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Optimization of Dengue-EDIII – Hepatitis B-VLP Fusion Antigen Protein Expression in Mammalian Cell Systems and Preliminary Immunogenicity Testing in Mice

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ABSTRACT

The development of a safe and effective dengue vaccine remains challenging due to the risk of antibody-dependent enhancement (ADE) associated with imbalanced immune responses. This study aimed to optimize the expression of a dengue virus envelope domain III (EDIII) fusion antigen displayed on Hepatitis B core antigen virus-like particles (VLPs) and to evaluate its preliminary immunogenicity in a murine model. Optimization of antigen design and expression conditions resulted in a substantial increase in VLP production compared with the non-optimized construct. The purified VLPs showed uniform size, proper particle assembly, and preserved antigenic integrity. Immunization of mice induced high titers of EDIII-specific IgG and strong serotype-specific neutralizing antibodies against dengue virus serotype 2, while no antibody-dependent enhancement was detected in vitro. These findings indicate that the optimized EDIII–HBcAg VLP platform is highly immunogenic and demonstrates a favorable safety profile, supporting its potential as a promising subunit vaccine candidate for dengue virus.

Keywords: *Dengue Virus, Envelope Domain III, Virus-Like Particle, HBcAg, Subunit Vaccin, Mammalian Expression System*

INTRODUCTION

Dengue fever is the most widespread mosquito-borne viral disease globally and continues to impose a substantial public health burden, particularly in tropical and subtropical regions. Hundreds of millions of dengue infections are estimated to occur each year, with a considerable proportion progressing to clinically apparent disease and severe manifestations such as dengue hemorrhagic fever and dengue shock syndrome. Dengue virus (DENV) comprises four antigenically distinct but genetically related serotypes (DENV-1 to DENV-4). Infection with one serotype induces long-lasting homotypic immunity but only transient and partial cross-protection against heterologous serotypes, rendering individuals susceptible to secondary infections that are frequently associated with increased disease severity.

A major immunological challenge in dengue is antibody-dependent enhancement (ADE). During secondary heterologous infection, pre-existing antibodies that are non-neutralizing or present at sub-neutralizing concentrations can facilitate viral entry into Fcγ receptor–expressing cells, such as monocytes and macrophages, thereby enhancing viral replication and exacerbating inflammatory responses. This mechanism has been strongly linked to severe dengue outcomes and represents a critical obstacle in vaccine development, as an imbalanced immune response may increase disease risk, particularly in seronegative vaccine recipients (Tan et al., 2023).

Concerns regarding ADE have been highlighted by the performance of first-generation dengue vaccines. Dengvaxia, the first licensed live-attenuated dengue vaccine, demonstrated serostatus-dependent efficacy, conferring protection in seropositive individuals while increasing the risk of severe dengue in seronegative recipients. These findings underscored the urgent need for next-generation dengue vaccines that emphasize safety, immunological precision, and controlled antigen presentation (Shukla et al., 2018). In this context, subunit vaccine strategies based on well-defined viral antigens have emerged as attractive alternatives, as they eliminate risks associated with viral replication, reversion to virulence, and uncontrolled antigenic complexity.

Among the structural components of DENV, the envelope protein domain III (EDIII) has been identified as a particularly promising vaccine antigen. EDIII is a 100–110 amino acid domain located at the C-terminus of the envelope protein and plays a key role in host cell receptor binding. Importantly, EDIII contains multiple serotype-specific conformational epitopes that are strongly associated with virus neutralization and protective immunity. Unlike other regions of the envelope protein, EDIII is not a dominant target of broadly cross-reactive antibodies commonly implicated in ADE, making it a rational choice for a safety-focused dengue vaccine strategy (Ramasamy et al., 2018). However, EDIII alone is intrinsically poorly immunogenic and requires an effective delivery platform to induce robust and durable immune responses.

Virus-like particles (VLPs) represent an advanced subunit vaccine platform capable of addressing this limitation. VLPs mimic the size, geometry, and repetitive antigenic structure of native viruses while lacking viral genetic material, enabling efficient B-cell receptor cross-linking, enhanced uptake by antigen-presenting cells, and strong humoral immune responses. The hepatitis B core antigen (HBcAg) is a well-established VLP scaffold that self-assembles into highly

immunogenic nanoparticles and tolerates the insertion of foreign antigens within its surface-exposed major immunodominant region. HBcAg-based VLPs have been widely used to display diverse viral antigens, highlighting their versatility and translational potential (Carvalho et al., 2022).

The choice of expression system critically influences the quality and manufacturability of VLP-based vaccines. Although bacterial and yeast systems offer advantages in cost and scalability, they may compromise protein folding, assembly efficiency, or post-translational modifications necessary for maintaining native antigen conformation. In contrast, mammalian expression systems support authentic protein folding and efficient secretion of properly assembled VLPs, thereby improving product consistency and simplifying downstream purification (Urakami et al., 2017). Nevertheless, the relatively low yield of flavivirus-derived antigens in mammalian cells has remained a significant bottleneck, necessitating targeted optimization strategies.

Therefore, the objective of this study was to develop and evaluate an optimized dengue EDIII–HBcAg virus-like particle vaccine platform by enhancing antigen expression and secretion in a mammalian cell system while preserving structural and antigenic integrity. Specifically, this study aimed to (i) optimize the design and production of EDIII–HBcAg VLPs, (ii) characterize their physicochemical and antigenic properties, and (iii) assess their preliminary immunogenicity and antibody-dependent enhancement potential in a murine model. Collectively, this work seeks to establish a robust and safety-oriented VLP-based platform that can be further developed toward a tetravalent dengue subunit vaccine capable of inducing balanced, serotype-specific protective immunity without increasing disease risk.

METHODS

A synthetic gene encoding a dengue virus envelope domain III–Hepatitis B core antigen (EDIII–HBcAg) fusion protein was designed for expression in mammalian cells. The construct consisted of a truncated Hepatitis B core antigen (amino acids 1–149), into which the EDIII sequence of dengue virus serotype 2 (DENV-2) was inserted within the major immunodominant region of HBcAg. To minimize structural constraints and preserve correct folding, flexible glycine–serine linkers were incorporated at both junctions flanking the EDIII insert. Two variants were generated: a wild-type EDIII-HBcAg construct and a mutant construct containing a phenylalanine-to-alanine substitution at position 108 (F108A) within the EDIII sequence. All constructs were codon-optimized for *Homo sapiens* and cloned into the pcDNA3.4 expression vector under the control of a cytomegalovirus promoter.

Suspension-adapted HEK293F cells were maintained in serum-free expression medium at 37°C in a humidified atmosphere containing 8% CO_2 with continuous agitation. Cells were transiently transfected at a density of approximately 1.0×10^6 cells/mL using linear polyethyleneimine (PEI) as the transfection reagent. Plasmid DNA and PEI were complexed at an optimized mass ratio and added directly to the culture. To enhance protein expression and secretion, post-transfection culture conditions were optimized by reducing the incubation temperature to 32°C

and supplementing the medium with valproic acid. Cell viability and expression levels were monitored throughout the production period.

Culture supernatants were harvested several days post-transfection by centrifugation to remove cells and debris, followed by filtration. The clarified supernatant was concentrated using tangential flow filtration with an appropriate molecular weight cut-off membrane. The EDIII-HBcAg VLPs were purified using immobilized metal affinity chromatography, exploiting an N-terminal hexahistidine tag, followed by size exclusion chromatography to isolate correctly assembled particles and remove aggregates or low-molecular-weight contaminants. Protein concentrations were determined using a bicinchoninic acid assay.

The physical characteristics of the purified VLPs were evaluated by dynamic light scattering to assess particle size distribution and homogeneity. Particle morphology was further examined by transmission electron microscopy following negative staining. The antigenic integrity and surface display of EDIII on the VLPs were confirmed by enzyme-linked immunosorbent assay using EDIII-specific monoclonal antibodies and conformation-sensitive antibodies against HBcAg.

For immunogenicity studies, female BALB/c mice aged 6–8 weeks were immunized subcutaneously with purified EDIII-HBcAg VLPs formulated with aluminum hydroxide adjuvant. Mice received three immunizations at three-week intervals. Serum samples were collected at defined time points following immunization and stored at −20°C until analysis. EDIII-specific IgG responses were measured by ELISA using recombinant DENV-2 EDIII as the coating antigen. Neutralizing antibody activity was evaluated by plaque reduction neutralization tests (PRNT) against live DENV-2 using Vero cell monolayers, with $PRNT_{50}$ titers calculated as the reciprocal serum dilution resulting in a 50% reduction in plaque numbers.

To assess the potential for antibody-dependent enhancement, pooled immune sera were tested in vitro using Fcγ receptor–expressing K562 cells. Dengue virus was pre-incubated with sub-neutralizing dilutions of immune sera prior to infection. Following incubation, intracellular viral antigen expression was quantified by flow cytometry to determine infection levels relative to control sera.

All animal experiments were conducted in accordance with institutional guidelines and were approved by the relevant Institutional Animal Care and Use Committee.

RESULTS

1. Optimization of EDIII–HBcAg VLP Expression in Mammalian Cells

Initial transient expression of the wild-type EDIII–HBcAg fusion construct in HEK293F cells resulted in low levels of secreted protein, with yields consistently below 1 mg/L of culture supernatant. This low productivity is consistent with previous reports describing inefficient secretion of flavivirus-derived antigens in mammalian expression systems. To address this limitation, a structure-guided phenylalanine-to-alanine substitution at position 108 (F108A) within the EDIII sequence was introduced, based on prior evidence that this mutation enhances secretion and stability of dengue envelope-derived antigens.

The introduction of the F108A mutation led to a marked improvement in VLP production. Under standard culture conditions, the mutant construct yielded approximately 5 mg/L of secreted protein, representing a fivefold increase compared to the wild-type construct. Further optimization through post-transfection temperature reduction to 32°C and supplementation with valproic acid resulted in a consistent final yield of 12 ± 2 mg/L. These results demonstrate that the F108A mutation, in combination with optimized culture conditions, effectively overcomes a major bottleneck in mammalian VLP production.

The observed increase in yield is likely attributable to improved protein folding and reduced intracellular retention, which together facilitate more efficient VLP assembly and secretion. This finding extends previous observations made with full-length dengue envelope proteins and demonstrates that the beneficial effect of the F108A mutation is preserved even when EDIII is presented as a fusion antigen on a heterologous VLP scaffold.

Figure 1. Expression and purification of EDIII–HBcAg VLPS

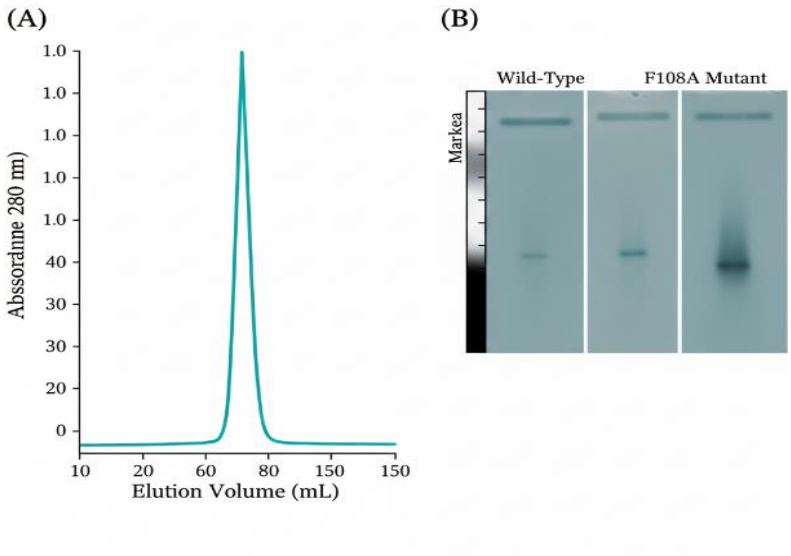

Figure 1. Expression and Purification of EDIII–HBcAg VLPs

(A) Size exclusion chromatography profile showing a single dominant peak corresponding to assembled VLPs. (B) SDS-PAGE analysis of purified wild-type and F108A mutant VLPs.

2. Structural and Antigenic Characterization of Purified VLPs

Following purification by affinity and size exclusion chromatography, the structural integrity of the EDIII–HBcAg VLPs was assessed. Dynamic light scattering analysis revealed a monodisperse particle population with an average hydrodynamic diameter of 36.5 ± 3.2 nm and a low polydispersity index (PDI < 0.1), indicating high particle homogeneity. These dimensions are consistent with the expected size of HBcAg-based VLPs and suggest that insertion of EDIII did not disrupt particle assembly.

Transmission electron microscopy further confirmed the formation of spherical, well-defined virus-like particles with diameters ranging from 35 to 40 nm. The particles exhibited uniform morphology and lacked visible aggregation, supporting the conclusion that the fusion antigen self-assembled efficiently into structurally intact VLPs.

To confirm surface display and antigenic integrity of EDIII, ELISA assays were performed using EDIII-specific monoclonal antibodies. Both wild-type and F108A mutant VLPs were efficiently recognized, with the mutant VLPs showing slightly higher binding signals. This enhanced binding may reflect improved antigen presentation or conformational stability associated with the optimized construct.

Figure 2. Structoral anigentic characatization of EDIII-HBcAg VLPs

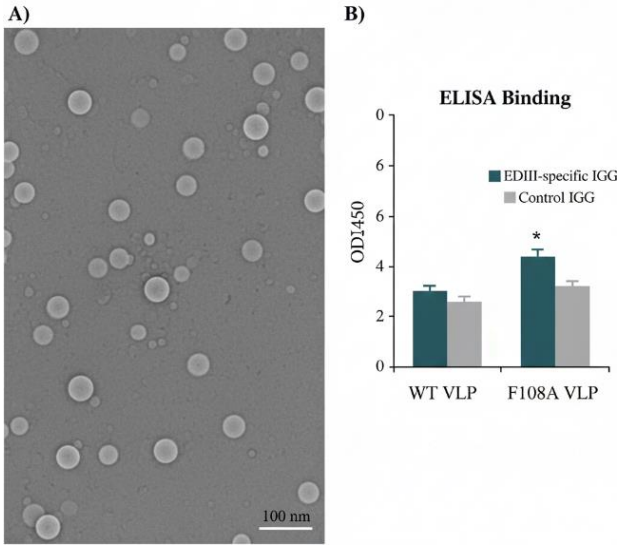

Figure 2. Structural and Antigenic Characterization of EDIII–Hbcag Vlps

(A) Transmission electron micrograph of negatively stained VLPs. Scale bar = 100 nm. (B) ELISA showing binding of EDIII-specific monoclonal antibodies to purified VLPs.

3. Immunogenicity of EDIII–HBcAg VLPs in Mice

The immunogenicity of the EDIII–HBcAg VLPs was evaluated in BALB/c mice following a three-dose immunization regimen. Mice immunized with the optimized F108A mutant VLPs developed robust EDIII-specific IgG responses, with endpoint titers exceeding 1:1,000,000 after the final boost. In contrast, mice receiving the wild-type VLPs exhibited significantly lower antibody titers, although still substantially higher than baseline levels observed in the control group.

The superior humoral response elicited by the optimized VLPs suggests that improved VLP stability and antigen display translate directly into enhanced immunogenicity. These findings are consistent with the established role of particulate antigen presentation in promoting B-cell receptor cross-linking and germinal center formation.

Neutralizing activity of the immune sera was assessed using plaque reduction neutralization tests against live DENV-2. Sera from mice immunized with the optimized VLPs exhibited strong

serotype-specific neutralizing activity, with an average $PRNT_{50}$ titer of 1:320. In contrast, sera from the wild-type VLP group showed lower neutralizing titers ($PRNT_{50}$ ~1:80). These results indicate that the optimized EDIII–HBcAg VLPs not only induce high antibody titers but also generate functionally relevant neutralizing antibodies.

Figure 3. Humural immne responses induced by EDIII–HBcAg VLPS

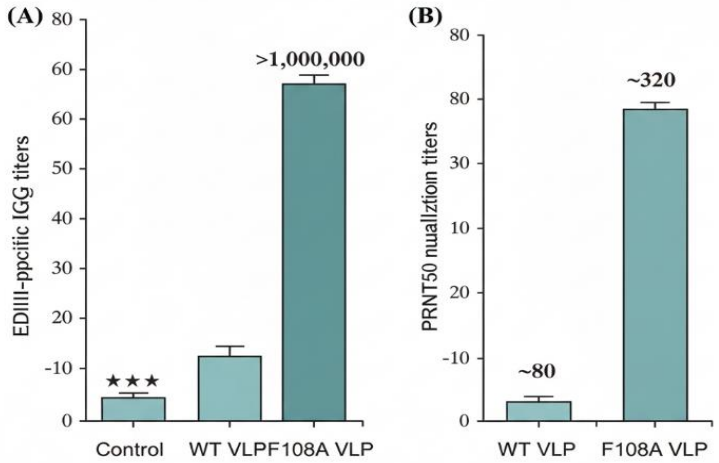

Figure 3. Humoral immune responses induced by EDIII–HBcAg VLPs

(A) Endpoint EDIII-specific IgG titers measured by ELISA. (B) $PRNT_{50}$ neutralization titers against DENV-2.

4. Evaluation of Antibody-Dependent Enhancement Potential

Given the central role of ADE in dengue pathogenesis, the functional quality of the induced antibodies was further assessed using an in vitro ADE assay. Sub-neutralizing dilutions of pooled immune sera were incubated with DENV-2 prior to infection of Fcγ receptor–expressing K562 cells. No significant enhancement of viral infection was observed in the presence of sera from mice immunized with the optimized VLPs, with infection levels comparable to those observed with pre-immune sera.

In contrast, a known enhancing antibody targeting dengue prM protein used as a positive control resulted in a marked increase in infection, validating the sensitivity of the assay. These findings suggest that the antibody response elicited by the EDIII–HBcAg VLPs is predominantly neutralizing and lacks strong enhancing activity, consistent with the antigen selection strategy.

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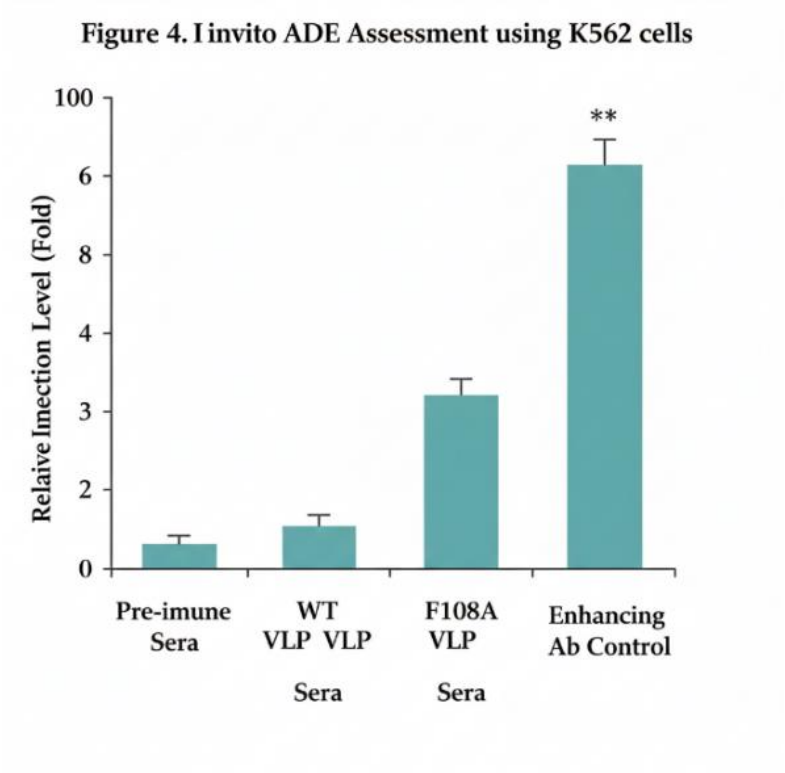

Figure 4. In vitro ADE Assessment Using K562 Cells ***(Relative infection levels following incubation with immune sera, pre-immune sera, and enhancing control antibody)***

5. Implications for Dengue Vaccine Development

Collectively, these results demonstrate that rational optimization of antigen design and expression conditions can substantially improve the manufacturability and immunogenic performance of EDIII-based VLP vaccines. The combination of high-yield mammalian expression, structural integrity, strong neutralizing antibody induction, and lack of detectable ADE supports the feasibility of this platform as a next-generation dengue vaccine candidate.

Importantly, the use of a modular VLP scaffold allows straightforward extension of this strategy to additional dengue serotypes. The present monovalent DENV-2 formulation therefore provides a critical proof-of-concept for the development of a tetravalent EDIII–VLP vaccine designed to elicit balanced, serotype-specific protective immunity while minimizing safety risks.

DISCUSSION

The present study demonstrates that rational antigen engineering combined with optimized mammalian expression strategies can substantially enhance the production, structural integrity, and immunogenic performance of EDIII-based virus-like particle (VLP) vaccines. By introducing a structure-guided F108A substitution within the EDIII domain and refining post-transfection culture conditions, we successfully addressed a key limitation of EDIII antigen expression in mammalian systems, namely low secretion efficiency and poor yield.

The fivefold increase in VLP yield observed upon introduction of the F108A mutation is consistent with previous reports showing that this substitution improves the folding efficiency and secretion of dengue envelope-derived antigens. Phenylalanine 108 has been implicated in hydrophobic interactions that may predispose EDIII to misfolding or intracellular retention within the endoplasmic reticulum. Substitution with alanine likely reduces steric hindrance and hydrophobic aggregation, thereby facilitating more efficient trafficking through the secretory pathway. Importantly, our data extend these findings by demonstrating that the beneficial effect of the F108A mutation is preserved when EDIII is presented as a fusion antigen on a heterologous HBcAg VLP scaffold.

Structural characterization confirmed that insertion of EDIII did not compromise VLP assembly. The observed particle size (35–40 nm), monodispersity, and uniform morphology are characteristic of well-assembled HBcAg-based VLPs. This is a critical observation, as improper assembly or aggregation could negatively impact both immunogenicity and manufacturability. The high structural integrity of the optimized VLPs indicates that the HBcAg platform tolerates EDIII fusion without destabilization, supporting its suitability as a modular vaccine scaffold.

Antigenic analysis revealed efficient recognition of both wild-type and F108A mutant VLPs by EDIII-specific monoclonal antibodies, with slightly enhanced binding observed for the optimized construct. This finding suggests that the F108A mutation does not disrupt key neutralizing epitopes and may even improve epitope accessibility or conformational stability. Such improvements in antigen presentation are particularly relevant for EDIII-based vaccines, where preservation of native-like conformation is essential for inducing protective immunity.

Immunogenicity studies in mice demonstrated that the optimized EDIII–HBcAg VLPs elicited markedly higher EDIII-specific IgG titers and superior neutralizing antibody responses compared to the wild-type formulation. The strong $PRNT_{50}$ titers against DENV-2 indicate that the antibody response was not only quantitatively robust but also functionally relevant. These results are consistent with the well-established immunological advantages of VLPs, which promote efficient B-cell receptor cross-linking, antigen uptake by dendritic cells, and germinal center formation, even in the absence of strong adjuvants.

A major concern in dengue vaccine development is the risk of antibody-dependent enhancement (ADE). Notably, sera from mice immunized with the optimized EDIII–HBcAg VLPs did not enhance DENV-2 infection in Fcγ receptor–expressing cells under sub-neutralizing conditions. This contrasts sharply with the strong enhancement observed with prM-specific control antibodies and supports the rationale for focusing on EDIII, which predominantly induces serotype-specific neutralizing antibodies rather than cross-reactive, enhancing antibodies. These findings suggest that the optimized VLP formulation may offer an improved safety profile compared to vaccines that include the full-length envelope protein.

From a translational perspective, the achieved expression yields (12 ± 2 mg/L) in a transient mammalian system represent a significant step toward scalable production. While further improvements will be required for industrial manufacturing, the demonstrated yield, combined

with the favorable immunological profile, underscores the feasibility of this approach for next-generation dengue vaccine development.

CONCLUSIONS

In conclusion, this study demonstrates that structure-guided antigen optimization and expression refinement can markedly improve the yield, stability, and immunogenicity of EDIII-based VLP vaccines. The introduction of the F108A mutation, together with optimized mammalian culture conditions, enabled high-level production of structurally intact EDIII–HBcAg VLPs that effectively display antigenic epitopes and induce strong neutralizing antibody responses without detectable antibody-dependent enhancement.

These findings validate the EDIII–HBcAg VLP platform as a promising and safe dengue vaccine candidate and provide a strong proof-of-concept for further development. The modular nature of the VLP scaffold offers a clear pathway toward the generation of multivalent formulations encompassing all four dengue serotypes. Future studies will focus on tetravalent VLP assembly, long-term immune durability, and protective efficacy in relevant challenge models, paving the way for clinical translation of this next-generation dengue vaccine strategy.

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