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# Evaluation of the stability of enterovirus 71 virus-like particle

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**Enterovirus 71 (EV71) is responsible for the outbreaks of hand-foot-and-mouth disease that caused significant mortality in children, but no vaccine is available yet. EV71 virus-like particle (VLP) is the empty capsid consisting of viral structural proteins but can elicit potent immune responses, rendering VLP a promising EV71 vaccine candidate. To evaluate whether VLP remains stable after long-term storage, which is crucial for advancing the VLP vaccine to the clinical setting, we evaluated the effects of NaCl concentration, buffers and temperatures on the VLP stability. We first validated the use of dynamic light scattering (DLS) for measuring the hydrodynamic diameter (≈30–35 nm) of VLP, which was close to the VLP diameter (≈25–27 nm) as measured by transmission electron microscopy (TEM). Using these techniques, we found that EV71 VLP remained stable for 5 months in sodium phosphate (NaPi) buffers with various NaCl concentrations. EV71 VLP also remained morphologically stable in NaPi, citrate and $TE^+$ buffers for 5 months, yet the enzyme-linked immunosorbent assay (ELISA) revealed that the VLP stored in citrate and $TE^+$ buffers partially lost the immunogenicity after 5 months. In contrast, the VLP stored in the NaPi buffer at 4°C remained stable macroscopically and microscopically for 5 months, as judged from the DLS, TEM and ELISA. The VLP stored at 25°C and 37°C also retained stability for 1 month, which would obviate the need of a cold chain during the shipping. These data altogether proved the stability of EV71 VLP and suggested that the VLP is amenable to bioprocessing and storage.**



[**Key words:** Baculovirus; Insect cell; Enterovirus 71; Virus-like particle; Stability]

Enterovirus 71 (EV71) belongs to the enterovirus family and shares similarity with other enteroviruses (e.g., poliovirus) in virus structure and replication process (1). Enterovirus has an icosahedral capsid (≈27 nm in diameter) consisting of VP1, VP2, VP3 and VP4 proteins (2,3). During the virus replication, translation of the RNA genome results in the production of a polyprotein comprising P1, P2 and P3 regions. The P3 region encodes nonstructural proteins such as 3CD protease, which can cleave P1 into VP1, VP3 and VP0. These 3 proteins spontaneously assemble into icosahedral procapsids and pack the RNA genomes into the provirions. At the final stage, VP0 is cleaved into VP2 and VP4 [for review see Lee and Chang (4) and McMinn (5)].

EV71 is a major causative agent responsible for the outbreaks of hand-foot-and-mouth disease (HFMD) in young children and infants in Asia-Pacific regions such as Taiwan, China, Malaysia, Japan and Vietnam (4,6–8). EV71 infection of children under 5 years of age may result in severe neurological complications such as aseptic meningitis, brain stem encephalitis and even death (9). The EV71 outbreaks in Taiwan resulted in 405 severe cases and 78 deaths in 1998 (10), and caused 373 severe cases and 14 deaths in 2008. In recent years the Asia-Pacific countries have experienced more frequent EV71-associated HFMD epidemics with high incidence of neurotropic complications and fatality rates (9). In particular, the 2008 outbreak in China resulted in 489,097 reported cases that included 1125 severe cases and claimed 126 lives. Since then, EV71 epidemics have recurred every year in China and led to more than 1 million HFMD cases and claimed the lives of hundreds of children each year (8). The increasing frequency of EV71 epidemics and high fatality rates underscore the urgent need to develop vaccines.

Currently, multiple forms of EV71 vaccines, including inactivated virus (11–13), VP1 subunit (14) and virus-like particle (VLP) (15,16), have been developed. The inactivated virus vaccine is intensively developed by a number of laboratories (13,17), companies (18,19) and research organizations (6,12,20,21), but inactivated virus vaccines may raise higher safety concerns. The VP1-based subunit vaccines are either not evaluated for vaccine efficacies or elicit poorer immune responses than the inactivated virus vaccines (4), probably because VP1 alone lacks certain conformation-dependent epitopes located at the junctions of structural proteins (22).

In contrast to the aforementioned vaccine forms, VLP is the empty particle consisting of viral structural proteins but devoid of viral nucleic acids. VLP is able to elicit broad and strong immune responses thanks to the preservation of capsid structure (23), thus VLP has become a promising vaccine platform (24). To develop the VLP-based EV71 vaccine, we assumed that EV71 VLP was able to form in a manner mimicking the natural virus assembly pathway. With this assumption, we unraveled that co-expression of EV71 P1

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and 3CD in insect cells led to the cleavage of P1 into VP1, VP3 and VP0, and the spontaneous VLP assembly (25). Therefore, we constructed a baculovirus Bac-P1-3CD capable of co-expressing P1 and 3CD (26). After Bac-P1-3CD infection of insect cells, harvest and purification, the dispersed EV71 VLP was indistinguishable from the authentic virus in size (25–27 nm), appearance (icosahedral) and surface epitopes (26). Immunization of mice with the EV71 VLP triggered potent EV71-specific humoral and cellular immune responses, and conferred protection to neonatal mice against lethal viral challenge (15). These data altogether demonstrated the potential of VLP as a vaccine against EV71.

One key determinant to the potent efficacy of VLP was the preservation of capsid structure because denatured VLP elicited lower immunogenicity and protected the mice from virus challenge to a lesser extent (15). As such, the handling and storage of VLP are critical because improper storage may result in the loss of intact VLP structure and immunogenicity, thus compromising the vaccine efficacy. For the development of VLP as a vaccine, here we sought to evaluate the stability of empty EV71 VLP under different conditions (salt concentrations, buffers and temperatures) for up to 5 months, using dynamic light scattering (DLS), transmission electron microscopy (TEM) and enzyme-linked immunosorbent assay (ELISA).

## MATERIALS AND METHODS

**Recombinant baculovirus and cell culture** The recombinant baculovirus Bac-P1-3CD was constructed previously (26) and contained two expression cassettes: P1 gene driven by the polyhedrin promoter and 3CD gene driven by the p10 promoter. Bac-P1-3CD was amplified by infecting Sf-9 cells, harvested, stored and titrated by end-point dilution method as described (27). The High Five (Invitrogen) insect cells were maintained in the shake flask at 27°C using Sf-900 II medium (Invitrogen).

**Production and purification of EV71 VLP** To produce the VLP, High Five cells were seeded to the stirred tank bioreactor (Biostat B, Sartorius) at $5 \times 10^5$ cell/ml, and were infected with Bac-P1-3CD at a multiplicity of infection (MOI) of 10 when the cell density reached $\approx 3 \times 10^6$ cell/ml. The cells were harvested by centrifugation when the viability dropped below 30% (yield ≈ 5–10 mg/L) and filtered through 0.22 μm filter. The supernatant was concentrated with a tangential flow filtration (TFF) unit (Labscale, Millipore) using a 1000 kD filter cassette and the buffer was exchanged to the 50 mM sodium citrate buffer (14.3 g/L $C_6H_5Na_3O_7 \cdot 2H_2O$, 0.26 g/L $C_6H_8O_7$, 5.84 g/L NaCl, pH 6.5). The VLP-containing solution was first purified by size-exclusion chromatography (Sephacryl S-400 HR, GE Healthcare) using the Fast Protein Liquid Chromatography (FPLC) system (Amersham).

The eluted sample containing the VLP was concentrated by TFF ultrafiltration using the 100 kD membrane cassette and was subjected to hydroxyapatite chromatography (CHT Ceramic Hydroxyapatite Type I 40 μm, Bio-Rad). The VLP was eluted using the 100 mM sodium phosphate (NaPi) buffer (5.1 g/L $Na_2HPO_4$, 8.84 g/L $NaH_2PO_4 \cdot H_2O$, pH 6.5) and concentrated by a 30 kD molecular weight cut-off ultracentrifugation unit (Centricon) to ≈300 μg/ml. The purified sample was analyzed by 12% SDS-PAGE and scanning densitometry to confirm the purity (26). Total protein concentration was measured using the Protein Assay kit (Bio-Rad).

**VLP samples preparation** To validate the DLS technique for the measurement of VLP size, the purified VLP was diluted with the 100 mM sodium phosphate (NaPi) buffer (pH 6.5) to different concentrations (6.25, 12.5 and 25 μg/ml). For the influence of NaCl concentrations on the VLP stability, NaPi buffers with different NaCl differentiations were prepared and mixed with the VLP solution (20:1 volumetric ratio, VLP concentration ≈ 15 μg/ml, total volume = 100 μl) to different final NaCl concentrations (0, 0.1 M, 0.5 M and 1.0 M). To assess how buffers affected the VLP stability, we prepared 3 buffers: NaPi (pH 6.5), citrate (14.3 g/L $C_6H_5Na_3O_7 \cdot 2H_2O$, 0.26 g/L $C_6H_8O_7$, 5.84 g/L NaCl, pH 6.5) and $TE^+$ (4.85 g/L Tris, 5.84 g/L NaCl, 0.19 g/L $MgCl_2$, 0.29 g/L EDTA, pH 7.4). The VLP was mixed with the buffer at a volumetric ratio of 1:20 (final VLP concentration ≈ 15 μg/ml). The VLP/buffer mixture was stored in the microfuge tube (Axygen) at different temperatures for different periods of time prior to analysis.

**Particle size measurement by dynamic light scattering (DLS)** The hydrodynamic diameter (referred to as size) of the VLP was measured using the particle analyzer (Zetasizer Nano ZS, Malvern Instruments). For measurement, 100 μl of VLP sample was loaded into the disposable low volume cuvette (Sarstedt, cat. no. 67.758.001) and mounted to the DLS chamber. After 1 min equilibration, the size distribution was obtained by the DLS measurement at 25°C. Each sample was measured 5 times with 10 runs in each measurement. For each condition, 3 independent VLP samples were measured. The average sizes of particles that were most predominantly present in the population were calculated from the measurements.

**Transmission electron microscopy (TEM)** The VLP samples were adsorbed onto Formvar/carbon-coated copper grids (TED Pella Inc., cat. no. 01800-F), negatively stained with 2% phosphotungstic acid (Sigma, cat. no. HT152) and were examined by a transmission electron microscope (H-7500, Hitachi).

**Enzyme-linked immunosorbent assay (ELISA)** The in vitro antigenicity of EV71 VLP was measured by sandwich ELISA as described (28). Briefly, each well in the 96-well plate was coated with 100 μl of protein A-purified rabbit anti-VLP polyclonal antibody (3.8 mg/ml, 1:10,000 dilution, provided by Prof. Bor-Luen Chiang) and incubated overnight. After 3 washes with PBST buffer (0.05% Tween 20 in PBS), the wells were blocked with PBST containing 1% bovine serum albumin (BSA) for 1 h. After washes, the VLP samples were added (100 μl/well) and incubated for 2 h. After washes, mouse monoclonal anti-EV71 IgG (1:500 dilution, Light Diagnostics Enterovirus 71 Reagent, Chemicon, cat. no. 3324) was added (100 μl/well). Following 2 h incubation and 4 more washes, goat anti-mouse horseradish peroxidase-conjugated IgG (Kirkegaard & Perry Laboratories) was added (100 μl/well) and incubated for 1.5 h. The color development was initiated by adding 100 μl TMB (tetramethyl benzidine) solution (Sigma) and terminated 15 min later by adding 50 μl $H_2SO_4$ (2 M). The optical density value at 450 nm ($OD_{450}$) was read with an ELISA reader (Dynex Technology).

## RESULTS

**EV71 VLP production** The EV71 VLP consisting of VP1, VP3 and VP0 was produced by infecting High Five cells with Bac-P1-3CD at MOI 10 with a yield of 5–10 μg/ml (15). After cell harvest, the culture supernatant was concentrated by tangential flow filtration and subjected to size-exclusion chromatography and a second hydroxyapatite chromatography. In the final step, the VLP was eluted with the sodium phosphate (NaPi) buffer, with a purity of ≈92%. The VLP was diluted to 300 μg/ml with NaPi buffer for the following assays (data not shown).

**Validation of VLP size measurement by DLS** One important indicator of the VLP stability is the size because VLP [e.g., VLP of hepatitis B virus (29) and human papillomavirus (30)] may aggregate or dissociate after long-term storage. The VLP aggregation/dissociation are manifested by the increase/decrease of particle size and can be readily measured by DLS (30). Therefore, we chose DLS to measure the hydrodynamic diameter (referred to as size thereafter) of EV71 VLP in order to evaluate the VLP stability.

To validate the VLP size measurement by DLS and determine the appropriate concentrations for measurement, the purified VLP was diluted with the NaPi buffer (the elution buffer at the final stage of VLP purification) to different concentrations (6.25, 12.5 and 25 μg/ml) and measured by DLS. The DLS measurements showed wide variations in VLP size at low VLP concentration (6.25 μg/ml), but exhibited consistent results at 12.5 and 25 μg/ml (data not shown). At 12.5 μg/ml, the average EV71 VLP size was ≈30–35 nm (Fig. 1A), which was close to the VLP diameter measured under TEM (≈25–27 nm, Fig. 1B). The similarity between the particle size measured by DLS (hydrodynamic diameter) and TEM (diameter) thus validated the use of DLS for EV71 VLP size measurement.

**Effect of NaCl concentrations on the VLP stability** It was shown that NaCl can prevent the VLP aggregation and effectively increase the VLP stability (30). Therefore, we examined how the NaCl concentrations influenced the VLP stability by measuring the particle size. NaPi buffers with different NaCl concentrations were prepared and mixed with the VLP to different final NaCl concentrations (0, 0.1 M, 0.5 M and 1.0 M). The VLP size was measured by DLS after storage at 4°C for 1 day (0 month) or for different periods of time (1, 3 and 5 months). The DLS data depicted that the average VLP size remained nearly constant within a narrow range (≈30–35 nm) at all time points and was independent of the NaCl concentrations (Fig. 2), suggesting the absence of VLP aggregation or degradation for 5 months even at high (1 M) or low (0 M) NaCl concentrations.

**Effect of buffers on the VLP stability** To assess how the VLP stability was influenced by buffers, we chose several buffers