

MATURE VIRUS-LIKE PARTICLES OF FLAVIVIRUSES

Technical Field

The present disclosure relates to virus-like particles (VLPs) or virus structural proteins of
5 flaviviruses incorporated with one or more modified forms that expression of which in the
transfected cells results in desired outcome. The VLPs disclosed in several embodiments are
preferably fully mature VLPs of flaviviruses. More importantly, the expressed VLPs is capable of
eliciting immune response in a mammal such as human subject upon administering
pharmaceutically effective dosage of the expressed VLPs to the mammal. The present disclosure
10 also includes a pharmaceutical or vaccine composition containing the expressed VLPs with or
without further modifications applicable for inducing active immunity in a subject against
flavivirus.

Background

15 Flavivirus comprise more than 70 different viruses, many of which are arthropod-borne and
transmitted by either mosquitoes or ticks. Flavivirus is a genus of viruses in the family Flaviviridae.
This genus includes the West Nile virus (WNV), dengue virus (DENV), Japanese encephalitis
(JEV), yellow fever virus (YFV), Zika virus (ZikV), tick-borne encephalitis virus (TBEV) and
several other viruses which may cause encephalitis or haemorrhagic diseases. Dengue fever is a
20 mosquito-borne disease caused by the dengue virus and has spread to most tropical and many
subtropical areas. The disease is caused by four closely related viruses, the Dengue virus type 1
(DENV-1), Dengue virus type 2 (DENV-2), Dengue virus type 3 (DENV-3) and Dengue virus
type 4 (DENV-4). Although Dengue virus is the most important flavivirus with respect to global
disease incidence, the development and use of vaccines against the virus has been hampered so
25 far by the theoretical risk of vaccine-related adverse events such as immune enhancement of
infection and the requirement to induce a long-lasting protective immune response against all four
dengue serotypes simultaneously.

Despite intensive research and development efforts to find an effective dengue vaccine in the past
30 decades, there is no reliable vaccine that can prevent dengue following infection by all four
serotypes of dengue virus. Recent phase IIb-III trials of Sanofi Pasteur's Dengvaxia in Asia and
Latin America revealed a moderately effective vaccine that is unable to provide protection against
diseases caused by DENV-2 and in seronegative volunteers (Sabcharoen et al, 2012; Capeding et

al, 2014; Villar et al, 2014; Hadinegoro et al, 2015). There is an urgent need to find an alternative dengue vaccine candidate that will be effective against DENV-2 as well as other serotypes, and safe for use in young and seronegative children. Dengue virus-like particles are a potential vaccine candidate that is generated by expressing the two viral envelope glycoproteins, prM and E, in
5 insect or mammalian cells. These two glycoproteins are synthesized as a polyprotein, which is cleaved at specific sites by host proteases; they then associate and assemble into virus-like particles with structural similarities to native virus particles although the virus-like particles do not contain internal core structure or viral genome (Shang et al, 2012). Subsequently, virus-like particles undergo post-translational modifications in the secretory pathway as they are exported
10 out of host cells in the same manner as virus particles in dengue virus-infected cells. As the generation of dengue virus-like particles is not dependent on active virus infection and intracellular replication of the viral genome, the prM and E genes employed in the expression cassette can be modified in a number of ways to enhance export, extracellular level and immunogenicity with lesser constraints than in the modifications of infectious virus particles. Recent understandings on
15 the structures of viral envelope proteins, intracellular and extracellular viral particle subpopulations, the antigenic composition of different types of viral particle, and the antibody responses to envelope proteins in dengue virus-infected persons are crucial to the manipulations aiming at improving dengue virus-like particles' potential as dengue vaccine.

20 It is well known that extracellular dengue virus particles are a mixture of particles with different maturation levels (Junjhon et al, 2010). Newly assembled, immature viral particles in the ER lumen contain equal quantities of the two envelope glycoproteins, prM and E, which associate as spikes comprising three pairs of non-covalently-linked prM-E heterodimers on the surface of particles (Zhang et al, 2003; Li et al, 2008). During export of particles through the secretory
25 pathway, low pH environment of the Golgi apparatus induces conformational changes of the prM and E proteins (Yu et al, 2008; Zheng et al, 2014), resulting in the dissociation of prM-E dimer and rearrangement of E to form E homodimeric molecules, and allowing cleavage of prM by an endoprotease enzyme, furin (Kuhn et al, 2002; Yu et al, 2008). Cleavage of prM at the pr-M junction generates a 99-residue pr peptide that non-covalently associates with the E dimer under
30 the low pH condition, and the membrane-associated M protein, which interact non-covalently with the underside of the E dimer in mature particles (Zhang et al, 2013). Upon release of particles, the pr peptide dissociates from extracellular particles, generating mature particles with smooth surface and increased infectivity. The presence of a cleavage-inhibitory acidic amino acid residue,

glutamic acid or aspartic acid, within the furin recognition site at the pr-M cleavage junction causes incomplete cleavage of prM, resulting in a mixed population of immature, partially mature as well as fully mature, M-containing particles in the extracellular compartment (Junjhon et al, 2008; 2010). Partially mature particles contain patches of E dimers co-existing with those of prM-E heterodimers (Plevka et al, 2011; 2014). The prM-containing immature and partially mature viral particles are less infectious than mature viral particles, but their infectivity against FcR γ + leukocytes can be enhanced in vitro by either anti-E antibodies or anti-prM antibodies at appropriate concentrations (Rodenhuis-Zybert et al, 2010; Richter et al, 2014).

10 Differences in the arrangement of prM/M and E in immature vs. mature particles are associated with changes in antigenicity. The E protein in immature particles (and the immature patch of partially mature particles) associates with prM in such a way that certain parts of the E molecule, such as the receptor-binding EDIII domain (Zhang et al, 2003), may not be readily accessible. Upon maturation, dissociation of E from prM and subsequent formation of E homodimer on mature particles (and the mature patch of partially mature particles) result in new epitopes that are formed by adjoining domains of two E molecules in the same dimer as well as between two or more E molecules in different dimers. Epitopes that are dependent on the quaternary structure of E dimers are targets of antibodies that strongly neutralize mature particles (Lok, 2016). In particular, the recently described E dimer-dependent epitopes, which are well conserved between serotypes of dengue virus (Dejniratisai et al, 2015; Rouvinski et al, 2015), can induce cross-reactive neutralizing antibodies that may be useful in the prevention of dengue in areas where several serotypes of dengue virus co-circulate. On the other hand, discrete epitopes found on the monomeric E protein, the form that associates with prM, tends to be hidden on mature particles, resulting in lower target availability that may reduce the activity of these antibodies in the neutralization of mature viral particles (Lok, 2016). A lack of quaternary structure-dependent epitopes on dengue virus-like particles that are not fully mature makes these particles potentially less useful as a vaccine candidate. A comparison of the immunogenicity of VLP generated without or with extensive (83%) prM cleavage showed greater ability of virus-like particles with high level of maturation to induce virus infectivity-neutralizing antibodies in the absence of adjuvant in mice (Suphatrakul et al, 2015).

15
20
25
30

Furthermore, dengue virus-like particles were generated by co-expressing the two envelope glycoproteins, prM and E, employing native viral nucleic acid sequence (Shang et al, 2012).

Dengue virus-like particles generated with using native prM sequence retain some amount of prM (Purdy and Chang, 2005; Konishi and Fujii, 2002; Urakami et al, 2017), which is associated with E in prM-E heterodimers found on the surface of immature and partially mature particles. In certain designs of dengue virus-like particles, the pr-M junction sequence had been modified to abolish prM cleavage by furin (Konishi et al, 2001; Konishi and Fujii, 2002; Yamaji, 2014), resulting in the generation of fully immature virus-like particles. Immature and partially mature virus-like particles, which are generated with native prM sequence or with prM cleavage-abolishing mutations, are capable of inducing prM-specific antibodies. Unlike E-specific antibodies, anti-prM antibodies generally do not neutralize virus infectivity and are unlikely to contribute to protection against dengue. On the other hand, anti-prM antibodies can facilitate infection of FcγR-bearing leukocytes by multiple serotypes of dengue virus (Dejniratisai et al, 2010; Smith et al, 2016) and may contribute to severe disease (Katzelnick et al, 2017). A lack of prM in dengue vaccine candidate is desirable (Dejniratisai et al, 2010; Flipse and Smit, 2015), but is difficult to achieve in the generation of particle-based vaccine candidate (i.e. by deleting the whole prM coding sequence from the prM+E expression cassette) because prM also serves as a chaperone for E protein in its folding in the endoplasmic reticulum (Konishi and Mason, 1993; Lorenz et al, 2002). In the absence of prM, an expression of E protein does not result in the release of membrane-associated particles (Konishi and Mason, 1993; Allison et al, 1995; Hsieh et al, 2014). Our previous attempt to reduce the amount of prM remaining on the extracellular dengue virus-like particles by substituting the acidic amino acid at the pr-M junction with an uncharged residue, such as alanine (Junjhon et al, 2008), in the (prM+E)-expressing vector results in an enhanced, but still incomplete, prM cleavage and the resultant particles can elicit anti-prM antibody response, albeit at reduced level (Suphatrakul et al, 2015). Although this previous design reduces a problem of anti-dengue prM antibody induction, there remains a limitation as quaternary structure-dependent epitopes (within and between E dimers) could not be formed fully in the remaining immature and partially mature particles.

In this design of dengue virus-like particles, the pr-less (M+E)-expressing vector is constructed for the generation of fully mature type of virus-like particles, which, as in the case of extracellular mature viral particles (Kuhn et al, 2002), are devoid of the external pr portion of prM. This modification maximizes the formation of quaternary structure-dependent epitopes on the particles as all E molecules are likely to engage in E homodimer formation. The presence of M allows for an interaction with E as in the mature viral particles (Zhang et al, 2013). As the pr portion of prM

contains discrete epitopes in which all known anti-prM monoclonal antibodies and natural anti-prM antibodies from dengue virus-infected persons and immunized animals have been mapped (Chan et al, 2012; Huang et al, 2006, 2008; Luo et al, 2013; Luo et al, 2015; Song et al, 2013; Smith et al, 2016; Wang et al, 2013), a lack of the pr portion of dengue virus prM in this design is also intended to prevent induction of anti-prM antibody response when this virus-like particle is employed as a vaccine in human. Deletion of the pr portion of prM, however, has an undesirable consequence that could affect yield. It is known that one function of prM, particularly the pr portion, is to suppress the fusion of virus particles with intracellular membrane during export (Zheng et al, 2010; Zheng et al, 2014). It is likely that, in the intended absence of the pr portion of prM, the mature virus-like particles could fuse with host cell membrane when they encounter the Golgi apparatus' low pH environment. To minimize acid-induced fusion of mature virus-like particles with Golgi apparatus membrane during export, a number of conserved and internally-located histidine residues in the M and E proteins, including histidine residues at positions 7, 102, 224, 284 (282 for DENV-3), 336 (334 for DENV-3), 357 (355 for DENV-3) and 392 (390 for DENV-3), are substituted with alanine or asparagine. This is based on previous findings that histidine serves as pH sensor, and protonation of histidine in the acidic environment leads to conformational changes of E and prM both during export (resulting in the conversion from prM-E heterodimer to E dimer) and during fusion with endosomal membrane in new virus-infected cells (resulting in the conversion from E dimer to E trimer) (Kampmann et al, 2006; Mueller et al, 2008; Fritz et al, 2008; Fuzo and Degreè, 2014; Zheng et al, 2014; Chaudhury et al, 2015). In this application, multiple histidine residues are substituted concurrently as single substitution of any histidine residue is unlikely to abrogate fusion (Nelson et al, 2009; Chaudhury et al, 2015) and increase yield (Christian et al, 2013). Histidine residues that can be accessed from the surface are not substituted to preserve the antigenicity of the E protein. Additional modifications of the E protein involve substitutions on the lateral side of the E dimer, including non-histidine residues at the positions 261 (259 for DENV-3), 263 (261 for DENV-3), 317 (315 for DENV-3) and 398 (396 for DENV-3) to increase the release of virus-like particles (Christian et al, 2013), and substitution at the fusion loop of E, including residues at the positions E107 or E108, to reduce the fusion potential of the E protein (Trainor et al, 2007; Christian et al, 2013; Urakami et al, 2017). Nonetheless, the expression of these virus-like particles in the extracellular level is still far from satisfying. Further modification on the dengue virus-like particles, M and E proteins, are therefore needed such that the increased extracellular virus-like particles may result in better or improved immune response in the subject being vaccinated with these virus-like particles.

Summary

The present disclosure, in several embodiments, aims to provide mature flavivirus-like particles that are able to induce or elicit active immune response in a subject after administering the particles to the subject under appropriate dosage. The mature particles are genetically modified
5 such that the expressed modified particles become more resistant against acid-induced conformational changes or fusion when these expressed particles are transported out of the host cells.

Another object of the present disclosure is to offer a chimeric peptide or virus structural protein
10 comprising a modified form of joined M and E proteins of flavivirus. Preferably, the signal sequence for the M protein and the C-terminal stem-anchor domain of the E protein in the disclosed chimeric peptide are substituted with the corresponding regions from the Defensin A protein of *Aedes aegypti* and the E protein of Japanese encephalitis virus, respectively, to enhance the level of extracellular virus-like particles.

Further object of the present disclosure includes offering a vaccine composition being configured to effectively react against flavivirus infection, or more particularly dengue virus infection, containing the above mentioned modified and/or chimeric peptides. Administering and exposing a subject with the disclosed vaccine composition having the modified peptides and/or chimeric for
20 a suitable period shall induce active immunization of the exposed subject.

At least one of the preceding objects is met, in whole or in part, by the present disclosure, in which one of the embodiments of the present disclosure relates to a virus-like particle capable of eliciting immune response in a mammal. The virus-like particles or peptides preferably comprise an amino
25 acids sequence substantially corresponding to a sequence setting forth in SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3 or SEQ ID NO. 4, which carries one or more human-made or artificial modifications or modified forms of dengue virus sequence. The modifications or modified forms may include conserved amino acid His at positions M7, E261, E282, and E317 of dengue virus sequence, which are replaced by amino acid Ala at positions 7, 336, 357 and 392 in SEQ ID NO.
30 1-3, or conserved amino acid His at positions M7, E259, E280 and E315 of dengue virus sequence are replaced by amino acid Ala at positions 7, 334, 355 and 390 in SEQ ID NO. 4.. It is crucial to note that the disclosed VLPs are being subjected to modifications or modified forms involving

combining the dengue virus M protein (free from the pr peptide) with the E protein at specific positions for acquiring better expression at the extracellular level.

- In some other embodiments, the modified forms further comprise replacement of conserved amino acid His at positions E27, E149 and E209 of dengue virus sequence with amino acid Asn at positions 102, 224 and 284 in SEQ ID NO. 1-3 or replacement of conserved amino acid His at positions E27, E149 and E207 of dengue virus sequence with amino acid Asn at positions 102, 224 and 282 in SEQ ID NO. 4.
- For other embodiments, the modifications further comprises any one or combination of replacement of amino acids Ser or Glu at position E186 of dengue virus sequence or amino acid Ser at position E184 of dengue virus sequence with amino acid Phe at position 261 in SEQ ID NO. 1-3 or at position 259 in SEQ ID NO. 4, respectively, replacement of amino acid Arg located at position E188 of dengue virus sequence or position E186 of dengue virus sequence with amino acid Leu at position 263 in SEQ ID NO. 1-3 or at position 261 in SEQ ID NO. 4, replacement of amino acid Asn or Val at position E242 of dengue virus sequence or amino acid Asn at position E240 of dengue virus sequence with amino acid Ser at position 317 in SEQ ID NO. 2-3 or position 315 in SEQ ID NO. 4, and replacement of amino acid Arg or Lys located at position E323 of dengue virus sequence or amino acid Lys position E321 of dengue virus sequence with amino acid Gln at position 398 of SEQ ID NO. 2-3 or position 396 of SEQ ID NO. 4.

- According to several embodiments, the virus-like particle is of flavivirus origin. More preferably, the flavivirus which the modified peptides or proteins generally derived from is dengue virus, Japanese encephalitis virus, Yellow fever virus, West Nile virus or Zika virus. For instance, in some embodiments, the signal sequence for the M protein and the C-terminal stem-anchor domain of the E protein in the disclosed chimeric peptide are substituted with the corresponding regions from the Defensin A protein of *Aedes aegypti* and the E protein of Japanese encephalitis virus, respectively, to enhance the level of extracellular virus-like particles.

- Another aspect of the present disclosure relates to an isolated polynucleotide encoding a virus-like particle or polypeptide capable of eliciting immune response in a subject upon administering the virus-like particle or polypeptide to the subject at a pharmaceutically effective dosage. More particularly, the virus-like particle or polypeptide comprises a modified form of M and E structural

proteins of flavivirus, wherein an amino acids sequence substantially corresponding to a sequence setting forth in SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3 or SEQ ID NO. 4, wherein conserved amino acid His at positions M7, E261, E282 and E317 of dengue virus sequence are replaced with amino acid Ala at positions 7, 336, 357, and 392 in SEQ ID NO. 1-3 or conserved amino acid His at positions M7, E259, E280 and E315 of dengue virus sequence are replaced with amino acid Ala at positions 7, 334, 355 and 390 in SEQ ID NO. 4. The alterations or modifications or modified forms shall render better expression of the protein in the extracellular level bringing forth better immunization towards the flavivirus in the subject being vaccinated using the disclosed composition.

10

To further increase the extracellular level expression, the disclosed particles may further comprise replacement of conserved amino acid His at positions E27, E149 and E209 of dengue virus sequence with amino acid Asn at positions 102, 224 and 284 in SEQ ID NO. 1-3 or replacement of conserved amino acid His at positions E27, E149 and E207 of dengue virus sequence with amino acid Asn at positions 102, 224 and 282 in SEQ ID NO. 4. Also, the modifications or modified forms further comprise any one or combination of replacement of amino acid Ser or Glu at position E186 of dengue virus sequence or amino acid Ser at position E184 of dengue virus sequence with amino acid Phe at position 261 of SEQ ID NO. 1-3 or at position 259 of SEQ ID NO. 4, respectively, replacement of amino acid Arg at position E188 of dengue virus sequence or position E186 of dengue virus sequence with amino acid Leu at position 263 in SEQ ID NO. 1-3, or at position 261 in SEQ ID NO. 4, respectively, replacement of amino acid Asn or Val at position E242 of dengue virus sequence or amino acid Asn at position E240 of dengue virus sequence with amino acid Ser at position 317 in SEQ ID NO. 2 -3 or position 315 in SEQ ID NO. 4, and replacement of amino acid Arg or Lys located at position E323 of dengue virus sequence or amino acid Lys position E321 of dengue virus sequence with amino acid Gln at position 398 of SEQ ID NO. 2-3 or position 396 of SEQ ID NO. 4.. In addition, the distal part of the E proteins (positions 472-570 of SEQ ID NO. 1-3 or positions 470-568 of SEQ ID NO. 4) is substituted with the corresponding sequence of Japanese encephalitis virus for some embodiments.

Further aspect of the present disclosure is directed to a vaccine composition effective against flavivirus such as dengue virus infection. Particularly, the vaccine composition comprises a pharmaceutically acceptable adjuvant; and modified flavivirus polypeptides or virus-like particles, which are immunologically active upon administering to a subject. The modified flavivirus

polypeptides have an amino acids sequence substantially corresponding to a sequence setting forth in SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3 or SEQ ID NO. 4. The amino acids sequence comprises a modified form or modification of dengue virus sequence, wherein conserved amino acid His at positions M7, E261, E282, and E317 of dengue virus sequence, are replaced by amino acid Ala at positions 7, 336, 357 and 392 in SEQ ID NO. 1-3, or conserved amino acid His at positions M7, E259, E280 and E315 of dengue virus sequence are replaced by amino acid Ala at positions 7, 334, 355 and 390 in SEQ ID NO. 4.

For a number of embodiments, the polypeptides in the vaccine composition possess more modified forms or modifications including replacement of conserved amino acid His at positions E27, E149 and E209 of dengue virus sequence with amino acid Asn at positions 102, 224 and 284 in SEQ ID NO. 1-3 or replacement of conserved amino acid His at positions E27, E149 and E207 of dengue virus sequence with amino acid Asn at positions 102, 224 and 282 in SEQ ID NO. 4.

15 **Brief Description of Drawings**

Figure 1 shows the modified amino acid sequence of flavivirus structural proteins (M and E) derived from DENV-1 and Japanese encephalitis virus and identified as SEQ ID NO. 1;

20 Figure 2 shows the modified amino acid sequence of flavivirus structural proteins (M and E) derived from DENV-2 and Japanese encephalitis virus and identified as SEQ ID NO. 2;

25 Figure 3 shows the modified amino acid sequence of flavivirus structural proteins (M and E) derived from DENV-3 and Japanese encephalitis virus and identified as SEQ ID NO. 4;

30 Figure 4 shows the modified amino acid sequence of flavivirus structural proteins (M and E) derived from DENV-4 and Japanese encephalitis virus and identified as SEQ ID NO. 3;

Figure 5 shows the modified nucleotide sequence encoding the flavivirus structural proteins (M and E) of DENV-1 virus-like particles and identified as SEQ ID NO. 5;

- Figure 6 shows the modified nucleotide sequence encoding the flavivirus structural proteins (M and E) of DENV-2 virus-like particles and identified as SEQ ID NO. 6;
- Figure 7 shows the modified nucleotide sequence encoding the flavivirus structural proteins (M and E) of DENV-4 virus-like particles and identified as SEQ ID NO. 7;
- Figure 8 shows the modified nucleotide sequence encoding the flavivirus structural proteins (M and E) of DENV-3 virus-like particles and identified as SEQ ID NO. 8;
- Figure 9 shows the intracellular expression of the viral protein E following transfection of mosquito cells with the expression vector for the expression of DENV-1, DENV-2, DENV-3, and DENV-4 virus-like particles that the E protein was demonstrated by indirect immunofluorescence assay with a mouse monoclonal anti-E antibody, 1D10, and Cy3-conjugated goat anti-mouse IgG antibody and nuclei were revealed by staining with DAPI;
- Figure 10 shows sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining of M and E proteins in fractions derived from purified DENV-1 mature virus-like particles that the virus-like particle was purified by 20%-55% sucrose step gradient centrifugation followed by rate zonal centrifugation, where the size of protein markers is indicated on the left;
- Figure 11 illustrates transmission electron micrographs of (a) purified DENV-1 virus-like particles, (b) purified DENV-2 virus-like particles, (c) purified DENV-3 virus-like particles and (d) purified DENV-4 virus-like particles;
- Figure 12 illustrates the neutralizing antibody response after the injection of DENV-2 virus-like particles in to 6 mice at weeks 0 and 12 that the reciprocal 50% plaque reduction neutralization titers (PRNT₅₀) of mouse sera obtained at different time points (weeks 0, 2, 4, 8, 12, 14 and 20) are plotted with each of the dots represents an individual mouse, where the line connects the geometric mean titer and error bars denote standard deviation;

Figure 13 illustrates the neutralizing antibody response in cynomolgus macaques after the prime- injection of a DENV-2 live attenuated virus on day 0, two booster injections with the DENV-2 virus-like particles on days 60 and 90, and a challenge injection with a DENV-2 wild type virus on day 120, where the reciprocal 50% plaque reduction neutralization titers (PRNT₅₀) of macaque sera obtained at different time points (days 0, 14, 30, 60, 74, 90, 104, 118, 134 and 150) are plotted with each of the dots and connecting line represent individual macaque.

Detailed Description

Hereinafter, the disclosure shall be described according to the preferred embodiments and by referring to the accompanying description and drawings. However, it is to be understood that referring the description to the preferred embodiments of the invention and to the drawings is merely to facilitate discussion of the various disclosed embodiments and it is envisioned that those skilled in the art may devise various modifications without departing from the scope of the appended claim.

Unless specified otherwise, the terms "comprising" and "comprise" as used herein, and grammatical variants thereof, are intended to represent "open" or "inclusive" language such that they include recited elements but also permit inclusion of additional, un-recited elements.

As used herein, the phrase "in embodiments" means in some embodiments but not necessarily in all embodiments.

The term "polypeptides" used herein throughout the disclosure refers to a chain of amino acids linked together by peptide bonds but with a lower molecular weight than protein. Polypeptides can be obtained by synthesis or hydrolysis of proteins. Few polypeptides can be joined together by any known method in the art to form a functional unit.

The term "polynucleotide" or "nucleic acid" as used herein designates mRNA, RNA, cRNA, cDNA or DNA. The term typically refers to oligonucleotides greater than 30 nucleotide residues in length.

The terms “partial” or “substantially corresponding to” used herein to describe the polypeptides or peptides throughout the disclosure refers to polypeptides or peptides that contain at least 70%, or more preferably at least 80%, sequential amino acids identical to the disclosed polypeptides or peptides as shown in Figure 1-4. These polypeptides though may not have the sequence fully
5 identical to disclosed polypeptides or peptides; these polypeptides may retain the active epitopes to react with the corresponding antibodies to elicit the desired immune response against flavivirus and these active epitopes are present on the peptides due to the sequence of the amino acids arranged in the peptides has about at least 70% to 90% similarity to the disclosed sequence by alignment.

10

As used herein, “a position corresponding to” or recitation that nucleotides or amino acid positions “correspond to” nucleotides or amino acid positions in a disclosed sequence, such as set forth in the Sequence listing, refers to nucleotides or amino acid positions identified upon alignment with the disclosed sequence to maximize identity using a standard alignment algorithm. By aligning
15 the sequences, one skilled in the art can identify corresponding residues.

20

The term “dengue sequence” or “dengue virus sequence” as used herein refers to a linear nucleotide or amino acid sequence or sequences of dengue virus, particularly a wild-type virus or a wild type of dengue virus having nucleotides or amino acids at position(s) corresponding to any
20 known or naturally occurred sequence of dengue virus. In the disclosure, the term “dengue sequence” or “dengue virus sequence” is also used interchangeably.

25

In the specification and claims of the present disclosure, the term “flavivirus structural protein” or “flavivirus peptide” or “flavivirus polypeptide” are used interchangeably referring to any peptide-based sequence derived from flavivirus that can be recognized by the immune system in a subject,
stimulates a cell-mediated immune response in a subject and/or stimulates the generation of antibodies in a subject.

30

The flavivirus selected in the present disclosure as the template or original structure or peptide-sequence structure for modification into the disclosed peptides or proteins may derive from Dengue virus, Yellow fever virus, Japanese encephalitis virus, West Nile virus and/or Zika virus. For some embodiments, one portion or region of the proteins derived from one virus type may be replaced or substituted by corresponding or similar region of other virus type. For example, the

signal sequence for the M protein of Dengue virus origin is removed and substituted with the corresponding regions originated from the Defensin A protein of *Aedes aegypti*. Similar modifications can be seen in other embodiments in which the C-terminal stem-anchor domain of the E protein originated from Dengue virus is replaced with corresponding region of the E protein from Japanese encephalitis virus. Such modifications involve moving or replacing one region of a protein of one virus type to protein of another virus type aims to improve yield of the expressed disclosed peptides or protein at the extracellular level. In accordance with several preferred embodiments, the flavivirus which the disclosed peptides originally or naturally acquired from is Dengue virus. The Dengue virus may be Dengue virus 1 including subtypes I to IV, Dengue virus 2 including subtypes Asian I, Asian II, Cosmopolitan, American and American/Asian, Dengue virus 3 including subtypes I to IV and Dengue virus 4 including subtypes I to III. Generally, Dengue virus structural protein consists of capsid protein, precursor membrane (prM) protein, membrane (M) protein and envelope (E) protein. For more embodiments, the disclosure proteins or peptides are mature virus-like particle comprises two of those structural proteins, namely the M protein and the E protein. The dengue virus structural protein may further comprise an amino acid corresponding to the initiation codon and a signal sequence to the amino terminus of the M sequence.

More specifically, some embodiments of the present disclosure provide a mature virus-like particle comprising a modified form of M and E structural proteins of flavivirus, wherein multiple amino acids are altered from its naturally occurred structure. These structural proteins can be expressed by using any known method in the field through a group of selected host cells or animal. One or more flavivirus structural proteins disclosed may be used for subject immunization as long as they spontaneously assemble into a particulate structure having active epitope for binding with the antibody to initiate the immunization process. For example, when eukaryotic cells expressing a gene encoding M and E proteins of dengue virus are cultured, the proteins are generated by the cells and assemble into virus-like particles and the virus-like particles can be collected from the cell culture media. Alternatively, chemical synthesis method can be employed as well giving rise to the interested structural protein identical or almost identical to the flavivirus structural proteins, M and E. Preferably, the created or expressed proteins of M and E are associated, joined, attached or held in a manner exposing the deliberately retain epitopes to yield the expected immune response in a subject.

The modified forms of M and E region of the flavivirus, or particularly dengue virus, amino acid sequences are represented by SEQ ID NO. 1-4. Viral structural proteins of various flaviviruses such as dengue virus types 1-4 have been identified and available at various public databases such as GenBank database. For example, Dengue virus type 1 (strain West Pac and strain 16007):
5 Accession No. U88535 and AF180817.1, Dengue virus type 2 (strain S1 vaccine and strain 16681): Accession No. M19197 and U87411.1, Dengue virus type 3 (strain Singapore 8120/95 and strain 16562): Accession No. AY766104 and KU725665.1 and Dengue virus type 4 (strain ThD4_0476_97 and strain H-241): Accession No. Y618988.1 and U18433.1. The information with respect to envelope protein including sequential amino acids arrangement of a flavivirus such
10 as dengue virus may be obtained from a database. By using the sequence alignment function in these databases, person skilled in the field shall be able to identify substantial length of SEQ ID NO. 1-4 being incorporated into a much longer sequence of a larger peptide or found a partial segment of SEQ ID NO. 1-4 in a shorter sequence of a relatively smaller peptide. Any modifications or modified forms of these longer or shorter sequences closely related to the
15 disclosed SEQ ID NO. 1-4 shall not depart from the scope of the present disclosure as long such modifications lead to better or improved induced immune response in the subject against flavivirus or dengue virus. It is important to note that the peptides expressed through the disclosed sequences are free of the pr portion of prM.

20 Accordingly, the disclosed peptides of SEQ ID NO. 1-4 are made or produced to include at least one amino acid alternation, preferably between amino acid position 7 and amino acid position 398, or between the positions determined as the above-identified positions when the amino acid sequence of a flavivirus or a fragment thereof is aligned with SEQ ID NO. 1-4. More specifically, the region comprising at least one amino acid alternations or modifications may be preferably
25 between amino acid position 7 and amino acid position 398 of SEQ ID NO. 1-4. Some embodiments of the expressed peptides may commonly comprise initiation codon Met and signalling peptide followed by M and E of Dengue Virus 1, Dengue Virus 2, Dengue Virus 3, or Dengue Virus 4 in some embodiments. The initiation codon, signalling region, peptide M and peptide E are preferably arranged in sequential tandem. For several embodiments, the distal part
30 of the E proteins is substituted or incorporated with the corresponding sequence of Japanese encephalitis virus. The presently disclosed protein, peptides or virus-like particle is capable of eliciting immune response in a mammal or subject comprising an amino acids sequence substantially corresponding to a sequence setting forth in SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID

NO. 3 or SEQ ID NO. 4. The disclosed amino sequence carries the desired modifications. For a number of embodiments, the modified form comprises an amino acids sequence substantially corresponding to an M and E sequence setting forth in SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3 or SEQ ID NO. 4, wherein conserved amino acid His at positions M7, E261, E282, and E317 of dengue virus sequence, are replaced by amino acid Ala at positions 7, 336, 357 and 392 in SEQ ID NO. 1-3, or conserved amino acid His at positions M7, E259, E280 and E315 of dengue virus sequence are replaced by amino acid Ala at positions 7, 334, 355 and 390 in SEQ ID NO. 4.

In more embodiments, the modified form further comprises replacement of conserved amino acid His at positions E27, E149 and E209 of dengue virus sequence with amino acid Asn at positions 102, 224 and 284 in SEQ ID NO. 1-3 or replacement of conserved amino acid His at positions E27, E149 and E207 of dengue virus sequence with amino acid Asn at positions 102, 224 and 282 in SEQ ID NO. 4. Also, the modified forms or the modifications on the disclosed sequences further comprises any one or combination of replacement of amino acids Ser or Glu at position E186 of dengue virus sequence or amino acid Ser at position E184 of dengue virus sequence with amino acid Phe at position 261 in SEQ ID NO. 1-3 or at position 259 in SEQ ID NO. 4, respectively, or replacement of amino acid Arg at position E188 of dengue virus sequence or position E186 of dengue virus sequence with amino acid Leu at position 263 in SEQ ID NO. 1-3 or at position 261 in SEQ ID NO. 4, respectively, or replacement of amino acid Asn or Val at position E242 of dengue virus sequence or amino acid Asn at position E240 of dengue virus sequence with amino acid Ser at position 317 in SEQ ID NO. 2-3 or position 315 in SEQ ID NO. 4, or replacement of amino acid Arg or Lys located at position E323 of dengue virus sequence or amino acid Lys position E321 of dengue virus sequence with amino acid Gln at position 398 of SEQ ID NO. 2-3 or position 396 of SEQ ID NO. 4.

With the exception of comprising at least one amino acid alteration in the region, a flavivirus structural protein contained in the virus-like particle may be a naturally occurring viral structural protein or a modified protein thereof. In plurality of embodiments, the modified protein has at least 70%, 75%, 80%, 85%, 90%, 95% or 98% amino acid sequence identity to a naturally occurring viral structural protein including M and E proteins. For more embodiments, the modified protein is a mutant with potentially at most 10% of the amino acids corresponding the sequence of SEQ ID NO. 1-4 are deleted, substituted, and/or added to a naturally occurring viral structural

protein including M and envelope regions. The sequence identity may be determined by conventional methods.

The modified flavivirus structural protein may have at least 70%, 75%, 80%, 85%, 90%, 95% or 98% amino acid sequence identity to an amino acid sequence represented by any one of SEQ ID Nos. 1-4. Also, the modified flavivirus structural protein may be a mutant where at most 10% of the amino acids are deleted, substituted, and/or added based on the flavivirus structural protein having an amino acid sequence represented by any one of SEQ ID NO. 1-4.

The second aspect of the present disclosure is directed to an isolated polynucleotide or nucleic acid molecule comprising a nucleotide sequence encoding the virus-like particle setting forth in SEQ ID NO. 1-4 with or without modifications. Preferably, the isolated polynucleotide encoding a virus-like particle or polypeptide capable of eliciting immune response in a subject upon administering the virus-like particle or polypeptide to the subject at a pharmaceutically effective dosage. The virus-like particle, peptide or polypeptide encoded by the disclosed polynucleotide comprises a modified form of M and E structural proteins of flavivirus, wherein a nucleotide sequence of the modified form is substantially corresponding to a sequence setting forth in SEQ ID NO. 5, SEQ ID NO. 6, SEQ ID NO. 7 or SEQ ID NO. 8, or an amino acids sequence substantially corresponding to a sequence setting forth in SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3 or SEQ ID NO. 4, wherein conserved amino acid His at positions M7, E261, E282 and E317 of dengue virus sequence are replaced with amino acid Ala at positions 7, 336, 357, and 392 in SEQ ID NO. 1-3 or conserved amino acid His at positions M7, E259, E280 and E315 of dengue virus sequence are replaced with amino acid Ala at positions 7, 334, 355 and 390 in SEQ ID NO. 4.

In accordance with several embodiments of the disclosed nucleic acid molecule, it may encode as well the modifications of replacement of conserved amino acid His located at positions E27, E149 and E209 of dengue virus sequence with amino acid Asn at positions 102, 224 and 284 in SEQ ID NO. 1-3 or replacement of conserved amino acid His at positions E27, E149 and E207 of dengue virus sequence with amino acid Asn at positions 102, 224 and 282 in SEQ ID NO. 4. Similarly, for more embodiments, the modifications encoded in the disclosed nucleic acid molecule further comprises any one or combination of replacement of amino acid Ser or Glu at position E186 of dengue virus sequence or amino acid Ser at position E184 of dengue virus sequence with amino acid Phe at position 261 of SEQ ID NO. 1-3 or at position 259 of SEQ ID NO. 4, respectively, replacement of amino acid Arg at position E188 of dengue virus sequence or position E186 of

dengue virus sequence with amino acid Leu at position 263 in SEQ ID NO. 1-3, or at position 261 in SEQ ID NO. 4, respectively, amino acid Asn or Val at position E242 of dengue virus sequence or amino acid Asn at position E240 of dengue virus sequence with amino acid Ser at position 317 in SEQ ID NO. 2-3 or position 315 in SEQ ID NO. 4, , and replacement of amino acid Arg or Lys at position E323 of dengue virus sequence or amino acid Lys position E321 of dengue virus sequence with amino acid Gln at position 398 of SEQ ID NO. 2-3 or position 396 of SEQ ID NO. 4.

It is important to note that multiple variants of the polynucleotide can be used for encoding the modified proteins or peptide since a single amino acid can be encoded by more than one nucleotide codon. Representative example of the variants respectively encoding for proteins of SEQ ID NO. 1-4 are respectively illustrated in Figure 5-8 as SEQ ID NO.5-8. More importantly, for some embodiments, the present disclosure provides a nucleic acid molecule which is further modified from the nucleic acid molecule having a nucleotide sequence represented by any one of SEQ ID Nos.5-8. The modified nucleic acid molecule may have at least 70%, 75%, 80%, 85%, 90%, 95% or 98% nucleotide sequence identity to the nucleic acid molecule having a nucleotide sequence represented by any one of SEQ ID Nos.5-8. Also, the modified nucleic acid molecule may be a mutant where at most 10% of the amino acids are deleted, substituted, and/or added based on the nucleic acid molecule having a nucleotide sequence represented by any one of SEQ ID Nos.5-8.

Further embodiments of the disclosure contain an expression vector comprising the nucleic acid molecule described above. The vector optionally comprises an expression control sequence operably linked to the disclosed nucleic acid molecule. More specifically, the present disclosure provides an expression vector for flavivirus structural proteins, M and E that the expression vector carries a nucleotide sequence encoding the variants of protein setting forth in SEQ ID NO. 1-4 with the above-mentioned amino acid replacement. The nucleotide sequence carried by the expression vector can be any one of SEQ ID Nos. 5-8.

Third aspect of the present disclosure provides a composition or vaccine composition comprising the virus-like particle provided in the first aspect of the present application and/or the nucleic acid molecule provided in the second aspect of the present invention. Particularly, the vaccine composition comprises pharmaceutically acceptable adjuvant; and polypeptides or virus-like particles, which are immunologically active upon administering to a subject, having an amino acids sequence substantially corresponding to a sequence setting forth in SEQ ID NO. 1, SEQ ID

NO. 2, SEQ ID NO. 3 or SEQ ID NO. 4. Each of the disclosed sequences is incorporated with one or more modified forms or modifications of dengue virus sequence, wherein conserved amino acid His at positions M7, E261, E282, and E317 of dengue virus sequence, are replaced by amino acid Ala at positions 7, 336, 357 and 392 in SEQ ID NO. 1-3, or conserved amino acid His at positions
5 M7, E259, E280 and E315 of dengue virus sequence are replaced by amino acid Ala at positions 7, 334, 355 and 390 in SEQ ID NO. 4.

For more embodiments of the disclosed vaccine composition, the modified forms or modifications further comprise replacement of conserved amino acid His at positions E27, E149 and E209 of dengue virus sequence with amino acid Asn at positions 102, 224 and 284 in SEQ ID NO. 1-3 or
10 replacement of conserved amino acid His at positions E27, E149 and E207 of dengue virus sequence with amino acid Asn at positions 102, 224 and 282 in SEQ ID NO. 4.

In several more embodiments of the disclosed vaccine composition, the modified forms or modifications further comprise any one or combination of replacement of amino acids Ser or Glu
15 at position E186 of dengue virus sequence or amino acid Ser at position E184 of dengue virus sequence with amino acid Phe at position 261 in SEQ ID NO. 1-3 or at position 259 in SEQ ID NO. 4, respectively, replacement of amino acid Arg located at position E188 of dengue virus sequence or position E186 of dengue virus sequence with amino acid Leu at position 263 in SEQ ID NO. 1-3 or at position 261 in SEQ ID NO. 4, replacement of amino acid Asn or Val at position
20 E242 of dengue virus sequence or amino acid Asn at position E240 of dengue virus sequence with amino acid Ser at position 317 in SEQ ID NO. 2-3 or position 315 in SEQ ID NO. 4, and replacement of amino acid Arg or Lys located at position E323 of dengue virus sequence or amino acid Lys position E321 of dengue virus sequence with amino acid Gln at position 398 of SEQ ID NO. 2-3 or position 396 of SEQ ID NO. 4.

25

Furthermore, the pharmaceutically acceptable carrier and/or adjuvant can be, but are not limited to, AddaVax (InvivoGen). In addition to adjuvant or carrier, the pharmaceutical composition of the present disclosure may contain a single active ingredient or a combination of two or more active ingredients, as far as they are not contrary to the objects of the present disclosure. In a
30 combination of plurality of active ingredients, contents or concentration of the respective ingredients in the disclosed composition may be suitably varied in different embodiments in consideration of their therapeutic effects and safety. The term "combination" used herein means two or more active ingredients are administered to a subject simultaneously in the form of a single

entity or dosage, or those active ingredients are administered to a subject as separate entities either simultaneously or sequentially with no specific time limits, wherein such administration provides therapeutically effective levels of the two components in the body, preferably at the same time.

5 In the fourth aspect, the present disclosure provides a method of producing an antibody against a flavivirus or an antiserum containing a neutralizing antibody against a flavivirus comprising bringing a subject into contact with the virus-like particle provided in the first aspect of the present application and/or the nucleic acid molecule provided in the second aspect of the present disclosure in a manner which an immune response is elicited by such contact. The subject can be
10 a mammal such as human. The antibody produced by the disclosed method may be used for passive immunization against a flavivirus-causing pathogen infecting other subjects or mammals by administering produced antibody with a therapeutically effective dosage to the mammal. The administered antibody is effective against the flavivirus infecting the mammal or subject thus preventing or curing the mammal from flavivirus infection or any diseased state caused by the
15 flavivirus.

The antibody produced from other mammal other than human in the present disclosure can be further subjected to humanization process using a\one or more conventionally known techniques. Thus, in more embodiment, the method provided in this aspect further comprises the step of
20 humanizing a non-human mammal produced antibody. The antibody or humanized antibody acquired may be given to the human subject for preventing or eradicating flavivirus infection, treating or alleviating any diseased state or symptoms raised by flavivirus infection in the subject. Moreover, the antibody produced according to the disclosed method can be employed for in vitro selection of a subpopulation from immune cells such as B-cell and T-cell derived from the patient.
25 The selected subpopulation immune cells are subsequently re-administered to the patient to boost immunity against flavivirus infection.

Further embodiments are directed to antiserum containing the disclosed antibodies obtainable by the conventional manner. Specifically, blood samples are taken from the immunized non-human
30 animal, and the blood is processed to obtain the antiserum, i.e. the antibody-containing liquid component of the blood thereof. The non-human mammal is preferably selected from the group consisting of rat, mouse, hamster, pig, rabbit, horse, donkey, goat, sheep, guinea pig, lama, and non-human primate.

In the fifth aspect, the present disclosure set out a method of treating a disease or condition caused by flavivirus infection such as dengue fever in a subject. Preferably, the virus-like particle provided in the first aspect, the nucleotide molecule provided in the second aspect, or the composition provided in the third aspect is administered to the subject. By administering the above
5 modified virus-like particle, nucleotide molecule encoding the modified virus-like particle or vaccine composition to the subject, immune response or immunity against a flavivirus can be induced or enhanced or established in the subject. Thus, any diseased condition potentially caused by the flavivirus infection such as dengue infection can effectively be prevented or eradicated by the established immunity. In this aspect, the virus-like particle, nucleotide molecule or
10 composition may be administered to the patient specifically to an affected organ or systemically to the general immune system of the subject boosting overall resistance against flavivirus infection. Preferably, diseased state or symptoms caused by a flavivirus may be dengue, dengue fever, dengue haemorrhagic fever and severe dengue.

15 In further embodiments of the method treating flavivirus infection, the virus like particle can also be applied for immune therapy. The virus-like particle may be applied to ex vivo to cells derived from the patient or a human cell line which are subsequently administered to the patient.

In the sixth aspect, the present disclosure provides a method of producing the virus-like particle provided in the first aspect comprising culturing a cell which is expressing the virus-like particle
20 from a gene encoding for the same; and recovering the virus-like particles from the cell culture. Various host-vector systems may be used in the disclosed method of producing the virus-like particle. Eukaryotic cells can be used for the method provided by the fourth aspect of the present application. Examples of eukaryotic cells include, but are not limited to, insect cells (e.g. C6/36, Sf9 cells, High five cells), yeast cells (e.g. *Saccharomyces cerevisiae*) and mammalian cells (e.g.
25 CHO cells, human embryonic kidney 293F cells). Vector used for the method provided by the second aspect of the present application comprises a nucleic acid molecule encoding the virus-like particle to be expressed. Cells may be transfected with the vector using conventional methods (e.g. lipofection, electroporation). A skilled person can select culture medium. After the transfection, a
30 stably protein-producing clone of transfected cells may be selected, and virus-like particle can be produced in the cells and released into culture media. Virus-like particle may be recovered from the culture media and purified using ultracentrifugation and/or chromatography. It is important to note that the expressed and recovered proteins or particles are mature virus-like particles, which

cannot replicate in the subject being administrated with the recovered proteins and, therefore, can be safely applied as the vaccine composition.

The following examples are intended to further illustrate the disclosure, without any intent for the disclosure to be limited to the specific embodiments described therein.

Example 1

Expression of flavivirus structural proteins

Dengue virus types 1-4 and Japanese encephalitis virus envelope glycoproteins were used as viral structural proteins in the present experiments. In the dengue virus structural proteins used, at least one modification or modified form was introduced by one or more approach known in the field. More specifically, conserved amino acid His at positions M7, E261, E282, and E317 of dengue virus sequence, are replaced by amino acid Ala at positions 7, 336, 357 and 392 in SEQ ID NO. 1-3, or conserved amino acid His at positions M7, E259, E280 and E315 of dengue virus sequence or wild type are replaced by amino acid Ala at positions 7, 334, 355 and 390 in SEQ ID NO. 4. In further embodiments of the experiments conducted, the modified form further comprises replacement of conserved amino acid His at positions E27, E149 and E209 of dengue virus sequence or wild type with amino acid Asn at positions 102, 224 and 284 in SEQ ID NO. 1-3 or replacement of conserved amino acid His at positions E27, E149 and E207 of dengue virus sequence or wild type with amino acid Asn at positions 102, 224 and 282 in SEQ ID NO. 4. Still in some embodiment of the experiments performed, more modifications were made to any one or a combination of replacing an amino acid Ser or Glu at position E186 of dengue virus sequence or amino acid Ser at position E184 of dengue virus sequence or wild type with amino acid Phe at position 261 in SEQ ID NO. 1-3 or at position 259 in SEQ ID NO. 4, respectively, replacing of amino acid Arg located at position E188 of dengue virus sequence or position E186 of dengue virus sequence with amino acid Leu at position 263 in SEQ ID NO. 1-3 or at position 261 in SEQ ID NO. 4, substituting of amino acid Asn or Val at position E242 of dengue virus sequence or amino acid Asn at position E240 of dengue virus sequence with amino acid Ser at position 317 in SEQ ID NO. 2-3 or position 315 in SEQ ID NO. 4, altering of amino acid Arg or Lys located at position E323 of dengue virus sequence or amino acid Lys position E321 of dengue virus sequence or wild type with amino acid Gln at position 398 of SEQ ID NO. 2-3 or position 396 of SEQ ID NO. 4. Some embodiments of the modified viral structural protein studied in the present experiments were listed in Table 1 below.

Position in this construct		Position in the wild type virus protein		DENV-1		DENV-2		DENV-3		DENV-4	
DENV-1, -2 and -4	DENV-3	DENV-1, -2 and -4	DENV-3	Wild type	Virus-like particle	Wild type	Virus-like particle	Wild type	Virus-like particle	Wild type	Virus-like particle
7	7	M 7	M 7	His	Ala	His	Ala	His	Ala	His	Ala
102	102	E 27	E 27	His	Asn	His	Asn	His	Asn	His	Asn
224	224	E 149	E 149	His	Asn	His	Asn	His	Asn	His	Asn
261	259	E 186	E 184	Ser	Phe	Ser	Phe	Ser	Phe	Glu	Phe
263	261	E 188	E 186	Arg	Leu	Arg	Leu	Arg	Leu	Arg	Leu
284	282	E 209	E 207	His	Asn	His	Asn	His	Asn	His	Asn
317	315	E 242	E 240	Thr	Thr	Asn	Ser	Asn	Ser	Val	Ser
336	334	E 261	E 259	His	Ala	His	Ala	His	Ala	His	Ala
357	355	E 282	E 280	His	Ala	His	Ala	His	Ala	His	Ala
392	390	E 317	E 315	His	Ala	His	Ala	His	Ala	His	Ala
398	396	E 323	E 321	Gln	Gln	Arg	Gln	Lys	Gln	Lys	Gln

To express the one or more modified viral structural proteins in insect cells, an M-E expression vector was transfected to C6/36 or Sf9 cells. After the transfection, intracellular E expression was examined in an indirect immunofluorescence assay, by using an anti-Dengue virus monoclonal antibody (clone 1D10) that specifically binds to the E protein and Cy3-conjugated goat anti-mouse Ig antibodies as the secondary antibody. The results are shown in Fig. 9.

Extracellular level of the E protein was assessed in a capture ELISA. Culture media from the transfected cells were harvested and the expression of the E protein was examined by using Dengue virus monoclonal antibody as the primary antibody and horse radish peroxidase-conjugated rabbit anti-mouse IgG antibody as the secondary antibody. Results are shown in Table 2 below.

Construct	Extracellular E protein concentration at indicated hour after transfection (µg/ml)		
	0 hr	24 hr	48 hr
Plasmid construct #1	0.097	0.172	0.386

Plasmid construct #2	0.014	0.171	0.381
----------------------	-------	-------	-------

Example 2

Preparation of a virus-like particle from stably flavivirus structural proteins-expressing cells

Expression vector for flavivirus M and E structural proteins used in Example 1 were used. In the same manner as Example 1, the vector was transfected to C6/36 or Sf9 cells. In the generation of stably expressing transfectants, the vector was transfected into the cells, and the recombinant transfected cells were selected with the use of blasticidin. Clones of drug-resistant transfectant were selected based on intracellular and extracellular expression of dengue virus E protein, employing a murine monoclonal antibody, 1D10, in indirect immunofluorescence and dot blot immunoassay formats. A transfectant clone with high level of extracellular E protein expression was expanded. Cell culture media were concentrated, and then subjected to purification by ultracentrifugation in sucrose gradient and/or potassium tartrate - glycerol gradient as described previously (Charoensri et al, 2014). The presence of the two envelope proteins, E and M, in the virus-like particle preparations was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining. The results are shown in Fig 10.

Morphological assessment was performed by negative staining using uranyl acetate and transmission electron microscopy. The results are shown in Fig 11. In the purified particulate fraction, generally round particles with some variations in size and shape, as expected from virus-like particles without the core structure, were observed.

Example 3

Antibody against Dengue virus in mice

Purified dengue virus-like particles obtained in Example 2 was used in this example. Six mice were immunized with 1 µg of the DENV-2 virus-like particles in 20% glycerol-NTE buffer (12 mM Tris [pH 8.0], 1 mM EDTA and 120 mM NaCl)- containing Squalene-oil-in-water adjuvant (AddaVax, InvivoGen) by subcutaneous injection at weeks 0 and 8. The serum was assayed for neutralizing antibody to DENV-2 by employing a prototypic DENV-2 strain 16681. Anti-DENV neutralizing antibodies in the immunized mice sera were detected by a plaque reduction neutralization test (PRNT) on Vero cells. The endpoint titer was calculated as the highest serum dilution tested that reduced the number of plaques forming unit by at least 50% (PRNT₅₀). The

results are shown in Fig 12. The virus-like particles were able to induce high levels of neutralizing antibodies in immunized mice.

Example 4

5 Antibody against Dengue virus in monkeys

Purified dengue virus-like particle obtained in Example 2 was used in this example. Six cynomolgus macaques were immunized with monovalent live-attenuated dengue virus at day 0. They were boosted with the 10 µg of virus-like particles in 20% glycerol-NTE buffer (12 mM Tris [pH 8.0], 1 mM EDTA and 120 mM NaCl) containing Squalene-oil-in-water adjuvant (AddaVax, InvivoGen) by subcutaneous injection twice on days 60 and 90. Macaques were then challenged with a DENV-2 wild type virus on day 120. The sera were assayed for neutralizing antibody to DENV-2 employing a DENV-2 prototypic strain 16681 and Vero cells in a plaque reduction neutralization test (PRNT). The endpoint titer was calculated as the highest serum dilution tested that reduced the number of plaques forming unit by at least 50% (PRNT₅₀). The results are shown in Fig 13. The virus-like particles were able to induce high levels of neutralizing antibodies to DENV-2 in live attenuated virus-primed macaques and protected them from infection following challenge with a DENV-2 wild type virus.

Example 5

20 Preparation of a Pharmaceutical Composition Comprising Dengue Virus-Like Particle

Dengue virus-like particles were prepared according to Example 2. To prepare a pharmaceutical composition which is a vaccine composition, 10 µg of purified virus-like particles were mixed with 0.1 ml of NTE buffer containing 12 mM Tris (pH 8.0), 1 mM EDTA and 120 mM NaCl.

25 It is to be understood that the present disclosure may be embodied in other specific forms and is not limited to the embodiments described above. However, modification and equivalents of the disclosed concepts such as those which readily occur to one skilled in the art are intended to be included within the scope of the claims which are appended thereto.

References

- Allison SL, Stadler K, Mandl CW, Kunz C, Heinz FX. Synthesis and secretion of recombinant tick-borne encephalitis virus protein e in soluble and particulate form. *J Virol* 1995; 69: 5816-20.
- 5 Capeding MR, Tran NH, Hadinegoro SR, Ismail HI, Chotpitayasunondh T, Chua MN, et al. Clinical efficacy and safety of a novel tetravalent dengue vaccine in healthy children in Asia: a phase 3, randomised, observer-masked, placebo-controlled trial. *Lancet* 2014; 384: 1358-65.
- Chan, A. H., Tan, H. C., Chow, A. Y., Lim, A. P., Lok, S. M., Moreland, N. J., et al. A human
10 prM antibody that recognizes a novel cryptic epitope on dengue E glycoprotein. *PLoS ONE* 2012; 7: e33451.
- Charoensri N, Suphatrakul A, Sriburi R, Yasanga T, Junjhon J, Keelapang P, et al. An optimized expression vector for improving the yield of dengue virus-like particles from transfected insect
15 cells. *J Virol Methods* 2014; 205: 116-23.
- Chaudhury S, Ripoll DR, Wallqvist A. Structure-based pKa prediction provides a thermodynamic basis for the role of histidines in pH-induced conformational transitions in dengue virus. *Biochem Biophys Rep* 2015; 4: 375-85.
20
- Christian EA, Kahle KM, Mattia K, Puffer BA, Pfaff JM, Miller A, et al. Atomic-level functional model of dengue virus envelope protein infectivity. *Proc Natl Acad Sci (USA)* 2013; 110: 18662-7.
- 25 Dejnirattisai W, Jumnainsong A, Onsirisakul N, Fitton P, Vasanawathana S, Limpitikul W, et al. Cross-reacting antibodies enhance dengue virus infection in humans. *Science* 2010; 328: 745-8.
- Dejnirattisai W, Wongwiwat W, Supasa S, Zhang XK, Dai XH, Rouvinsky A, et al. A new class of highly potent, broadly neutralizing antibodies isolated from viremic patients infected with
30 dengue virus. *Nat Immunol* 2015; 16: 170-7.
- Flipse J, Smit JM. The complexity of a dengue vaccine: a review of the human antibody response. *PLoS Negl Trop Dis* 2015; 9: e0003749.
- 35 Fritz R, Stiasny K, Heinz FX. Identification of specific histidines as pH sensors in flavivirus membrane fusion. *J Cell Biol* 2008; 183: 353-61.

- Fuzo CA, Degreè L. The pH dependence of flavivirus envelope protein structure: insights from molecular dynamics simulations, *J Biomolecular Structure Dynamics* 2014; 32: 1563-74.
- Hadinegoro SR, Arredondo-Garcia JL, Capeding MR, Deseda C, Chotpitayasunondh T, Dietze R,
5 et al. Efficacy and long-term safety of a dengue vaccine in regions of endemic disease. *N Engl J Med* 2015; 373: 1195–206.
- Hsieh SC, Tsai WY, Nerurkar VR, Wang WK. Characterization of the ectodomain of the envelope protein of dengue virus type 4: expression, membrane association, secretion and particle formation
10 in the absence of precursor membrane protein. *PLoS ONE* 2014; 9: e100641.
- Huang KJ, Yang YC, Lin YS, Huang JH, Liu HS, Yeh TM, et al. The dual-specific binding of dengue virus and target cells for the antibody-dependent enhancement of dengue virus infection. *J Immunol* 2006; 176: 2825–32.
15
- Huang KJ, Cheng YT, Lin YS, Huang JH, Liu HS, Yeh TM, et al. Anti-prM antibody as an autoantibody in dengue virus infection. *Am J Infect Dis* 2008; 4: 59-67.
- Junjhon J, Lausumpao M, Supasa S, Noisakran S, Songjaeng A, Saraithong P, et al. Differential
20 modulation of prM cleavage, extracellular particle distribution, and virus infectivity by conserved residues at nonfurin consensus positions of the dengue pr-M junction. *J Virol* 2008; 82: 10776-91.
- Junjhon J, Edwards TJ, Utaipat U, Bowman VD, Holdaway HA, Zhang W, et al. Influence of pr-M cleavage on the heterogeneity of extracellular dengue virus particles. *J Virol* 2010; 84: 8353-
25 8.
- Kampmann T, Mueller DS, Mark AE, Young PR, Kobe B. The role of histidine residues in low pH-mediated viral membrane fusion. *Structure* 2006; 14: 1481-7.
- Katzelnick LC, Gresh L, Halloran ME, Mercado JC, Kuan G, Gordon A, et al. Antibody-
30 dependent enhancement of severe dengue disease in humans. *Science* 2017; 358: 929–32.
- Konishi E, Fujii A, Mason PM. Generation and characterization of a mammalian cell line continuously expressing Japanese encephalitis virus subviral particles. *J Virol* 2001; 75: 2204-12.

- Konishi E, Fujii A. Dengue type 2 virus subviral extracellular particles produced by a stably transfected mammalian cell line and their evaluation for a subunit vaccine. *Vaccine* 2002; 20: 1058-67.
- 5 Konishi E, Mason PW. Proper maturation of the Japanese encephalitis virus envelope glycoprotein requires cosynthesis with the premembrane protein. *J Virol* 1993; 67: 1672-5.
- Kuhn RJ, Zhang W, Rossmann MG, Pletnev SV, Corver J, Lenches E, et al. Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. *Cell* 2002; 108: 717-25.
- 10 Li L, Lok SM, Yu IM, Zhang Y, Kuhn RJ, Chen J, et al. The flavivirus precursor membrane-envelope protein complex: structure and maturation. *Science* 2008; 319: 1830-4.
- Lok SM. The interplay of dengue virus morphological diversity and human antibodies. *Tr*
- 15 *Microbiol* 2016; 24: 284-93.
- Lorenz IC, Allison SL, Heinz FX, Helenius A. Folding and dimerization of tick-borne encephalitis virus envelope proteins prM and E in the endoplasmic reticulum. *J Virol* 2002; 76: 5480-91.
- Luo YY, Feng JJ, Zhou JM, Yu ZZ, Fang DY, Yan HJ, et al. Identification of a novel infection-enhancing epitope on dengue prM using a dengue cross-reacting monoclonal antibody. *BMC*
- 20 *Microbiology* 2013; 13: 194.
- Luo Y, Guo X, Yan H, Fang D, Zeng G, Zhou J, et al. Comprehensive mapping infection-enhancing epitopes of dengue pr protein using polyclonal antibody against prM. *Appl Microbiol*
- 25 *Biotechnol* 2015; 99: 5917-27.
- Mueller DS, Kampmann T, Yennamalli R, Young PR, Kobe B, Mark AE. Histidine protonation and the activation of viral fusion proteins. *Biochem Soc T* 2008; 36: 43-5.
- 30 Nelson S, Poddar S, Lin T-Y, Pierson TC. Protonation of individual histidine residues is not required for the pH-dependent entry of West Nile virus: evaluation of the "histidine switch" hypothesis. *J Virol* 2009; 83: 12631-5.
- Plevka P, Battisti AJ, Junjhon J, Winkler DC, Holdaway HA, Keelapang P, et al. Maturation of
- 35 flaviviruses starts from one or more icosahedrally independent nucleation centers. *EMBO Reports* 2011; 12: 602-6.

- Plevka P, Battisti AJ, Sheng J, Rossmann MG. Mechanism for maturation-related reorganization of flavivirus glycoproteins. *J Structural Biol* 2014; 185: 27–31.
- Purdy DE, Chang GJJ. Secretion of noninfectious dengue virus-like particles and identification of
5 amino acids in the stem region involved in intracellular retention of envelope protein. *Virology* 2005; 333:239-50.
- Richter MK, da Silva Voorham JM, Torres Pedraza S, Hoornweg TE, van de Pol DP, Rodenhuis-
Zybert IA, et al. Immature dengue virus is infectious in human immature dendritic cells via
10 interaction with the receptor molecule DC-SIGN. *PLoS One* 2014; 9: e98785.
- Rodenhuis-Zybert IA, van der Schaar HM, da Silva Voorham JM, van der Ende-Metselaar H, Lei
HY, Wilschut J, et al. Immature dengue virus: a veiled pathogen? *PLoS Pathog* 2010; 6: e1000718.
- 15 Rouvinski A, Guardado-Calvo P, Barba-Spaeth G, Duquerroy S, Vaney MC, Kikuti C, et al.
Recognition determinants of broadly neutralizing human antibodies against dengue viruses.
Nature 2015; 520: 109-13.
- Sabchareon A, Wallace D, Sirivichayakul C, Limkittikul K, Chanthavanich P, Suvannadabba S,
20 et al. Protective efficacy of the recombinant, live-attenuated, CYD tetravalent dengue vaccine in
Thai schoolchildren: a randomised, controlled phase 2b trial. *Lancet* 2012; 380: 1559–67.
- Suphatrakul A, Yasanga T, Keelapang P, Sriburi R, Roytrakul T, Pulmanusahakul R, et al.
Generation and preclinical immunogenicity study of dengue type 2 virus-like particles derived
25 from stably transfected mosquito cells. *Vaccine* 2015; 33: 5613-22.
- Shang W, Liu J, Yang J, Hu Z, Rao X. Dengue virus-like particles: construction and application.
Appl Microbiol Biotechnol 2012; 94: 36-46.
- 30 Smith SA, Nivarthi UK, de Alwis R, Kose N, Sapparapu G, Bombardi R, et al. Dengue virus prM-
specific human monoclonal antibodies with virus replication enhancing properties recognize a
single immunodominant antigenic site. *J Virol* 2016; 90: 780–9.
- Song KY, Zhao H, Li SH, Li XF, Deng YQ, Wang HJ, et al. Identification and characterization of
35 a linearized B-cell epitope on the pr protein of dengue virus. *J Gen Virol* 2013; 94: 1510–6.

- Trainor NB, Crill WD, Roberson JA, Chang GJ. Mutation analysis of the fusion domain region of St. Louis encephalitis virus envelope protein. *Virology* 2007; 360: 398-406.
- 5 Urakami A, Ngwe Tun MM, Moi ML, Sakurai A, Ishikawa M, Kuno S, et al. An envelope-modified tetravalent dengue virus-like-particle vaccine has implications for flavivirus vaccine design. *J Virol* 2017; 91: e01181-17. doi:10.1128/JVI.01181-17.
- Villar L, Dayan GH, Arredondo-Garcia JL, Rivera DM, Cunha R, Deseda C, et al. Efficacy of a tetravalent dengue vaccine in children in Latin America. *N Engl J Med* 2015; 372: 113-23.
- 10 Wang Z, Li L, Pennington JG, Sheng J, Yap ML, Plevka P, et al. Obstruction of dengue virus maturation by fab fragments of the 2H2 antibody. *J Virol* 2013; 87: 8909-15.
- Yamaji H. Suitability and perspectives on using recombinant insect cells for the production of virus-like particles. *Appl Microbiol Biotechnol* 2014; 98: 1963-70.
- 15 Yu IM, Zhang W, Holdaway HA, Li L, Kostyuchenko VA, Chipman PR, et al. Structure of the immature dengue virus at low pH primes proteolytic maturation. *Science* 2008; 319: 1834-7.
- Zhang Y, Corver J, Chipman PR, Zhang W, Plevnev SV, Sedlak D, et al. Structures of immature flavivirus particles. *EMBO J* 2003; 22: 2604-13.
- 20 Zhang X, Ge P, Yu X, Brannan JM, Bi G, Zhang Q, et al. Cryo-EM structure of the mature dengue virus at 3.5-Å resolution. *Nat Struct Mol Biol* 2013; 20: 105-10.
- 25 Zheng A, Umashankar M, Kielian M. In vitro and in vivo studies identify important features of dengue virus pr-E protein interactions. *PLoS Pathogens* 2010; 6: e1001157.
- Zheng A, Yuan F, Kleinfelter LM, Kielian M. A toggle switch controls the low pH-triggered rearrangement and maturation of the dengue virus envelope proteins. *Nat Comm* 2014; 5: 3877.
- 30

Claims

1. A virus-like particle capable of eliciting immune response in a mammal comprising a modified form of M and E structural proteins of flavivirus,
wherein the modified form comprises an amino acids sequence corresponding to an M and E sequence setting forth in SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3 or SEQ ID NO. 4,
wherein conserved amino acid His at positions M7, E261, E282, and E317 of dengue virus sequence, are replaced by amino acid Ala at positions 7, 336, 357 and 392 in SEQ ID NO. 1-3, or conserved amino acid His at positions M7, E259, E280 and E315 of dengue virus sequence are replaced by amino acid Ala at positions 7, 334, 355 and 390 in SEQ ID NO. 4.
2. The virus-like particle of claim 1, wherein the modified form further comprises replacement of conserved amino acid His at positions E27, E149 and E209 of dengue virus sequence with amino acid Asn at positions 102, 224 and 284 in SEQ ID NO. 1-3 or replacement of conserved amino acid His at positions E27, E149 and E207 of dengue virus sequence with amino acid Asn at positions 102, 224 and 282 in SEQ ID NO. 4.
3. The virus-like particle of claim 1, wherein the modified form further comprises any one or combination of replacement of amino acids Ser or Glu at position E186 of dengue virus sequence or amino acid Ser at position E184 of dengue virus sequence with amino acid Phe at position 261 in SEQ ID NO. 1-3 or at position 259 in SEQ ID NO. 4, respectively, or replacement of amino acid Arg at position E188 of dengue virus sequence or position E186 of dengue virus sequence with amino acid Leu at position 263 in SEQ ID NO. 1-3 or at position 261 in SEQ ID NO. 4, respectively, or replacement of amino acid Asn or Val at position E242 of dengue virus sequence or amino acid Asn at position E240 of dengue virus sequence with amino acid Ser at position 317 in SEQ ID NO. 2-3 or position 315 in SEQ ID NO. 4, or replacement of amino acid Arg or Lys at position E323 of dengue virus sequence or amino acid Lys at position E321 of dengue virus sequence with amino acid Gln at position 398 of SEQ ID NO. 2-3 or position 396 of SEQ ID NO. 4.
4. An isolated polynucleotide encoding a virus-like particle or polypeptide capable of eliciting immune response in a subject upon administering the virus-like particle or polypeptide to the subject at a pharmaceutically effective dosage, the virus-like particle or polypeptide

comprising a modified form of M and E structural proteins of flavivirus, wherein a nucleotide sequence of the modified form is corresponding to a sequence setting forth in SEQ ID NO. 5, SEQ ID NO. 6, SEQ ID NO. 7 or SEQ ID NO. 8, or an amino acid sequence of the modified form is corresponding to a sequence setting forth in SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3 or SEQ ID NO. 4, wherein conserved amino acid His at positions M7, E261, E282 and E317 of dengue virus sequence are replaced with amino acid Ala at positions 7, 336, 357, and 392 in SEQ ID NO. 1-3 or conserved amino acid His at positions M7, E259, E280 and E315 of dengue virus sequence are replaced with amino acid Ala at positions 7, 334, 355 and 390 in SEQ ID NO. 4.

5. The isolated polynucleotide of claim 4, wherein the modified form further comprises replacement of conserved amino acid His at positions E27, E149 and E209 of dengue virus sequence with amino acid Asn at positions 102, 224 and 284 in SEQ ID NO. 1-3 or replacement of conserved amino acid His at positions E27, E149 and E207 of dengue virus sequence with amino acid Asn at positions 102, 224 and 282 in SEQ ID NO. 4.
6. The isolated polynucleotide of claim 4, wherein the modified form further comprises any one or combination of replacement of amino acid Ser or Glu at position E186 of dengue virus sequence or amino acid Ser at position E184 of dengue virus sequence with amino acid Phe at position 261 of SEQ ID NO. 1-3 or at position 259 of SEQ ID NO. 4, respectively, or replacement of amino acid Arg at position E188 of dengue virus sequence or position E186 of dengue virus sequence with amino acid Leu at position 263 in SEQ ID NO. 1-3, or at position 261 in SEQ ID NO. 4, respectively, or replacement of amino acid Asn or Val at position E242 of dengue virus sequence or amino acid Asn at position E240 of dengue virus sequence with amino acid Ser at position 317 in SEQ ID NO. 2 -3 or position 315 in SEQ ID NO. 4, or replacement of amino acid Arg or Lys at position E323 of dengue virus sequence or amino acid Lys at position E321 of dengue virus sequence with amino acid Gln at position 398 of SEQ ID NO. 2-3 or position 396 of SEQ ID NO. 4.
7. A vaccine composition comprising: pharmaceutically acceptable adjuvant; and polypeptides or virus-like particles, which are immunologically active upon administering to a subject, having an amino acids sequence corresponding to a sequence setting forth in SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3 or SEQ ID NO. 4, wherein the amino acids sequence comprises a modified form of dengue virus sequence, wherein conserved amino

acid His at positions M7, E261, E282, and E317 of dengue virus sequence, are replaced by amino acid Ala at positions 7, 336, 357 and 392 in SEQ ID NO. 1-3, or conserved amino acid His at positions M7, E259, E280 and E315 of dengue virus sequence are replaced by amino acid Ala at positions 7, 334, 355 and 390 in SEQ ID NO. 4.

8. The vaccine composition of claim 7, wherein the modification further comprises replacement of conserved amino acid His at positions E27, E149 and E209 of dengue virus sequence with amino acid Asn at positions 102, 224 and 284 in SEQ ID NO. 1-3 or replacement of conserved amino acid His at positions E27, E149 and E207 of dengue virus sequence with amino acid Asn at positions 102, 224 and 282 in SEQ ID NO. 4.
9. The vaccine composition of claim 7, wherein the modifications further comprises any one or combination of replacement of amino acids Ser or Glu at position E186 of dengue virus sequence or amino acid Ser at position E184 of dengue virus sequence with amino acid Phe at position 261 in SEQ ID NO. 1-3 or at position 259 in SEQ ID NO. 4, respectively, or replacement of amino acid Arg at position E188 of dengue virus sequence or position E186 of dengue virus sequence with amino acid Leu at position 263 in SEQ ID NO. 1-3 or at position 261 in SEQ ID NO. 4, respectively, or replacement of amino acid Asn or Val at position E242 of dengue virus sequence or amino acid Asn at position E240 of dengue virus sequence with amino acid Ser at position 317 in SEQ ID NO. 2-3 or position 315 in SEQ ID NO. 4, or replacement of amino acid Arg or Lys at position E323 of dengue virus sequence or amino acid Lys position E321 of dengue virus sequence with amino acid Gln at position 398 of SEQ ID NO. 2-3 or position 396 of SEQ ID NO. 4.
10. A vaccine composition comprising pharmaceutically acceptable adjuvant; and a nucleotide sequence setting forth in SEQ ID NO. 5, SEQ ID NO. 6, SEQ ID NO. 7 or SEQ ID NO. 8 respectively corresponding to amino acids sequence SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3 and SEQ ID NO. 4.

ABSTRACT

The present disclosure associates to a modified and expressed virus-like particles. Particularly, the virus-like particle is capable of eliciting immune response in a mammal upon administering a pharmaceutically efficient dosage to the mammal. The virus-like particle comprises a modified form of M and E structural proteins of flavivirus. Further, the virus-like particle comprises an amino acids sequence substantially corresponding to a sequence setting forth in SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3 or SEQ ID NO. 4, wherein conserved and internally located His at multiple positions of the M and E proteins are substituted with uncharged residues, and other secretion-enhancing substitutions are introduced.