



## Morphological characterization of a plant-made virus-like particle vaccine bearing influenza virus hemagglutinins by electron microscopy



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### ABSTRACT

Plant-made virus-like particle (VLP) vaccines that display wild-type influenza hemagglutinin (HA) are rapidly advancing through clinical trials. Produced by transient transfection of *Nicotiana benthamiana*, these novel vaccines are unusually immunogenic, eliciting both humoral and cellular responses. Here, we directly visualized VLPs bearing either HA trimers derived from strains A/California/7/2009 or A/Indonesia/5/05 using cryo-electron microscopy and determined the 3D organization of the VLPs using cryo-electron tomography. More than 99.9% of the HA trimers in the vaccine preparations were found on discoid and ovoid-shaped particles. The discoid-shaped VLPs presented HA trimers on their outer diameter. The ovoid-shaped VLPs contained HA trimers evenly distributed at their surface. The VLPs were stable for 12 months at 4 °C. Early interactions of the VLPs with mouse dendritic and human monocytoïd (U-937) cells were visualized by electron microscopy after resin-embedding and sectioning. The VLP particles were observed bound to plasma membranes as well as inside vesicles. Mouse dendritic cells exposed to VLPs displayed classic morphological changes associated with activation including the extensive formation of dendrites. Our findings demonstrate that plant-made VLPs bearing influenza HA trimers are morphologically stable over time and raise the possibility that these VLPs may interact with and activate antigen-presenting cells in a manner similar to the intact virus.

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## 1. Introduction

Influenza infection is a major global health threat [1,2]. Estimates vary but 5–15% of the world's population is infected annually resulting in massive morbidity and as many as 500,000 deaths in a 'good year' [1]. Currently, vaccine production relies primarily on fertilized chick embryos, with strain choices for southern and northern hemisphere formulations based largely on the viruses circulating in the opposite hemisphere during the previous influenza season [3]. This approach typically requires several months to adapt circulating strains for optimal growth in eggs and to generate large quantities of vaccine [4]. Furthermore, this process can introduce mutations that interfere with vaccine

efficacy [5]. New approaches for vaccine production are needed both to better target wild-type viruses and to increase the speed of production in case of a pandemic.

Using transient *Agrobacterium* transfection of a distant relative of the tobacco plant (*Nicotiana benthamiana*), Medicago Inc., is developing candidate virus-like particle (VLP) vaccines for influenza [6,7]. The VLPs are composed of recombinant wild-type viral hemagglutinin (HA) incorporated in a pleomorphic lipid envelope derived from the plant cell membrane [6]. Viral HA accounts for >98% of the VLPs protein content and the VLPs do not contain any detectable nucleic acid of viral, bacterial, or plant origin [8]. The scalability of the plant-based platform and the speed of recombinant vaccine production (weeks rather than months) offer real advantages in epidemic, pre-pandemic, and pandemic situations. These plant-made VLPs have proved to be immunogenic in both animal models and human clinical trials [6,7,9], eliciting durable

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and cross-reactive antibody production as well as robust poly-functional T cell responses [10,11].

The general size and shape of the plant-made VLPs, the form assumed by the HA itself (e.g.: monomer, dimer, trimer), the number of HA molecules per particle and the distribution of HA on the VLP surface could all plausibly influence VLP-immune cell interactions and the immune response [12–14]. We therefore sought to better characterize two VLP vaccines bearing either a seasonal (H1-VLP: A/California/7/2009) or an avian strain HA (H5-VLP: A/Indonesia/5/05). Using cryo-electron microscopy (cryo-EM) and cryo-electron tomography (cryo-ET), we performed a detailed morphological analysis of the vaccines and their stability during storage at 4 °C over a year. We determined the shape and size of the VLPs as well as the positioning of the HA trimers on their surface. We also visualized VLPs interacting with immune cells using histological electron microscopy. Overall, we found that both the H1 and H5 candidate vaccines contained mainly discoid or ovoid-shaped VLPs with HA trimers on their surface. When added to either murine or human antigen presenting cells (APC) *in vitro*, the VLPs were observed to interact with the plasma membrane where they were rapidly internalized and induced ultrastructural changes consistent with cell activation.

## 2. Methods

### 2.1. Virus-like particles

The monovalent VLPs used in the current study were clinical-grade material produced by Medicago Inc (Quebec, QC) as previously described [7]. The native HA sequences targeted were derived from A/California/7/2009 (H1-VLP: 441 µg/mL based on HA content) or A/Indonesia/5/05 (H5-VLP: 209 µg/mL based on HA content). VLP stock solutions were maintained in aliquots in phosphate-buffered saline (PBS: pH 7.4) at 4 °C in the dark.

### 2.2. Negative stain preparation

The VLP samples were diluted with PBS pH 7.4–100 µg/mL based on estimated HA content. 5 µL of the diluted VLP solution was placed for 30 s on 200 Hex grids (EMS Inc., Hatfield, PA, USA), formerly carbon coated and glow-discharged. The grids were washed twice with 5 µL of distilled water for 30 s followed by two incubations with a solution of 1.5% uranyl formate (EMS Inc.). Excess fluid was removed and samples were left to air dry. The grids were imaged commonly on a Tecnai T12 (FEI Inc., Hillsboro, OR, USA). This microscope and all others used for this study are located at the Facility for Electron Microscopy Research at McGill University.

Several approaches were used to facilitate visualization of individual VLPs for the long-term stability analyses including (1) Trituration × 15 by pipette (tip inner-diameter of 0.018 in.); (2) passages through a Hamilton micro-syringe (5 µL RN syringe, with a 26 s bevel tip, inner diameter 0.005 in.); (3) Inversion × 10; (4) vortexing 30 s.

### 2.3. Observation of VLPs by two dimensional cryo-EM

The VLP vaccine preparations were observed using cryo-EM upon reception, and after storage at 4 °C every 3 months over a 12 month period. At each time point, 5 µL of either H1-VLP or H5-VLP were placed for 1 min on a Quantifoil R2/2 copper grid (EMS Inc.) previously glow-discharged for 30 s. Excess fluid was removed and the sample was plunged frozen in liquid ethane at –180 °C using a Vitrobot (FEI Inc.). The chamber was set to 100% humidity at 4 °C. Grids were stored in liquid nitrogen until imag-

ing. The prepared VLP samples were visualized on a Tecnai F20 microscope (FEI Inc.). At each time point, 100–150 images from one grid were recorded at 29,000× magnification and a defocus of –9 µm using a Ultrascan 4 k × 4 k CCD Camera (Gatan Inc., Pleasanton, CA, USA).

The micrographs were analyzed using Image J software [15]. Overall, 4666 H1-VLPs and 3424 H5-VLPs (~1000 particles every 3 months) were classified according to shape and size. The average diameters and standard deviations were calculated and the statistical significance was determined with One-way ANOVA.

### 2.4. Observation of VLPs using cryo-electron tomography (cryo-ET)

The frozen hydrated VLP samples were imaged in a Titan Krios 300 kV Cryo-STEM (FEI Inc.). Images were recorded on a Falcon 2 Direct Detection Device (DDD) (FEI Inc.) at a pixel size of 3.6 Å at the specimen level. Tilt series were collected using the Volta Phase Plate (FEI Inc., Oregon, USA) and the Tomography software (FEI Inc., Oregon, USA). Data were recorded from +60° to –60° in 2° increments at the nominal magnification of 22,500× (pixel size at the specimen level of 3.7 Å) and a defocus level of –0.5 µm. The images were binned by a factor of two prior to processing. 3D reconstructions were calculated using the Imod Etomo package suite [16] and its simultaneous iterative reconstruction technique (SIRT) procedure with 15 iterations. The distance between adjacent HA trimers was measured by recording the coordinates in the tomogram of points located at the center of the tail and head regions of adjacent trimers. For visualization, individual VLPs were selected from the reconstructed tomogram data. Filtering with Nonlinear Anisotropic Diffusion with IMOD package suite [17] was completed. Contours were displayed using UCSF Chimera 1.10.2 [18], and coloured using Adobe® Photoshop® CS4 software (San Jose, CA, USA).

### 2.5. Particle size by dynamic light scattering (DLS)

DLS was performed using a Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) and Zetasizer software (Version 7.11) for data analysis. VLP samples were analyzed undiluted at a total protein concentration between 400 and 600 µg/mL. A volume of 0.6 mL was dispensed into a standard polystyrene semi-micro cuvette and measured in triplicate. The intensity-average size distributions from each set of scans were averaged for each sample.

### 2.6. Particle size by nanoparticle tracking analysis (NTA)

NTA measurements were performed using a NanoSight LM20 instrument (Malvern Instruments) consisting of a microscope (×20 magnification), onto which is mounted a video camera, and a LM20 unit (sample unit) with a laser light source (405 nm). Following the manufacturer's instructions, the samples were diluted with PBS to reach a particle concentration of approximately 1.0<sup>9</sup> particles/mL. Each sample was analyzed in triplicate. The NanoSight LM20 records 90 s sample videos which were then analyzed with the Nanoparticle Tracking Analysis (NTA) software (version 2.3).

### 2.7. Histological electron microscopy

Mouse dendritic cells (DCs) were differentiated from mouse bone marrow and cultured as previously described [19]. Cells were plated on Lab-Tek 8-well permanox chamber slides (EMS Inc., Hatfield, PA, USA) at the concentration of 1 million cells per mL and left to adhere to the plates for 30 min at room temperature (RT). Cells were then washed with serum free media and either incubated with 20 µg/mL of H1-VLP at RT for 60 min, or incubated with 15 µg/mL of H1-VLP at 4 °C for 30 min before fixation with 2.5%

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