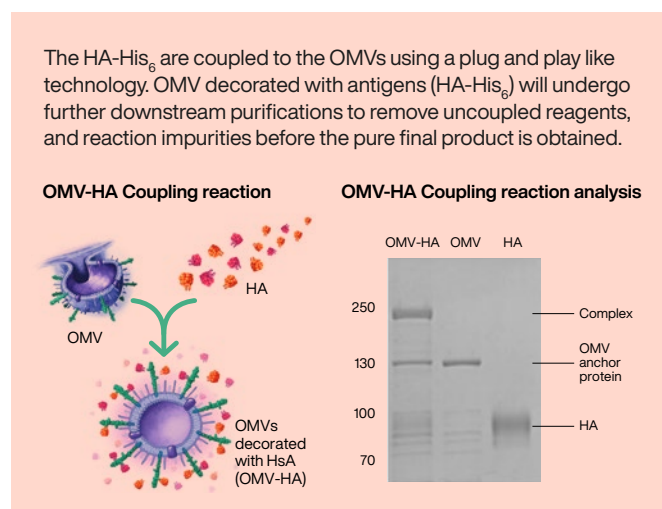


Figure 6. An illustration of the coupling procedure.

Out of 2.5 L supernatant, 12.8 mg pure OMV-HA complex was obtained to be used in the mice immune response experiments. This quantity is more than sufficient for the mice experiments.

If the vaccine candidate demonstrates a positive effect (high efficacy) in the mice studies, the next phase can be initiated. In most cases, the His tag would be removed, and another chromatography step would be developed prior to development of the GMP manufacturing process.

Conclusion – manufacturing process set in PoC phase

The optimization of the purification process for antigen, HA-His₆, was successful, proving to be fast, efficient and straightforward (1-4 in the summary illustration; Figure 7). In the PoC phase, the use of His-tagged proteins, if possible, is highly advantageous as it provides an easy and reliable capture system. In this study, only pre-packed columns were used to further speed up the process and ensure consistent reliable results.

- Speed is of essence in early discovery (PoC phase)
- Pre-packed columns are convenient to use
- Optimizations are important to obtain desired purity and yield
- In PoC studies it is advantageous to have a good biopharmaceutical-supplier collaboration

Optimization of IMAC process

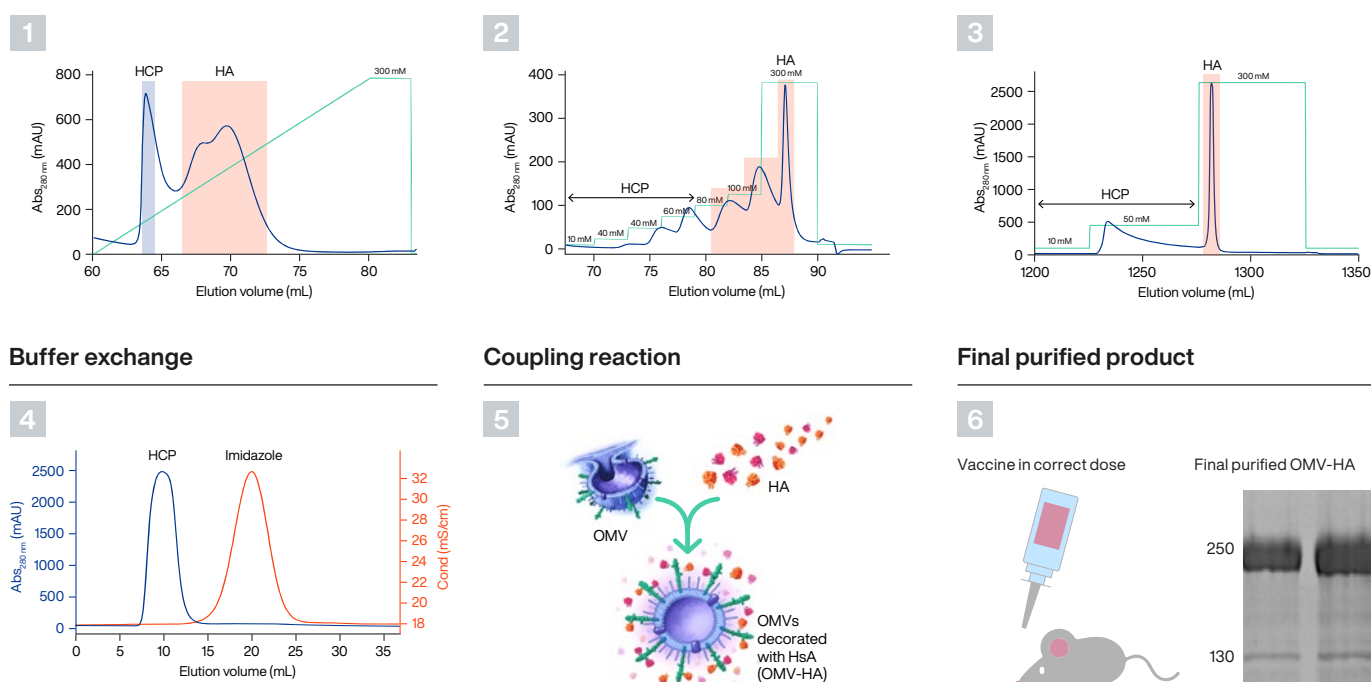


Figure 7. Overview of the PoC study for the OMV-HA vaccine candidate.

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