

## **Structural heterogeneity of dengue virus particles: implications for vaccine design**

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Dengue virus infection continues to pose serious public health threat in tropical regions around the world. Existing countermeasures against mosquito vectors of dengue viruses are limited and largely ineffective in the long term basis. A tetravalent live, attenuated vaccine candidate is currently tested in phase III clinical trial. Additional candidates in various forms are being generated with a common goal of obtaining safe, efficacious and accessible dengue vaccine for general use. Recent studies on the structure of dengue virus particles and specific immunological responses induced during natural infection reveal a complex mixture of extracellular virus particles at different maturation levels that may play an essential role the generation of predominantly cross-reactive B cell responses in infected individuals. Applications of these findings in the design of newer vaccine candidates will be crucial to the development of effective dengue vaccine in the future.

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### **Introduction**

Dengue virus infection in human causes a spectrum of illnesses, ranging from undifferentiated fever, dengue fever and dengue hemorrhagic fever/dengue shock syndrome [1]. Transmission of dengue viruses occurs predominantly in tropical and sub-tropical countries around the world with an increasing trend in recent decades. Currently dengue is endemic in more than 100 countries in Africa, the Americas, the Eastern Mediterranean, South-east Asia and the Western Pacific [2]. The most recent estimate by World Health Organization reveals that up to 2.5 billion people-over 40% of the world's population-are

now at risk from dengue [2]. In each year there may be 50-100 million dengue infections worldwide, causing enormous economic burden [3-6]. Existing public health countermeasures against mosquito vectors of dengue viruses are limited and largely ineffective in the long term. The development of vaccine against dengue has been difficult with only one tetravalent live, attenuated vaccine candidate undergoing a phase III efficacy trial in human at the present [7]; the result will be available in a few years from now. Meanwhile, additional candidates are being generated with the use of recombinant DNA and production

technologies [8], but the physicochemical and biological features that need to be incorporated in a safe and efficacious vaccine against dengue remains unclear. Recent progresses in the understanding of dengue virus structure and heterogeneity, immune responses during natural infection, and pathophysiology of dengue hemorrhagic fever will be crucial in the development of effective dengue vaccine for general use in the future.

### Structure of dengue virus particles

Dengue viruses are RNA viruses in the genus *Flavivirus* of the family *Flaviviridae* [9]. The single-stranded RNA genome encodes three virion-associated structural proteins (C, prM/M and E) and six non-structural proteins (NS1, 2A, 2B, 3, 4A, 4B and 5). Mature viral particles, about 50 nm in diameter, consist of an outer envelope of lipid bilayer and 180 copies of each of the two transmembrane proteins, M and E, and an internal nucleocapsid composed of C in association with an about 11-kb RNA molecule [10]. The major envelope glycoprotein, E, is responsible for receptor binding and fusion, and represents the target of antibody-mediated neutralization of viral infectivity. Structural domains of E are associated with these functions: the central domain I, the elongated dimerization domain II with a hydrophobic fusion loop, the receptor-binding globular domain III (EDIII), the stem-anchor region, and the transmembrane region [9, 11]. Short hinge regions between domains I-II and domains I-III allow considerable movement of these domains [12], particularly during acid-induced fusion of viral envelope with intracellular membrane [13]. The second envelope protein, M, is derived by an intramolecular furin-mediated proteolytic cleavage of prM [14], which serves as a chaperone of E during assembly and plays an important role in preventing premature fusion of the virus envelope to host cell membrane during exit of virus particles [15]. Genome-containing mature viral particles are infectious; however, the ratio of infectious particles to genome-containing particles in a few viral preparations that had been

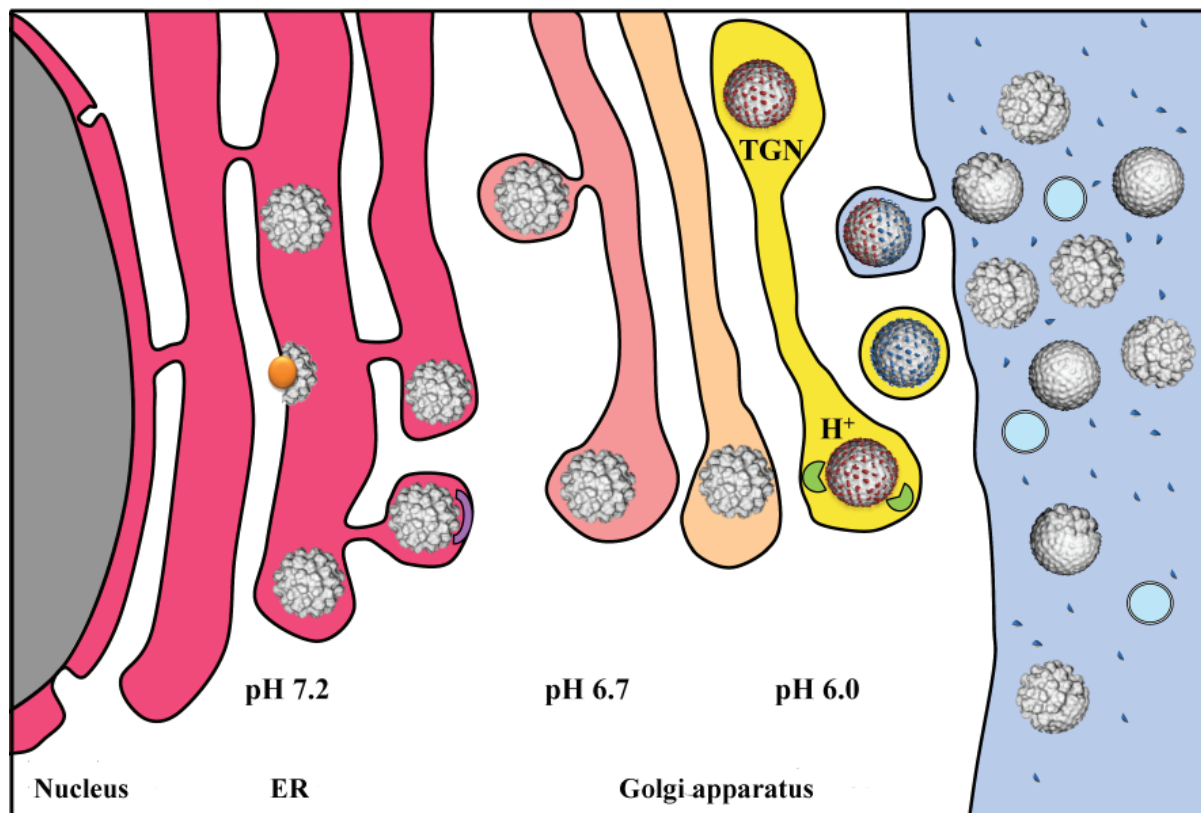
examined appears to be quite low [16-18].

Other types of dengue viral particles include non-infectious subviral particles of about 30 nm in diameter that are devoid of nucleocapsid [17]. Based on the analysis of particles generated from cells transfected with prM and E of a related flavivirus, tick-borne encephalitis virus, the subviral particles contain 30 pairs of E dimer, which are arranged in a different (T=1) icosahedral configuration [19]. In addition, large virus-like particles without nucleocapsid are generated during the expression of envelope proteins of tick-borne encephalitis virus and West Nile virus in mammalian cells [20, 21]. While small subviral particles represent a minor population of extracellular particles in a dengue serotype 2 prototype virus preparation derived from mosquito cells [17], large virus-like particles remain to be documented during dengue virus replication. The proportion of virion-sized particles and small subviral particles can be affected by variations in the cleavage efficiency of prM [17].

Four serologically distinct types of dengue virus are distinguished based on the neutralization test. Nucleotide sequence analysis of the genome reveals about 40% differences that are distributed in both coding and non-coding portions of the genome. An epitope common to all flaviviruses is located at the fusion loop in domain II [22], likely due to the conservation of fusion loop sequence in the E protein. Other epitopes include those that are detected in all four dengue virus serotypes (complex-specific epitope), some serotypes (subcomplex-specific epitope), a particular serotype (type-specific epitope), or among some genotypes of a serotype. These epitopes are located in domains I-II as well as in domain III of the E protein.

### Pathway of particle maturation

During dengue virus replication in infected host cells, viral particles are assembled by budding of viral structural components and associated cellular membrane into the lumen of endoplasmic reticulum (Figure 1). Nascent spherical “immature”



**Figure 1.** Schematic representation of dengue virus maturation in the vesicular transport pathway. Assembly of immature particles occurs in the endoplasmic reticulum. Subsequent transport and maturation via proteolytic cleavage of prM requires the activity of class II ADP-ribosylation factor (shown in purple) and furin (green), respectively. Changes in the conformation of envelope proteins that occur in the acidic Golgi apparatus generate smooth particles with uncleaved forms of prM (red stripes) at the intradimeric interface. Following cleavage of prM by furin, pr peptides (blue stripes) remain non-covalently bound with particles during exit through acidic intracellular compartment. Upon encountering physiologic pH in the extracellular milieu, pr peptides dissociate from the particles. Extracellular particles include virion-sized particles at various maturation levels (immature particles - spiky, mature particles - smooth, and partially mature - mosaic) as well as virus-like particles (circles without detail). TGN, trans-Golgi network.

particles of about 60 nm in diameter contain 60 spikes of non-covalently linked heterotrimers of prM and E that are arranged in an icosahedral configuration [23]. The rough surface of nascent viral particles reflects the slanting axis of E as well as the protruding N-terminal pr portion of prM, which forms a cap-like structure partially covering the fusion loop at the tip of E domain II [23,24]. In this conformation, the receptor-binding domain III of E is located at the base of spikes adjacent to lipid bilayer. Likely as a result of such arrangement of prM and E, the immature particles do not bind efficiently with host cells

and are poorly infectious [25].

Immature particles are transported through the vesicular transport pathway in a process that requires specific recognition of prM with class II ADP-ribosylation factors [26]. Low pH environment of the Golgi apparatus induces conformation changes and rearrangement of the two envelope glycoproteins [27]. Dissociation of prM-E heterotrimers in acid-primed immature particles generates head-to-tail homodimers of E lying in a flattened configuration in parallel with the lipid bilayer [28]. Isolated prM molecules at the intradimeric interface undergo site-specific

proteolytic cleavage that is catalyzed by furin, a trans-Golgi network resident proprotein convertase [14]. Following cleavage, the N-terminal pr peptides remain non-covalently bound with particles during their passage through acidic cellular compartment while the membrane-associated M protein is hidden by the overlying lattice of E dimers [10,28]. Association of prM with E in immature particles and subsequent changes that occur following cleavage of prM play a critical role in preventing low pH-triggered E-mediated fusion of viral envelope with internal cellular membrane [29].

In the extracellular milieu, mature viral particles display smooth surface as pr peptides dissociate from the particles upon encountering physiologic pH [10,28]. The basic protein subunit in the form of head-to-tail E homodimer is arranged in groups of three parallel dimer sets in a “heringbone” pattern that follows an icosahedral configuration [10]. In the mature particles, domain III of E becomes exposed as its position shifts toward the surface. Differences in the local arrangement of the E dimers create distinct environments for EDIIIs present at the five-fold vertices, around the five-fold axes, and at the three-fold axes of rotational symmetry that may influence their interactions with cellular receptors and specific antibodies. Binding of a lectin, DC-SIGN, on the surface of dendritic cells to the Asn67 N-glycosylation site on two adjacent E dimers in mature particles may interfere with the receptor-binding function of the EDIIIs that are clustered at the five-fold vertices to a lesser extent than those at other sites [30]. An epitope recognized by a monoclonal antibody, 1A1D-2, on EDIII is partially hidden at room temperature conditions, but becomes recognizable following incubation at 37 °C. Only EDIIIs at the 5-fold and 3-fold axes of symmetry, however, can be bound by 1A1D-2 Fab fragment [31]. Binding of 1A1D-2 results in changes in the position of Fab-bound E monomers relative to the unbound counterparts, indicating that symmetrical arrangement of E lattice in mature particles is not static but capable

of accommodating some movements of individual E monomer [31].

## Partial maturation

While cleavage of prM by furin is indispensable for viral infectivity, it is not a prerequisite for the release of particles from infected cells. In a recent example, particles with very low level of infectivity can be released from infected cells lacking furin function [32]. During dengue virus infection of commonly employed host cells in vitro, it has been observed repeatedly that extracellular dengue virus particles contain some uncleaved prM molecules [33], and the extent of prM cleavage appears to be variable among the four serotypes (R. Sriburi and N. Sittisombut, unpublished results). Site-directed mutagenesis studies revealed that partial prM cleavage in dengue viruses reflects the cleavage-inhibitory influence of a highly conserved acidic residue in the furin target sequence at the pr-M junction [16,17]. At the level of individual particle, prM molecules are cleaved in a progressive manner [34]. As a result, extracellular dengue virus particles are heterogeneous with regards to the distribution of prM. In addition to fully immature particles with 180 prM molecules/particle and prM-less mature particles, “partially mature” particles displaying distinct regions of rough surface and smooth surface are detected with cryo-electron microscopy [34,35]. On these partially mature particles, groups of prME heterotrimeric spikes (as found in immature particles) are found in distinct cluster that is separated from surrounding area occupied by the E homodimeric lattice (as in mature particles) [36]. Estimation of the proportion of various types of particle of a DENV-2 prototypic strain by direct counting of cryo-EM images of virion-sized particles reveals a large fraction of partially mature particles [34]. Overall, prM-containing particles may represent up to 90% of the extracellular particles found in a mosquito cell-derived virus preparation [34]. It is unclear whether prM-containing particles are present extracellularly during natural infection in humans.

Under appropriate *in vitro* conditions, prM-containing particles of dengue virus can infect susceptible host cells. In the presence of anti-prM antibody, immature particles released from cells lacking furin are able to productively infect leukocytes through an interaction of the Fc portion of particle-bound antibody with cell surface FcR molecules [25,37]. Other anti-prM antibodies that cross-react with a cellular protein, heat shock protein 60, can also mediate dengue virus infection of cells lacking FcR [38]. Furthermore, enhancement of the infectivity of immature particles is observed with an antibody recognizing the fusion loop epitope in E protein that is present in immature particles [39]. These evidences indicate an ability of prM-containing viral particles in establishing infection when entry occurs via non-receptor-mediated pathways.

### Antibody responses in human

During natural dengue virus infection, human B cell responses against viral structural proteins are directed predominantly toward cross-reactive epitopes on prM and E [37,40-42]. Anti-prM antibodies are non- or weakly neutralizing [37,41]; on the contrary, they can mediate infection of leukocytes by dengue virus *in vitro* and may contribute to infection enhancement during secondary dengue virus infection. While serotype-specific antibodies recognizing EDIII epitopes are strongly neutralizing in *in vitro* testings, these antibodies are uncommonly detected in infected individuals and do not contribute to neutralization of dengue virus by immune sera [43,44]. The underlying basis for functional differences between group-reactive antibodies and type-specific antibodies was studied employing anti-EDIII murine monoclonal antibodies. Neutralization of DENV-2 by antibodies recognizing group- and sub-complex-reactive epitopes in EDIII requires a greater level of antibody occupancy as compared with those specific for adjacent serotype-specific epitope [45-48]. Rapid waning of cross-reactive immunity observed in primarily dengue-infected individuals may reflect the need for high anti-

body levels for effective neutralization via cross-reactive epitopes during subsequent infection by heterologous dengue serotypes.

As distinct conformations and oligomeric states of prM and E, and their associated different surfaces for antibody recognition, are present in dengue viral particles at different maturation levels, the preponderance of cross-reactive anti-prM and anti-E B cell responses generated in infected individuals suggests that prM-containing immature and partially mature particles play a major role in the stimulation of antibody response during natural infection. Partial maturation and the resultant retention of prM in a large fraction of extracellular viral particles may have been selected during evolution to evade host humoral immunity [49], possibly by favoring the stimulation of infection-enhancing prM-specific and other cross-reactive E-recognizing antibodies while minimizing the production of strongly neutralizing antibodies against the receptor-binding EDIII. A consequence of antibody-dependent infection enhancement due to cross-reactive antibodies is thought to allow the co-circulation of multiple dengue serotypes in nature [50].

### Implications for vaccine design

Various dengue vaccine candidates in the pipeline differ widely in their physico-chemical and biological properties, and methods of production [8]. From the structure point of view, these candidates take the forms of particles containing whole prM+E molecules, or subunit preparations of isolated E ectodomain or only the EDIII portion (with or without carrier protein). Particulate preparations include inactivated viruses; live, attenuated viruses; and (prM+E)-expressing plasmids that allow assembly of virus-like particles. While the particulate preparations provide organized structures that resemble infectious viruses, their prM content and surface structure may elicit the production of antibodies recognizing prM- and cross-reactive E epitopes in the similar manner as that found during natural infection. On the other hand, subunit preparations may help avoid the

stimulation of anti-prM response and its possible consequences. However, a recent study comparing different West Nile virus immunogens indicates that immunization with particulate preparation may be more desirable than using isolated EDIII in inducing the neutralizing antibody response [51]. While isolated EDIII stimulates the production of higher levels of EDIII-binding antibodies, greater neutralizing antibody response was achieved with using particulate preparations as not all EDIII-binding antibodies from the immunization with isolated EDIII can get access to corresponding epitopes in infectious virus particles that are employed as targets in the neutralization testing [51]. Despite a low level of EDIII-binding antibodies generated with immunization with particles, these antibodies may focus on surface exposed epitopes that are more relevant to neutralization [51].

It is possible that high prevalence of B cell responses against prM and other cross-reactive E epitopes during natural infection contribute to severe outcomes during secondary dengue virus infections. Levels of circulating antibody specific for an epitope on prM has been shown to correlate with disease severity in dengue virus-infected patients [52]. Attempts aiming at the reduction of anti-prM antibody response following immunization could help to minimize vaccine-induced enhancement of subsequent dengue virus infections [37]. Additionally, a live, attenuated dengue vaccine with minimal prM content may be less susceptible to infection enhancement mediated by pre-existing anti-prM antibody when given to recipients who experienced previous dengue virus infections. Conceivably, this may be achieved by presenting the immune system with minimal level of immature/partially mature particles while maximizing the proportion of mature particles in the vaccine preparation. However, most, if not all, neutralizing antibodies against dengue virus exhibit infection-enhancing activities at low concentration [41]. Infection-enhancing activity appears to be inherent to antibodies that recognize dengue envelope proteins. Thus, it may be more beneficial to maximize the stimulation of

neutralizing antibody response than to minimize the production of enhancing antibodies in vaccine recipients. When vaccine recipients are stimulated simultaneously with a tetravalent vaccine that elicit strong and durable neutralizing responses, the generation of cross-reactive enhancing antibodies in such vaccinees may not be as relevant as in individuals getting infected in a sequential manner in nature.

Partial maturation of virus particles not only results in the release of various types of virion-sized particles at different maturation levels but also affects the proportion of virion-sized particles and smaller subviral particles [17,34]. Mutant dengue viruses genetically engineered to be less susceptible to prM cleavage are enriched with subviral particles as well as immature/partially mature virion-sized particles [17,34]. Interestingly, Ohtaki et al identified West Nile virus-like particles of different sizes generated from stably transfected CHO cell clones [21]. Large (40-50 nm in diameter) mature particles that are enriched with M protein induce higher neutralizing antibody response and, at limiting antigen doses, more potent protection against live virus challenge in mice than smaller (20-30 nm) prM-containing particles [21]. Greater protection against infectious viruses following immunization with large particles may be due to differences in the arrangement of envelope proteins between large and small particles that affect the fine specificity and overall quality of the antibody responses.

Design of dengue vaccine candidates in the future may need to take into account the influence of form (particulate vs. subunit preparations) as well as size (large vs. small particles) and the maturation state of particles (immature/partially mature vs. mature) on the generation of desirable antibody responses. How various structural forms of dengue vaccine candidates with their associated different conformations of envelope proteins affect and shape the immune response in vaccinees remains to be fully elucidated. Knowledge from such studies will be crucial in guiding the testing and selection of dengue vaccine candidates for optimal use in the real life situation.

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