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Fig. 2 Immunofluorescence staining of C6/36 cells. Intracellular E protein in C6/36 cells was detected by using an anti-E antibody, 1D10, and Cy3-conjugated goat anti-mouse IgG antibody. Nuclei were stained with DAPI. C6/36 cells infected with a DENV-2 strain 16681 and uninfected C6/36 cells were employed as the positive control and negative control, respectively. DIC, differential interfereence contrast.

Detection of E protein in the particulate fraction following ultracentrifugation

The culture media of several stably expressing clones were pooled, concentrated and subjected to ultracentrifugation employing two sets of sucrose density gradients. Following the first centrifugation using 20 g% and 55 g% step sucrose gradient, the E protein was detected by dot blot immunoassay to be present in the 20 - 55g% sucrose interface as well as in the 20 g% sucrose fraction and the culture media fraction (data not shown). Materials from the 20 g% and 55 g% sucrose interface were subjected to a rate zonal separation using 5-25 g% sucrose gradient, and all fractions were dotted on nitrocellulose membrane for the detection of E protein by dot immunoassay
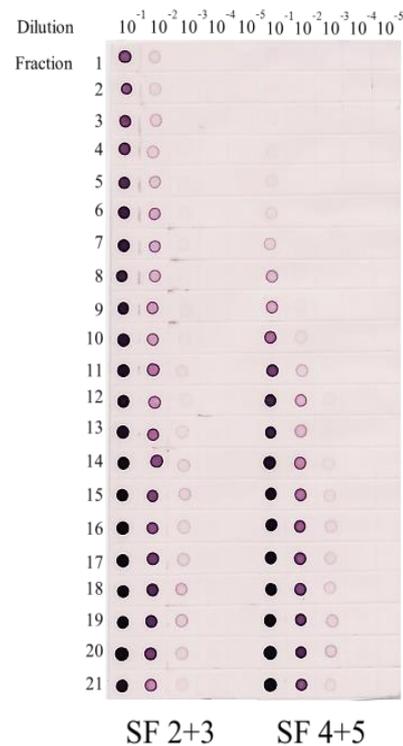

Fig. 3 Dot blot immunoassay. The fractions 2+3 and 4+5 from the step sucrose gradient were subjected to rate zonal separation using 5-25 g% sucrose gradient and the fractions 1-21 dotted at several dilutions onto nitrocellulose membrane. The E protein was detected by an anti-E monoclonal antibody, 1D10, and alkaline phosphatase-conjugated goat anti-mouse IgG antibody.

employing the monoclonal antibody 1D10. As shown in Fig. 3, the E protein was detected in all fractions with relatively higher concentration in the fractions 13-19 from the bottom of the tube. The migration of the E protein in this rate zonal separation was similar to that of the subviral particles of DENV-2 (Junjhon et al., 2008), suggesting that the chimeric E protein is present in the form of subviral particle-like materials.
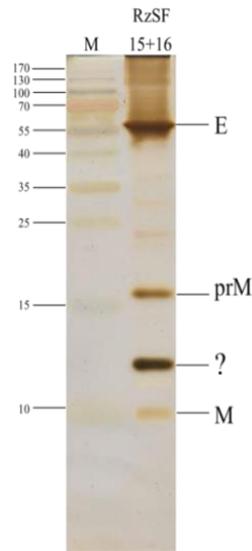
Fig. 4 SDS-PAGE and silver staining. The rate zonal sucrose fractions 15-16 were separated by electrophoresis in a 0.1% SDS, 5-15% polyacrylamide gel in non-reducing condition and stained with silver nitrate. Sizes (in kD) of the protein marker bands are indicated.

Chimeric E proteins assembled into spherical particles

The protein component of the rate zonal fractions 15-16 was examined by electrophoresis in the 5-15% polyacrylamide gel and staining with silver nitrate. Three major bands that were detected with the size of approximately 50, 20 and 10 kDa (Fig. 4) correspond to the expected size of the three viral proteins E (54 kDa), prM (19 kDa) and M (8 kDa) (Chang, 1997). An additional band of about 12 kDa may represent an unknown host/serum protein or an M dimer. The extent of prM cleavage of the prM protein in this preparation as calculated from the adjusted prM and M band density was 41%. This prM cleavage is higher than that of DENV-1 (Keelapang et al., 2013) but less than that of the DENV-2 VLPs employed as the backbone (Suphatrakul et al., 2015).
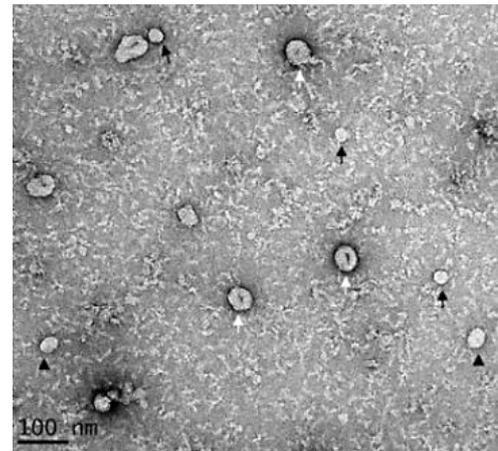

Fig. 5 Electron microscopy visualization of E protein-containing fraction. Symbols indicate particle of different sizes: small (black arrow), medium (black triangle) and large (white arrow).

This result indicates that the substituted EDIII domain derived from DENV-1 down modulated the cleavage of prM. Whether these proteins were able to assemble into particles was next examined by negative staining and transmission electron microscopy.

Electron microscopy study of the fractions 15+16 revealed spherical particles of various sizes (Fig. 5), the majority of which is distributed into two class sizes: small (26-30 nm, 48.8%) and medium (35-38 nm, 13.2 %) (Fig. 6). In addition, there were larger particles of 42-60 nm that did not cluster into distinctive peaks. Small particles of 26-30 nm were similar to DENV-2 native subviral particles (Junjhon et al., 2008) and DENV-2-JE chimeric virus-like particles with minimal prM cleavage (Charoensri et al, 2014; Suphatrakul et al., 2015). The medium size particles may represent an artifact resulting from the contraction of the larger particles during negative staining as these particles are devoid of the capsid
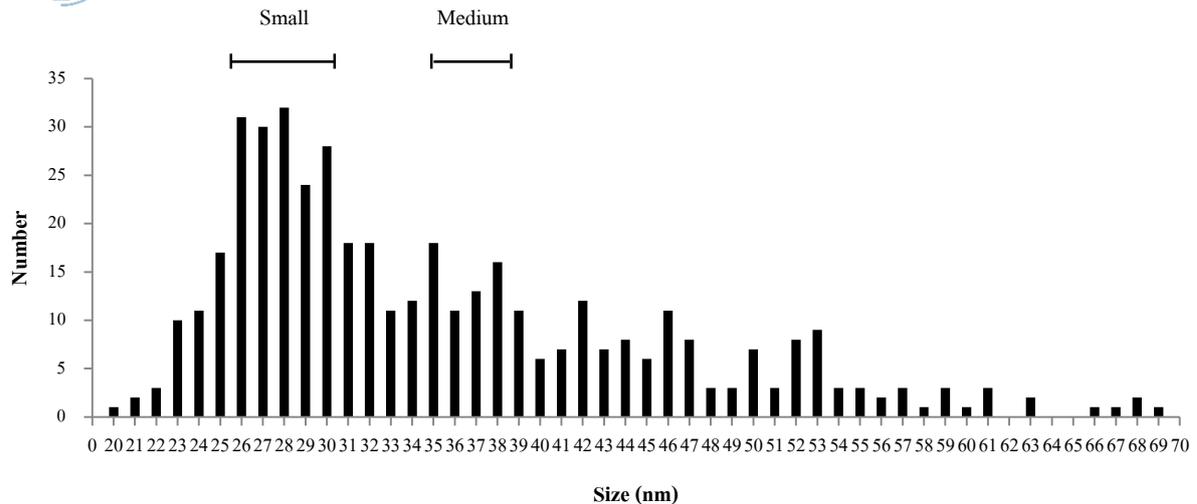
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Fig. 6 Frequency distribution of particles according to size (*n*=441). Particles were separated into classes of 1 nm increments based on the mean diameter and the frequency at each increment plotted against the diameter. Bars indicate the peak frequency of the three size classes.

protein, or a distinct class of particles. Similar broad distribution of virus-like particles had been described previously with tick born encephalitis virus (Ferlenghi et al., 2001). Taken together, these results indicate the assembly of virus-like particles of various sizes and subsequent release of the particles from transfected C6/36 cells.

Acknowledgements

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