Supporting Information

Alumina-encapsulated vaccine formulation with improved thermostability and immunogenicity

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1. Experimental

Preparation of boehmite colloids. As the previous description,[1] 2.837 g of $Al(i\text{-}Pro)_3$ (Sigma Aldrich, #229407) was added in 51 ml water at 85°C in an air-open flask and hydrolyzed for 45 min. Then, 1.67 ml of 1 M HCl was added and the temperature was increased to 95°C. The flask was kept in open air for 3 h to enable isopropanol evaporation, and then placed under reflux for 16 h. The reflux apparatus was removed and water was evaporated until the total solution volume was reduced to less than 25 ml, which was finally re-adjusted to 25 ml to ensure the concentration of 15 mg Al^{3+} /ml.

Zeta potential. The examinations were performed by a Zetasizer Nano S (Malvern).

Cells and viruses. As the previous description,[2] human rhabdomyosarcoma (RD) cells were obtained from American Type Culture Collection (ATCC) and were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO_2. Human EV71 strain A12 was isolated from hand, foot, and mouth disease patients. Both *in-vitro* and *in-vivo* characterizations demonstrated that the strain was nonvirulent in suckling mice with ideal attenuated characteristics. All virus stocks were prepared and titrated in RD cells and stored at -70°C until the use.

Vaccine encapsulation. The boehmite colloid suspension was added into the vaccine solutions (10^6–10^8 PFU/mL) to reach the final Al^{3+} concentration of 225 μg/mL. The mixture was mixed and incubated at 37°C for 1 h.

Electron microscopy. The suspension containing examples were dropped onto carbon-coated copper TEM grids (400 meshes, Agar Scientific) and then dried at room temperature. The observations were performed using a JEM-1200EX instrument (JEOL) and the EDX analyses were conducted using an S-4800 instrument (HITACHI).

One-step quantitative real-time RT-PCR (qRT-PCR). RNA of samples was extracted with RNA Purelink RNA mini kit (Ambion), and one-step quantitative real-time RT-PCR was used to quantify the amount of viral RNA by using One Step PrimeScript RT-PCR Kit (Takara) with EV71 specific forward premiers (Fwd, 5'-GGCCATTTATGTGGGTAACTTTAGA-3'; Rev, 5'-CGGGCAATCGTGTCACAAC-3') and probe (5'-FAM-AAGACAGCTCTCGCGACTT GCTCGTGBQH1-3'). Absolute quantification of RNA was calculated according to the standard curve, which was generated by serially diluting a RNA solution of determined titer in RNase-free water.

Plaque assays. RD cells at 90% confluence in 12-well plates were infected with 400 μL of serially diluted virus solutions. After 1 h, the infected cells were washed and then incubated for 3 days in DMEM supplemented with 2% FBS and 1% low melting point agarose. The cells were fixed with 4% formaldehyde and stained using a crystal violet solution (1% crystal violet, 0.85% NaCl, and 2% formaldehyde).

Thermal stability tests. EV71, EV71-Alum or EV71@NanoAlum were incubated at 25°C or 42°C and the samples were collected periodically. The infectivities were determined using plaque assays as previously described.

Indirect immunofluorescence assays (IFA). RD cells at 90% ~100% confluences were infected with EV71, EV71-Alum or EV71@NanoAlum at the desired multiplicity of infection (M.O.I.). The infected cells were fixed at 12 h post infection with precooled acetone at -20 °C for 30 min. The infected cells were washed with PBS and then incubated with polyclonal antibodies against EV71 for 1 h; the cells were then washed with PBS and incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibodies. After washing the infected cells with PBS,

DAPI was added, and the cells were incubated at room temperature for 5 min to stain the nuclei. Fluorescence was detected after PBS washing.

Growth curves. Viral growth curves in RD cells were determined by seeding EV71, EV71@NanoAlum in RD cells at 90% ~100% confluence in 24-well plates at the desired M.O.I. The supernatants were collected at intervals, and their viral titers were examined using plaque assays.

Animal assessments. Animal experiments were approved by and performed in strict accordance with the guidelines of the Animal Experiment Committee of the State Key Laboratory of Pathogen and Biosecurity. Groups (5 mice for each group) of BALB/c mice were subcutaneous immunized with EV71, EV71-Alum and EV71@NanoAlum respectively. Sera were collected at 14 d post-infection. All samples had the same initial virus titers (200 μL, 10^6 PFU/mL). Serum IgG and neutralization antibodies were detected by ELISA and micro neutralization assays, respectively.

Microneutralization tests. Mouse serum was serially diluted (2-fold each time) in DMEM, starting at 1:8. The virus suspensions (200 μL at 100 PFU) were mixed with 200 μL of diluted sera, and the mixtures were incubated at 37°C for 1 h. The mixtures were then added to 90% confluent RD cells and incubated for 3 days. The appropriate serum, viruses, and cell controls were included in the tests. End point titers were calculated according to the Karber method, as previously described.[3]

Enzyme-linked immunosorbent assays (ELISAs). EV71 specific serum IgG antibodies were detected using indirect ELISAs in 96-well flat-bottomed plates (Costar) coated with EV71, which was diluted 1:100 in 0.1 M carbonate/bicarbonate buffer (pH 9.6). The plates were coated overnight at 4°C. After a blocking step with 2% BSA in PBST, the plates were incubated with serially diluted sera in duplicate wells for 1 h at 37°C. Peroxidase-conjugated horse anti-mouse IgG diluted in PBST (1:5000) was added, and the plates were incubated at 37°C for 45 min. TMB substrate was then added. The reaction was ended by adding 2M H_2SO_4 to the wells. The absorbance of the plates was determined at 492 nm.

ELISPOT assays. IFN-γ ELISPOT mouse kits (BD Biosciences) were used according to the manufacturer's instructions. Briefly, 96-well filtration plates were coated overnight at 4°C with capture monoclonal antibody; the plates were then washed and blocked with RPMI-1640 medium containing 1% L-glutamine and 10% FBS for 2 h at room temperature. Splenocytes in RPMI-1640

(5 x10^5 cells/well) were added and EV71 was subsequently added as the stimulation antigen; the plates were cultured for approximately 20 h at 37°C and 5% CO_2. After washing steps with water and PBST, the plates were incubated with biotinylated detection antibody at room temperature for 2 h. Then, plates were incubated with HRP-conjugated streptavidin at room temperature for 1 h after washing three times with PBST. Spots were revealed using an AEC substrate reagent kit (BD Bioscience) at room temperature and counted using an Immunospot Reader (Cellular Technology).

Statistical analyses. The statistical significance of antibody titers differences among different groups was analyzed using Student's t-test as implemented in SPSS software. The results with error bars were expressed as means ±standard deviations.

2. Figures

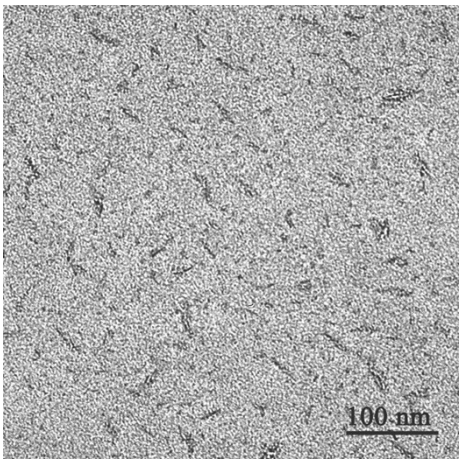

Fig. S1 TEM image of nano alumina clusters. They had typical length of 10-50 nm and width of 5-10 nm and these products were the same as the previously reported ones.[1]

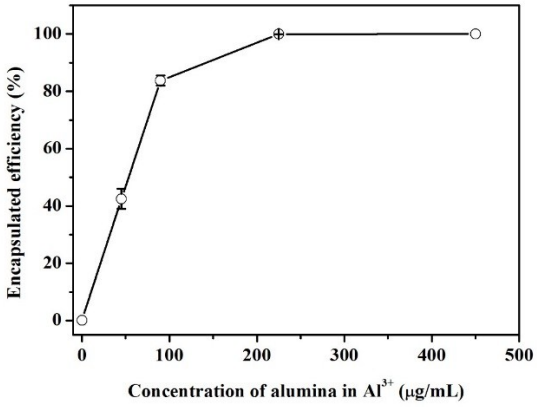

Fig. S2 Encapsulation efficiency of EV71 within nano alumina phase and the optimal concentration of Al^{3+} was 225 μg/mL.

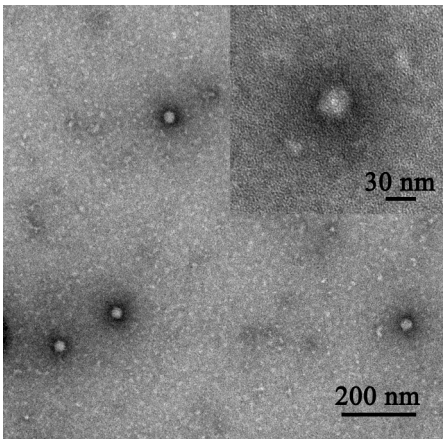

Fig. S3 Typical TEM image of negative stained EV71 virus particles, which had a diameter of about 30 nm.

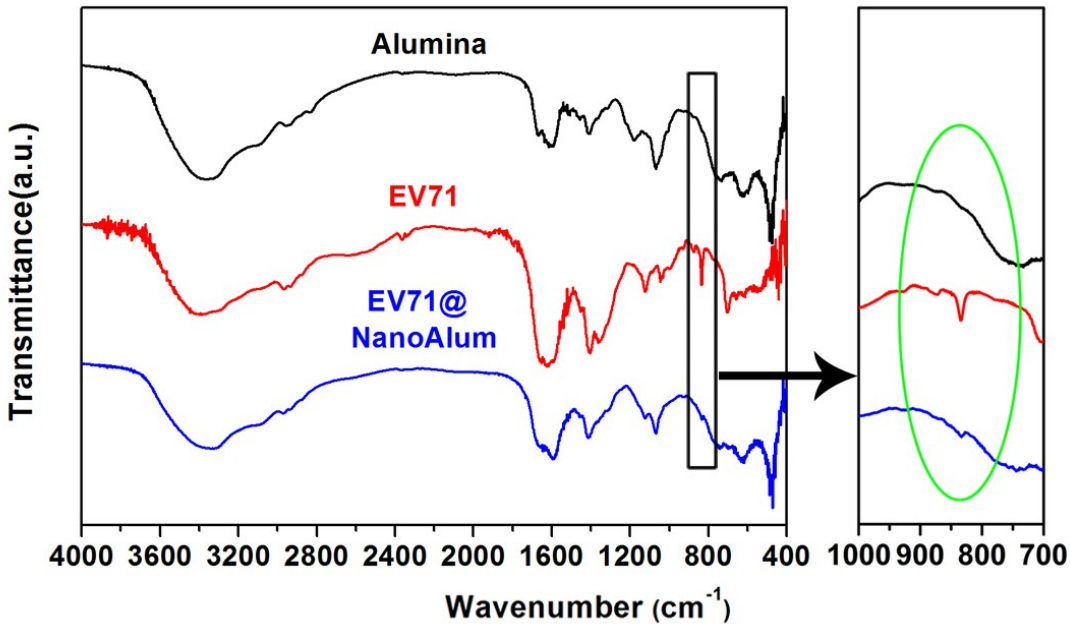

Fig. S4 FT-IR spectra of alumina, EV71 and EV71@NanoAlum. The signals of 400~830 cm^{-1} were contributed by the typical finger-print band of alumina. The peaks at 1050~1070 cm^{-1} were associated with the symmetric stretching vibrations of the Al-O-H bonds. The peak in EV71 at 847 cm^{-1} is unique, which was found in EV71@NanoAlum as well.

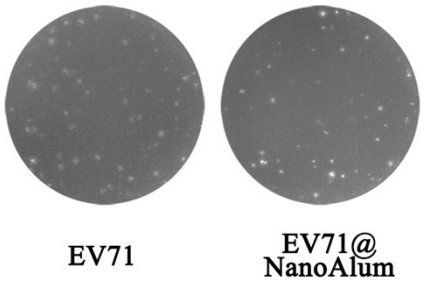

Fig. S5 Plaque morphologies of EV71 and EV71@NanoAlum in RD cells were similar.

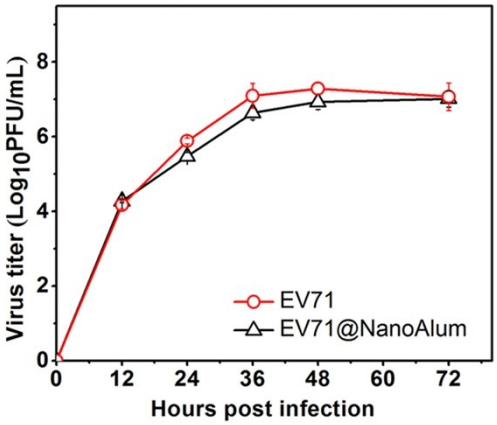

Fig. S6 Growth curves of EV71 and EV71@NanoAlum in RD cells.

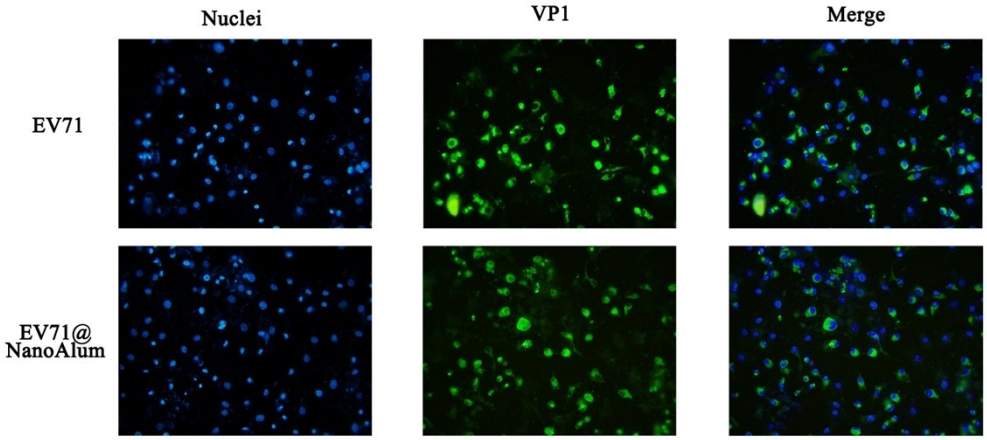

Fig. S7 Indirect immunological fluorescence of EV71 and EV71@NanoAlum (0.1 MOI) at 12 h.

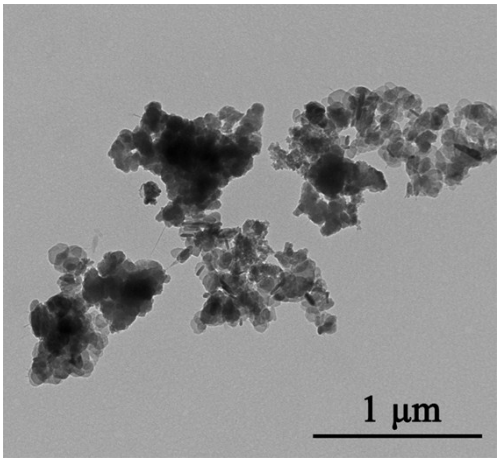

Fig. S8 TEM image of the commercial alumina adjuvant material and the relatively large particles were aggregated with the size of ~1 μm.

Reference

1. A. Rutenberg, V. V. Vinogradov, D. Avnir, *Chem. Commun*., 2013, **49**, 5636.

2. G. Wang, H.-J. Wang, H. Zhou, Q.-G. Nian, Z. Song, Y.-Q. Deng, X. Wang, X.-F. Li, S.-Y. Zhu, C.-F. Qin, *ACS nano*, 2015, **9**, 799-808.

3. E. Traggiai, S. Becker, K. Subbarao, L. Kolesnikova, Y. Uematsu, M. R. Gismondo, B. R. Murphy, R. Rappuoli, A. Lanzavecchia, *Nat. Med*., 2004, **10**, 871-875.