

Viral Nanoparticles as Antigen Carrier: Influence of Shape on Humoral Immune Responses *in Vivo*

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Electronic Supplementary Information

1. Materials

Estriol, succinic anhydride, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC·HCl), N-hydroxysuccinimide (NHS), bovine serum albumin (BSA), protein maker, complete Freund's adjuvant, incomplete Freund's adjuvant, DMEM medium, HRP-conjugated second antibody, other chemicals and cell culture reagents were local commercial products and used as received. All organic solvents were dried and distilled before used.

2. Measurements

Ultracentrifugation was performed at the indicated rpm values with a Beckman Optima L-80 XP ultracentrifuge equipped with 50.2 Ti rotor. Transmission electron microscopy (TEM) analysis was carried out by dropping 20 μ L aliquots of each sample at a concentration of 0.1 mg/mL onto 300-mesh carbon-coated copper grids. Then the grids were stained with 20 μ L of 2% uranyl acetate and viewed with JEOL JEM-1011 microscope. Fast Protein Liquid Chromatography (FPLC) analysis was performed on an AKTA explorer (GE Healthcare) instrument with HiTrap Q Sepharose FF exchange column. 0.05 M potassium phosphate buffer (pH 7.4) was used as the low-salt buffer and 1.0 M NaCl in 0.05 M potassium phosphate as high-salt eluent. Zeta potential was measured by a Malvern Zetasizer Nano ZS. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was prepared in house with 12% acrylamide separating gel (30:1 acrylamide to bisacrylamide) with 5% acrylamide stacking gel. ELISA microplate absorbance was measured with ELx808 absorbance microplate readers (BioTek).

3. Purification of TMV-EPMK and CPMV

TMV-EPMK and wild-type CPMV were isolated from infected tobacco leaves following previously established protocols^{1,2}. Secondary leaves of tobacco plants and cowpea plants were inoculated with TMV-EPMK and CPMV, respectively. Freshly harvested or frozen (stored at -80 °C) infected leaves were crushed and homogenized in potassium phosphate buffer (0.1 M, pH 7.8 for TMV-EPMK and pH 7.0 for CPMV) with β -mercaptoethanol (0.2-0.3%). The mixture was filtered through 2 layers of cheesecloth and centrifuged at 9000 rpm (Sorvall, SLA1500) for 15 min before the supernatant was treated with a 1:1 ratio of CHCl₃:n-butanol while stirring on ice for 30 min. The aqueous portion was collected and TMV-EPMK or CPMV was precipitated by the addition of polyethylene glycol 8K (8% wt/v) and NaCl (0.2 M). The resultant pellets were resuspended in potassium phosphate buffer (0.01 M, pH 7.8 for TMV-EPMK and pH 7.0 for CPMV), layered over a 20% wt/wt sucrose solution and ultracentrifuged at 42,000 rpm (Beckman 50.2 Ti) at 4 °C for 2.5 h. Pure TMV-EPMK or CPMV pellet was resuspended overnight in potassium-phosphate buffer (0.01 M, pH 7.8 for TMV-EPMK and pH 7.0 for CPMV). The virus concentration was checked with UV-vis spectroscopy and determined by absorbance at 260 nm (0.1 mg/mL virus gives an absorbance of 0.3 for TMV-EPMK and 0.8 for CPMV). The integrity of the virus morphology was confirmed by TEM.

4. Synthesis of estriol 3-hemisuccinate (E3-3-HS)

Estriol 3-hemisuccinate (E3-3-HS) was synthesized according to the reported method with some improvements³. Estriol (500 mg, 1.734 mmol) was dissolved in 30 mL of dry pyridine. Under the protection of argon atmosphere, succinic anhydride (174 mg, 1.734 mmol) in 10 mL of dry pyridine was added dropwise over 30 min to the rapidly stirring solution. Then the reaction was allowed to proceed at 45 °C for 48 h under argon. The solvent was removed under vacuum and the resulting precipitate was purified by silica column chromatography using CH₃OH/ CHCl₃ (1/100), CH₃OH/ CHCl₃ (1/20), CH₃OH/ CHCl₃ (1/10) as successive eluent to yield a white solid (146 mg, 22%). ¹H NMR (400 MHz, CD₃OD/CDCl₃) δ (ppm): 7.10 (d, J= 8.0 Hz, 1H), 6.62 (d, J= 8.0 Hz, 1H), 6.55 (s, 1H), 2.80 (m, 2H), 2.65 (m, 4H), 2.25 (m, 2H), 1.96 (m, 2H), 1.85 (m, 2H), 1.72 (m, 2H), 1.45 (m, 5H), 0.84 (s, 3H).

5. Synthesis of TMV-EPMK-E3 and CPMV-E3

The viruses were stored in 0.01 M potassium phosphate buffer (pH 7.8) at a concentration of about 10 mg/mL. 20 mg TMV-EPMK or CPMV was added to a mixture of E3-3-HS (20 mg, about 50-fold excess relative to the viral subunit), EDC·HCl (191.7 mg, 1 mM) and NHS (115 mg, 1 mM), in 10 mL mixed solution of buffer and dimethyl sulfoxide (DMSO) (v/v=80:20). The reaction was performed at 4 °C for 48 h. After dialysis in buffer to eliminate most of the excess small-molecule reagents, the products was purified by ultracentrifugation at 42,000 rpm for 2.5 h with Beckman 50.2 Ti rotor and the resulting transparent pellets were redissolved in 2 mL 1×PBS buffer. The modified virus concentration were measured using a modified Lowry protein assay kit (Pierce). The yield of TMV-EPMK-E3 and CPMV-E3 synthesis were 60%. The resulting conjugates were characterized by TEM, SDS-PAGE, zeta potential and ion exchange FPLC.

6. General procedure for mouse immunization

Pathogen-free BALB/c male mice age 6-8 weeks were obtained from MabCom, Inc. and maintained in Institute of Biotechnology, DaQing. All animal care procedures and experimental protocols have been approved by the MabCom, Inc.. Groups of three BALB/c mice were injected subcutaneously under the scruff on day 0 with 0.05 mg TMV-EPMK-E3 and CPMV-E3 constructs as emulsions in complete Freund's adjuvant (0.1 mL, Sigma). Boosters were given subcutaneously under the scruff on days 14 and 28 with the constructs as emulsions in incomplete Freund's adjuvant (0.1 mL). Blood (~0.2 mL/mouse) was collected from each mouse on days 35. Sera from each group of mice were isolated and pooled.

7. General procedure for cell fusion

Cell fusion was performed for producing monoclonal antibodies. Boosted animal was sacrificed by CO₂ asphyxiation. Spleen was separated and teased into a single-cell suspension. Debris and disperse cells were removed by passage through a fine-mesh metal screen and red blood cells (RBC) was lysed with 0.83% NH₄Cl. Spleen cells was washed and suspended in DMEM medium. Mixed SP2/0 cells and spleen cells at a 1:1 ratio and then centrifuged cell mixture 4 min at 500 × g, room temperature. Pre-warmed 50% PEG was added to the mixed-cell pellet drop-by-drop and then filled the tube with pre-warmed DMEM. After stayed at 37 °C for 10 min, then the mixed-cell was centrifuged 3 min at 500 × g at room temperature. Discarded the supernatant and suspended the cell pellet into 10% serum HAT DMEM and then added 280μL of suspension to each well of a 96-well flat-bottom plate. The plate was incubated overnight in a humidified 37 °C, 5% CO₂ incubator. On days 2, 3, 4, 5, 7, 9, and 11, aspirated half the volume of each well and added fresh DMEM-20/HEPES/pyruvate/HAT. On day 14, fed cells with complete DMEM-20/HEPES/pyruvate/HT. On day 15 and subsequently, fed cells using complete DMEM-20/HEPES/pyruvate without HAT or HT. When most of the growing cells demonstrate 10% to 25% confluence 2 days later, screening was performed to identify hybridomas by testing cell culture supernatants for the presence of the desired antibody and hybridoma was expanded to produce monoclonal antibodies.

8. General procedure for ELISA

For antibody titer measurement, a 96-well microplate was first coated with a solution of BSA-E3 in PBS buffer (0.5 μg/mL) and then incubated overnight at 4 °C. The plate was then washed four times with PBS/0.5% Tween-20 (PBST), followed by the addition of 1.5% (w/v) BSA in PBS to each well and incubated at room temperature for one hour. The plate was washed again with PBST, and mouse sera were added in 100-fold dilution at first well and

followed a 1:4 serial dilution in 0.1% BSA/PBS. The plate was incubated for two hours at 37 °C and washed. A 1:8000 diluted horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (Jackson) in 0.1% BSA/PBS was added to each well, respectively. The plate was incubated for one hour at 37 °C, washed, and a solution of 3,3',5,5'-tetramethylbenzidine (TMB) was added. Color was allowed to develop for 20 min, and then a solution of 0.5 M H₂SO₄ was added to quench the reaction. The optical density was then measured at 450 nm. Each experiment was repeated at least three times, and the average of the quadruplicate was used to calculate the titer. Errors of each measurement were typically within 10%. Antibody titers were defined by cut-off value.

For antibody specificity measurement, the 96-well microplate was first coated with TMV-EPMK, TMV-EPMK-E3, CPMV and CPMV-E3, respectively. After blocking with BSA, a serial dilution of anti-E3 monoclonal antibody were added and then followed the general ELISA measurement.

Table S1. Physicochemical parameters of VNP-E3

	diameter (nm)	surface area (nm ²)	volume (nm ³)	specific surface area (nm ⁻¹)
TMV-EPMK-E3	300 × 18	16956	76302	0.22
CPMV-E3	29	2641	12764	0.21

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