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COMMUNICATION

Viral Nanoparticles as Antigen Carrier: Influence of Shape on Humoral Immune Responses *in Vivo*

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Viral nanoparticles (VNPs) can serve as effective carriers for small molecular haptens with improved humoral immune responses in mice, which is dependent on their shapes. We observed that rod-shaped VNPs elicited higher antibody titers with high specificity compared to spherical VNPs.

Developing safer and more effective vaccines is crucial to maintaining public health[1]. Traditionally, therapeutic peptides or other small-molecular haptens were often attached to a carrier with strong immunogenicity, such as keyhole limpet hemocyanine (KLH)[2], to improve the immune response[3]. However, these protein carriers cannot control the display pattern of haptens, an essential feature to crosslink B cell receptors and consequently promote B cell maturation[4, 5]. Meanwhile, KLH always induced undesired immune response to itself[6] which might cause the exclusion of vaccine[7] and suppression of antibodies that are specific to target antigens[8, 9]. As a result, KLH and similar proteins often failed to elicit strong immune responses, especially for many antigens that have small size and weak immunogenicity[10].

Recently, viral nanoparticles (VNPs) have been employed as powerful platforms for presenting haptens[11]. VNPs, which are composed of subunit proteins that self-assemble into capsid with a highly ordered manner and encapsulate genomic materials inside the capsid[12, 13], were shown to be highly immunogenic[14]. With the well-defined structural feature, VNPs have been used as carriers to present antigens through genetic and chemical modifications[15, 16]. Multivalent antigens displayed on the surface of VNP at high density and in highly repetitive arrays[4, 17], were available to stimulate immune response by crosslinking B cell receptors and activating B cells responses consequently[18]. In particular, plant viruses, which generally are non-infectious to human[19, 20], are becoming more attractive platforms for vaccine development[21, 22]. For example, through genetically modification, epitopes from murine hepatitis virus and foot-and-mouth disease virus have been successfully displayed on tobacco mosaic virus (TMV)[23, 24]. Cowpea mosaic virus (CPMV) has been used to display epitopes from Mink enteritis virus and Canine parvovirus[25, 26]. Both TMV and CPMV have been extensively studied for bacterial vaccine by displaying the outer membrane protein F of Pseudomonas aeruginosa[27, 28]. In addition, plant viruses have also been employed to display carbohydrate antigens with weak immunogenicity via chemical linkages[29]. Several groups have reported that a tumor associated carbohydrate antigen termed as Tn antigen could be attached to the exterior surfaces of TMV and CPMV through chemical conjugation and the resulting conjugates could elicit a high-titer antibody response which could recognize Tn antigens presented on the cancer cell surface[30-32].

On the other hand, it has been shown that the shape of nanoparticles plays an important role in their biobehaviors including the body clearance[33], tissue distribution[34] and cellular uptake[35]. It has been hypothesized that the morphology of nanoparticles could influence the transport to lymphoid organ and uptake by antigen presenting cells (APCs), thereby affect the quality and quantity of the *in vivo* immune response [36]. For VNP platforms, they normally share two basic structures with either rod-shape as TMV and potato virus X, or spherical shape as CPMV and bacteriophage Qβ[15]. To test the influence of VNP shapes on immunoresponses, a lysine mutant of TMV (TMV-EPMK) with 300 nm long and 18 nm in diameter which contains a functional lysine residue inserted between Met 1 and Ser 2 at *N*-terminus of its coat protein[37] was used as the rod-shaped structure (**Figure 1A**). Wild-type CPMV, a ~ 29 nm spherical VNP was used as the comparison **(Figure 1B)**. In this work, a small molecule, estriol (E3), was used as a model antigen (hapten) due to its low immunogenicity, while TMV-EPMK and CPMV were chemically attached to E3 through an amidation reaction **(Figure 1C)**[38, 39]. The conjugates were injected into mice to examine how the immune response could be affected by the shape of VNP carriers.

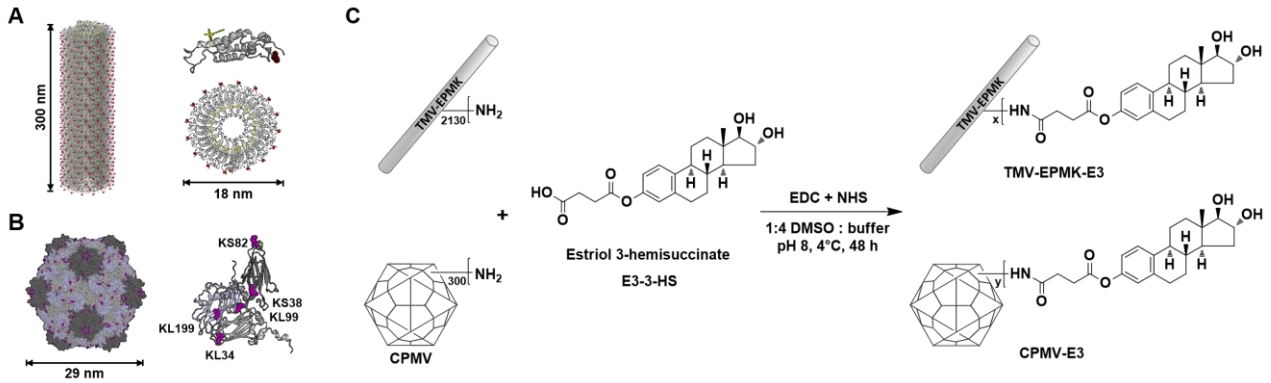

Figure 1 (A) Structures of TMV-EPMK (left) and its cross section (bottom right), coat protein (upper right) with lysine insertion marked in red. (B) Structures of CPMV (left) and its subunits (right) with reactive lysine on the exterior surface marked in purple. Models were generated using PyMol (www.pymol.org) with coordinates obtained from the RCSB Protein Data Bank (www.pdb.org). (C) Synthesis of TMV-EPMK-E3 and CPMV-E3 conjugates.

As shown in **Figure 1C**, small molecules E3-3-HS bearing carboxylic groups were attached to the exterior surface of VNPs through activation with EDC and NHS. The resulting conjugates TMV-EPMK-E3 and CPMV-E3 were purified by ultracentrifugation and analyzed by transmission electron microscopy (TEM) to confirm that the viral particles still kept intact structure upon E3 conjugation (**Figure 2A, B**). The covalent conjugation of E3 on the surface of VNPs was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis (**Figure 2C**). Based on SDS-PAGE analysis, we estimated ~50% modification efficiency of TMV-EPMK-E3, which was consistent to the reported modification of TMV-EPMK with fluorescein[38]. The coat protein dimer of TMV-EPMK-E3 probably resulted from the crosslinking of neighbor coat proteins by inserted Lysine and Glutamic acid. About two E3 haptens were attached to per CPMV subunit, which was also consistent with the literature report[39]. In addition, zeta potential measurement indicated that E3 modified viruses have increased negative zeta potential values, i.e. -37.6 ± 1.3 mV for TMV-EPMK-E3 conjugates and -35.3 ± 0.6 mV for CPMV-E3 conjugates, in comparison with -27 ± 0.5 and 20.8 ± 1.3 mV of unmodified viruses respectively (**Figure 2D**). It can be attributed to the derivatization of the positively charged lysine residues on the exterior surface of viruses by E3. Ion exchange FPLC showed that the conjugation of E3 on viruses enhanced the interaction with an anion exchange column due to the increased negative surface charge (**Figure 2E, F**), which indicated the property of virus was changed by the substitution of E3 on virus.

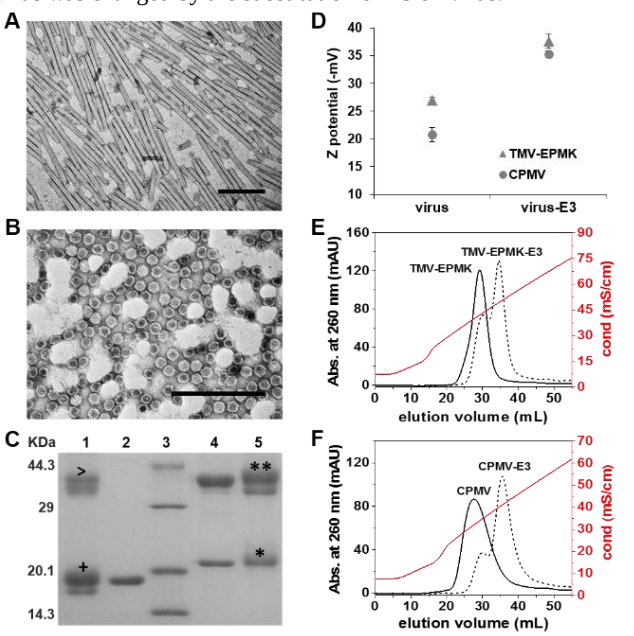

Figure 2. TEM images of (A) TMV-EPMK-E3 and (B) CPMV-E3. Scale bars indicate 200 nm. (C) SDS-PAGE analysis of TMV-EPMK-E3 (lane 1), TMV-EPMK (lane 2), protein markers (lane 3), CPMV (lane 4) and CPMV-E3 (lane 5). Coat protein of TMV-EPMK-E3 (+) and large subunit (**) and small subunit (*) of CPMV-E3 showing mass shift with E3 conjugation. ">" indicates TMV coat protein dimer. (D) Zeta potential of TMV-EPMK-E3 and CPMV-E3. Anion exchange FPLC analysis of (E) TMV-EPMK and TMV-EPMK-E3, (F) CPMV and CPMV-E3. Absorbance was monitored at 260 nm for (E) and (F).

To test the influence of VNPs shape on the antibody production *in vivo*, groups of three BALB/c male mice were immunized subcutaneously with 0.05 mg either TMV-EPMK-E3 or CPMV-E3 conjugates in complete Freund's adjuvant[40] at day 0. Booster immunizations were performed subcutaneously on days 14 and 28 with the same amount in incomplete Freund's adjuvant emulsion. Bleed were collected from mice on days 38 and sera were isolated, in which the specific E3 antibody levels were analyzed by enzyme-linked immunosorbent assay (ELISA). Results showed that both conjugates elicited specific E3 antibody in mice. However, the anti-E3 antibody from TMV-EPMK-E3 immunized mice could be detected at much higher dilution than that from CPMV-E3 immunized mice (**Figure 3A**). The antibody titer from TMV-EPMK-E3 immunized mice was as high as 130,000, whereas CPMV immunized mice showed 11.4-fold lower titer with 11,400 only (**Figure 3B**). The higher titer obtained with TMV-EPMK-E3 highlighted the advantage of rod-shaped TMV which could elicit a stronger antibody response in mice compared to spherical CPMV.

In order to determine antibody specificity of interaction with E3 antigen and viral nanoparticle platform, we performed cell fusion to produce hybridomas which could secrete monoclonal antibody (mAb)[41]. The mAbs were purified and then measured by ELISA coating with TMV-EPMK, CPMV, TMV-EPMK-E3 or CPMV-E3, respectively. Results showed that mAbs produced by TMV-EPMK-E3 immunized mice could specifically recognize E3 antigen displayed on both TMV-EPMK and CPMV, without interaction with TMV-EPMK carrier (**Figure 3C**). Whereas, CPMV-E3 failed to elicit the specific mAbs against E3 exclusively, which showed strong affinity with E3 as well as CPMV carrier (**Figure 3D**).

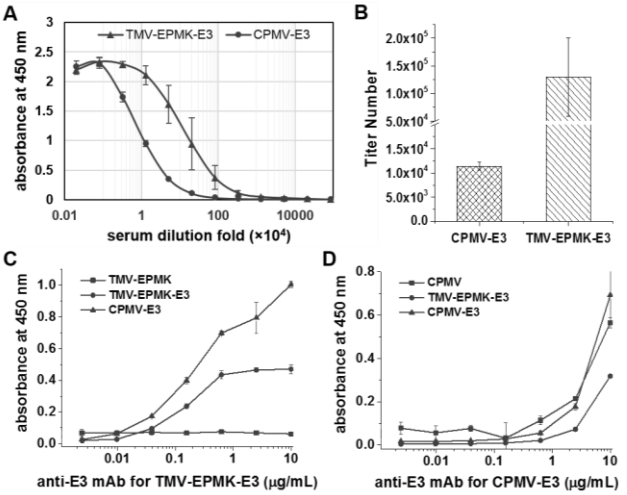

Figure 3. (A) ELISA of the specific antisera from mice immunized with TMV-EPMK-E3 and CPMV-E3. The microplate was coated with BSA-E3 conjugate. (B) ELISA E3 antibody titers. (C) Antibody specificity elicited by TMV-EPMK-E3. (D) Antibody specificity elicited by CPMV-E3. Inset indicates the microplate coating.

Although we hypothesized that the shape of carriers could have great influences on the antibody titer and specificity, to conclude that the immune response depended on the one sole parameter appeared difficult because other different physicochemical parameters, such as protein sequence, surface charge, surface area and volume, etc., might also play an important role. Firstly, TMV-EPMK and CPMV are made up of different protein sequences which may induce difference in immune response. Several groups have reported that both CPMV and TMV had the helper T cell epitopes and could serve as carriers to elicit immune response *in vivo*[30, 32], which implied that the different protein sequence might have limited influence on the resulted immunogenicity. Clearly the difference of shape and size between TMV-EPMK and CPMV lead to big difference in their surface area and volume. As reported, the specific surface area, which was total surface area

per single nanoparticle volume had an inversely proportional relationship to antibody production[42]. The specific surface areas of both VNPs were similar and estimated to be very close to 0.2 (**Table S1**).

It was believed that the clearance and physiological tissue distribution of injected vaccines would affect the immune response that it elicits[36]. Manchester and co-workers reported that spherical CPMV were cleared rapidly from plasma in mice and the majority of the injected CPMV were trapped in the liver but only fewer in spleen by 30 min[43]. Interestingly, cowpea chlorotic mottle virus (CCMV) and heat shock protein (Hsp) which possess the spherical structure as CPMV, also behaved similarly like CPMV *in vivo*[44]. However, the rod-shaped TMV, reported by Huang and co-workers, accumulated more in spleen than in liver with a quick clearance from bloodstream within 40 min and no overt toxicity[45]. Steinmetz and co-workers also reported that, the filamentous Potato virus X showed higher location in spleen than in liver compared to spherical CPMV[46]. These reports demonstrated that rod-shaped or filamentous VNPs with high aspect ratio accumulated predominantly in spleen, while the spherical particles located more in liver. Meanwhile, even with the same proteins and comparable surface charge, spherical TMV-SNPs which generated from thermal denaturation of TMV rods showed faster clearance from tissue than rod-shaped TMV[47]. In this case, the accumulation of rod-shaped TMV nanoparticles in spleen and slower clearance from tissue would increase the opportunity of uptake by macrophage and presenting of antigen to immune cells[48].

Furthermore, the rod-shaped TMV particle has a high aspect ratio and a relatively flat surface along long axis, which could display antigens in a highly organized 2D pattern with the same epitope pointing to the same orientation. As comparison, spherical CPMV always displays antigens toward different 3D orientations. Moreover, since cells are almost 10 times larger than TMV and CPMV, the surface area of rod-shaped TMV along long axis that contact to cell surface is larger than that of spherical CPMV. When interacted with immune cells, antigens presented on TMV with the higher ordered 2D pattern would activate B cells much more effectively and specifically. Finally, the efficient interaction with macrophage and effective activation of B cells enhanced the immune response powerfully and elicited higher antibody titer with specific recognition to antigen, which explained why rod-shaped TMV could induce a higher specific E3 antibody titer.

In summary, we demonstrated that plant VNPs were promising platform to improve the immunogenicity of small molecule antigen. Moreover, the shape of viral nanoparticle had obvious influence on the antibody titer and specificity. Our data suggested that rod-shaped TMV was more effective in eliciting higher antibody titers in mice compared to spherical CPMV. The monoclonal antibodies induced by rod-shaped TMV are more specific for small molecular antigens. However, further studies are still needed to test different haptens (antigens) with different structural features. In addition, for practical applications, a better mechanistic investigation is necessary to evaluate how the shape of a nanoparticle-based antigen carrier will influence the *in vivo* immunoresponses.

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Notes and references

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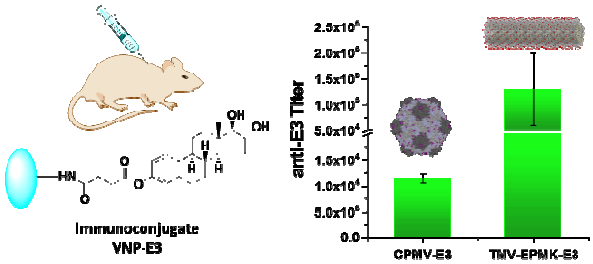

Rod-shaped viral nanoparticles sever as effective carriers for small molecular haptens with improved humoral immune responses *in vivo*.