Hepatitis E virus truncated capsid protein as a potential carrier of exogenous antigens for the development of chimeric virus-like particle vaccines

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Abstract

Background: Virus like particle (VLP), a multiprotein structure which is assembled automatically, can stimulate robust immune responses due to an appropriate size, repetitive epitopes, and a structure similar to native virions. Utilizing VLPs as vaccine carriers to present exogenous antigens is a promising and challenging field in vaccine design. Hence, this study aims to investigate the potential of Hepatitis E virus (HEV) truncated capsid protein as a VLP carrier presenting foreign antigens in vaccine design.

Results: S and M domains of HEV ORF2 protein (aa112-455) were selected as an optimal carrier (CaSM). The exogenous antigen Seq8 containing three immunogenic domains from three different foot-and-mouth disease virus (FMDV) strains was linked to the C-terminal of CaSM to construct a chimeric VLP vaccine candidate (CaSM-Seq8). Morphological analysis showed that CaSM-Seq8 self-assembled into VLPs with a diameter of approximately 26 nm, similar to the VLPs of CaSM alone but smaller than native HEV virions. Further, the thermal stability and the proteolysis resistance of Seq8 were enhanced when carried by CaSM. The antigenicity analysis revealed a more robust reactivity against anti-FMDV specific antibodies when Seq8 was presented on the CaSM particles. Upon injection into mice, anti-FMDV IgGs induced by CaSM-Seq8 appeared earlier, increased faster, and maintained higher levels for a longer time than those induced by Seq8 antigen alone or a commercial inactivated FMDV vaccine.

Conclusions: This study demonstrates the potential of the HEV truncated capsid protein VLPs as a presenting-platform of exogenous antigens in vaccine design and promising preliminary results on chimeric VLP vaccine against foot-and-mouth disease.

Background

HEV is an icosahedral, spherical particles, with a diameter of 27-32nm, belonging to alphavirus subgroup [1]. Its genome is a single-stranded RNA composed of approximately 7.2kb, including three ORFs, a 5’-methylguanine cap and a 3’ poly(A) stretch [2]. ORF1 encodes the non-structural proteins (pORF1) which are cleaved from a polyprotein; the ORF2 encodes the capsid protein (pORF2) which includes the majority of the neutralizing epitopes and seems to be the best candidate for the development of recombinant protein vaccines and diagnostic tests [3]; the ORF3 protein encodes a multi-functional protein (pORF3), involved in virion egress, formation of quasi-enveloped virions, and regulation of signaling pathways [2, 3].

The HEV capsid protein contains three domains, namely S domain (aa129-319), M domain (aa320-455), and P domain (aa456-606), all of which play distinct structural roles [4]: The S and M domains could form an internal scaffold shell ending by a long proline-rich hinge which provides flexible mobility for the P domain that contains the dominant neutralizing epitopes and the key motifs for the interaction with the cellular receptors; such structure therefore, allows an optimal presentation of the this functional P domain as a protruding spike from the surface of the particle formed by the S and M domains (Fig. 1A-D) [4, 5].

Previously, it has been demonstrated that a truncated protein of pORF2 (aa112-660) expressed using a baculovirus system, could form VLPs similar to the native HEV virion [6]. Further, the HEV capsid protein could self-assemble even with N-terminal truncations up to aa125 and a C-terminal truncation up to aa602, indicating that the essential elements for the self-assembly the HEV capsid protein are located between 125-602 [7]. Moreover, other key structural features of the HEV ORF2 protein have been established: (i) the N-terminal region (aa1-112) plays a key role in the assembly of p595 (aa112-608) into T=3 VLPs and this region is also involved in the packaging of the viral genome[4, 8];(ii) the S domain is indispensable for the formation of T=1 VLPs[8]; (iii) an N-terminal truncation up to aa367 is the largest truncation that maintained the self-assembly ability as it has been shown with the recombinant vaccine p239, while the E2 (aa394-606) and E2S (aa459-606) proteins could only form hexamers and dimers, respectively [8, 9].

A virus-like particle, is a vacant multiprotein structure and doesn't contain virial genome, which is formed by the self-assembly of several copies of a given protein expressed in a given expression system [10, 11]. VLPs can stimulate robust humoral and cellular immunity responses via several mechanisms, including mimicking the structure and conformation of the native virus, possessing repetitive elements, and larger dimensions [12-14]. Given these unique characteristics, VLPs became interesting in vaccine design and development, employed as platforms presenting exogenous antigens [13, 15] and as carriers to deliver nucleic acids, drugs, and proteins [16].

Given the above mentioned features of the HEV ORF2 particles and the importance of the VLPs as carriers and antigen-presenting platforms, in this study we looked into the potential of the ORF2 VLPs as a platform for presenting exogenous antigens by maintaining the S and M domains that would form the core of the particle and substituting the protruding P domain by and exogenous chimeric antigen (Fig. 1E, F). Previously, several chimeric HEV VLPs were designed by inserting foreign antigens at different positions: inserting a 18 amino acids peptide of HIV-1 gp120 into HEV between aa485 and aa486 [15]; inserting a myc-tag/FLAG-tag/HA-tag between aa488 and aa489 [17]; adding a B cell epitope tag consisting 11 amino acids to the C-terminal [18]; linking hepatitis A virus VP1 (aa 24–171) to the C-terminal of p431-615 [19]; fusing HEV ORF3 p70-123 to ORF2 p112-608 [20]. However, in all these studies the inserted fragments were usually either short (only linear epitopes) or attached to the C-terminal of the P domain which would limit their flexibility and their full exposure [21].

Herein, we investigated an antigen-carrier VLPs model "S domain-M domain-exogenous antigens" (CaSM-EAg), exploiting the structural characteristics of the HEV capsid, and using a chimeric protein (Seq8) as a foreign antigen. This latter has been designed according to previous reports [22-25], and contains three different neutralization epitopes of three different foot-and-mouth virus (FMDV) strains. Using molecular cloning technology, the chimeric VLPs were (CaSM-Seq8) were produced in Escherichia coli, purified, and their structural characteristic as well as their physical properties, antigenicity, and immunogenicity were analyzed.

Results

- **Design and Construction of HEV ORF2 and Seq8 recombinant proteins**

The target genes (*NcoI*-CaSM-*XhoI*, *NcoI*-CaSM-Seq8-*XhoI,* and *NcoI*-Seq8-*XhoI*) were successfully amplified (Fig. 2A) and inserted into the pET28a vector. Then, the expression constructs were used to transform competent E. coli BL21 cells. After the selection of the target clones by endonuclease digestion and DNA sequencing, the protein expression was induced. The results revealed that CaSM, Seq8, and CaSM-Seq8 were highly over-expressed and the soluble fractions were successfully purified (Fig. 2B).

- **Assessment of thermal stability and proteolysis resistance**

Physical properties, like thermal stability and resistance to proteolysis, are important in vaccine design. Therefore, we sought to study whether these properties would be optimized when the Seq8 antigen is presented on CaSM particles. According to the thermal stability results (Fig. 2C), CaSM was stable at 4℃ and 37℃, and only slight degradation occurred after 5 and 7 days of storage at 37℃; meanwhile, CaSM-Seq8 was also very stable at both 4℃ and 37℃. By contrast, the Seq8 antigen alone was extremely thermo-labile with a complete degradation after 1 day at 37℃ and an obvious gradual degradation at 4℃ from day 1 to day 7 post-incubation. This indicates that indeed the CaSM could enhance the thermal stability of the presented foreign antigen.

Next, we studied whether the carrier CaSM would facilitate exogenous antigens to become resistant to digestion (pepsin and trypsin). As a result (Fig. 2D), CaSM and CaSM-Seq8 were resistant to pepsin but vulnerable to trypsin; however Seq8 was vulnerable to both pepsin and trypsin and was digested totally. This result indicated that our carrier CaSM could enhance the resistance of presented antigens to pepsin. Therefore, our carrier CaSM would have potential application in the oral vaccine development.

- **Computational analysis of the self-assembly of the expressed proteins**

According to the predictions of GalaxyWeb Homomer server [26], CaSM had the ability to form pentamers which in turn assembled into decamers, like the native HEV T=1 or T=3 VLPs (Fig. 3A, B). Furthermore, when the P domain was substituted by Seq8, CaSM-Seq8 showed the same assembly pattern than the CaSM protein (Fig. 3C). However, Seq8 could only assemble into a dimer (Fig. 3D). Therefore, these pentamers and decamers, acting as intermediates in the formation of HEV VLPs, are primarily indicators supporting our hypothesis that S and M domains are the basis for the assembly of ORF2 VLPs, and P domain could be replaced by an exogenous antigens.

- **Morphological assessment of the target proteins using electron microscopy**

The purified proteins were visualized under a transmission electron microscope, and their radius were analyzed using Image Pro Plus. The results indicated that CaSM could form VLPs with a radius of 13.03±1.739nm (Fig. 4A), and CaSM-Seq8 could also self-assemble into particles with a radius of 12.91±1.823nm, with similar size and shape than the CaSM VLPs (Fig. 4B). However, on the Seq8 micrographs abundant smaller particles were (with a radius of 5.231±2.923nm) and fewer irregular large particles were observed (Fig. 4C). As a control, the same technique was used to calculate native HEV

virions radius (21.36±2.575nm) (Fig. 4D), slightly larger than the CaSM and CaSM-Seq8 VLPs. This could be due to the absence of the P domain in the former and the presence of a smaller entity than the P domain in the latter.

- **Antigenicity analysis**

First, we sought to computationally assess the antigenicity of Seq8 alone and when linked to the CaSM protein, based on the predicted 3D structure models. According to protrusion index (PI) calculation, most epitope site were more exposed in CaSM-Seq8 than in Seq8 alone (Fig. 5A). Since the Seq8 antigen contains three different immunogenic domains (3 G-H loops), the AUC of each domain was calculated to compare the exposure of the whole epitopes and the results indicated that the degree of exposition of domains I and III was significantly higher in CaSM-Seq8 than in Seq8, as tested the paired parametric t test (Fig. 5B). These results supported that these three domains were more protruded in CaSM-Seq8 than in Seq8, and suggested that CaSM-Seq8 may have a higher antigenicity than Seq8 alone.

Next, we sought to experimentally assess the antigenicity of CaSM-Seq8 and Seq8 proteins. as the results of Elisa (Fig. 6A-D), in almost all the dilution ratios, OD_{450} values of CaSM-Seq8 were higher than those of Seq8 when they were detected by FMDV pAbs, swine JMS+AsiaI strains immunized sera, and swine Mya98+Cathy immunized sera. Then, a significant difference was observed among the OD_{450} values of CaSM-Seq8, Seq8, and CaSM in two-way ANOVA. The followed Tukey test also revealed some significant difference when these proteins were tested in pairs. In the Western Blot grayscale comparison (Fig. 6E), CaSM-Seq8 bands had higher grayscale values than Seq8 bands. All the results supported that CaSM-Seq8 had a higher antigenicity than Seq8.

- **Immunogenicity analysis**

After the injection of CaSM-Seq8, Seq8 and FMDV inactivated vaccine into mice, the induced anti-FMDV IgG levels were monitored up to 10 weeks post-injection using an indirect ELISA (Fig. 6F). Antibody titers induced by CaSM-Seq8 were higher than those induced in all the other experimental groups. Moreover, FMDV-specific antibodies appeared earlier in CaSM-Seq8 immunized mice (approximately at week2) than in the commercial vaccine or Seq8 immunized mice (approximately at week4). Additionally, although the titer of commercial vaccine immunized sera and Seq8 immunized sera began to decrease approximately at week6 and week8, the titer of CaSM-Seq8 immunized sera remained at higher levels at week10. All these results supported the highest immunogenicity of CaSM-Seq8, and CaSM-Seq8 could be a potential and promising anti-FMDV vaccine.

Discussion

Hepatitis E virus, an icosahedral virion with a diameter of approximately 27-32nm, is an oral-transmitted virus causing acute viral hepatitis [1]. Its capsid protein is encoded by ORF2, and contains three structural domains: S domain (aa129-319), M domain (aa320-455) and P domain (aa456-606) forming the protrusion outside the particle [5, 27].

In the present study, the HEV truncated capsid protein (aa112-455) named CaSM, comprising the S and M domains as well as the linker linking the P domain to the M domain, has been selected to present exogenous antigens. The selection of this fragment was based on the structural features of these domains reported after the elucidation of high-resolution structures of the HEV capsid [4, 5, 27]. The S domain which is the most conserved region among HEV genotypes [28] formed an icosahedral shell serving as the base for the arrangement of the subsequent domains (M and P) [27]. Although HEV capsid seems to share similar folding and similar VLP morphology with other viruses such caliciviruses [29, 30], the arrangement of the M domain seems to be characteristic of the HEV capsid, by its strong interaction with the S domain allowing the enhancement of the VLPs stability [27]. Furthermore, unlike the P2 domain of caliciviruses that is inserted into the P1 domain (M domain) [29], the HEV capsid P domain is independent from SM-formed shell, linked to the C-terminal of the M domain through a long proline-rich linker that insures this independence and allows the flexibility of the P domain and its proper dimerization [4, 5, 27]. This is also another characteristic of the morphology of the HEV particles.

As an exogenous antigen, we designed the Seq8 chimeric protein, containing three immunogenic domains of FMDV VP1 protein (three VP1 G-H loops from three different FMDV strains) [22, 23]. Then, the Seq8 antigen was linked to the C-terminal of the CaSM protein to construct CaSM-Seq8. This stands as substitution of the P domain in the HEV capsid protein.

Next, through computational analysis, we predicted the oligomerization of the three proteins CaSM, CaSM-Seq8 and Seq8 and found that CaSM and CaSM-Seq8 could form pentamers and decamers, arranged similarly to those previously described as intermediates in the assembly of HEV VLPs [27]. This suggested that the substitution of the P domain would not interfere with the proper aggregation of the S and M domain domains of the CaSM-Seq8 and the formation of the desired chimeric VLPs.

To experimentally confirm these speculations, the three target proteins were overexpressed in E. coli, purified and visualized by transmission electron microscopy. On the micrographs, CaSM self-assembled into VLPs with a diameter of approximately 26nm similar in shape but smaller in size than particles observed on the Native HEV micrographs. This was as expected since the CaSM protein contains the amino acids that previous studies have already identified as essential for truncated HEV ORF2 proteins to form T=1 or T=3 VLPs, such as aa111, aa126 and aa367 [7, 8]. More interestingly, the CaSM-Seq8 chimeric protein also formed VLPs with approximately the same diameter and morphology than CaSM particles. These results along with the computational predictions points toward the conclusion that the substitution of the P domain with the exogenous Seq8 antigen did not affect the assembly of the HEV S/M domain into particles that present Seq8 as a protruding spike (Fig. 3). However, further determination of the high-resolution structure of CaSM-Seq8 chimeric VLPs is needed to confirm these speculations.

Further, we sought to evaluate whether the formation of the VLPs affected the antigenicity and immunogenicity of the inserted Seq8 antigen. The bioinformatics analysis revealed that the Seq8 epitopes were more exposed when attached to CaSM than when presented on Seq8 alone. This suggested that antigenicity of Seq8 would be enhanced in the chimeric VLPs. Indeed, the Western blot

and ELISA results showed that the immunoreactivity against FMDV-specific antibodies was stronger with the CaSM-Seq8. Likewise, after injection into mice, the chimeric VLPs induced higher anti-FMDV IgG levels that appeared earlier, increased faster and lasted longer than those induced by Seq8 alone or even the FMDV inactivated vaccine. These results indicated clearly that on one hand, the formed chimeric VLPs did not interfere with presentation of the Seq8 epitopes; and the optimal exposure of these epitopes enhanced the overall antigenicity/immunogenicity of Seq8 epitopes, on the other hand. These effects can be explained by the intrinsic characteristics of CaSM-carrier VLPs such as the inclusion of the proline-rich linker that would allow the protrusion of the foreign antigen and augment its conformational flexibility for an optimal interaction with the antibodies. Moreover, the large particulate form of the chimeric VLPs permitted currying repetitive copies of the target epitopes which can stimulate a more robust immune reactions [31]. Besides, numerous T cell epitopes (aa73–156; aa289–372; aa361–444) are located in S and M domains [32], which participate in activating a stronger cellular immune response.

Furthermore, it has been previously reported HEV VLPs could be used as an oral delivery system [15, 18]. Therefore, we investigated the thermal stability and proteolysis resistance the chimeric VLPs. As a result, both properties were enhanced when Seq8 was carried by CaSM particles. This indicated that CaSM, antigen presenting platform, had potential application in vaccine design, especially oral vaccines.

In conclusion, in this study we demonstrated that the S and M domains of the HEV capsid protein can self-assemble into VLPs, and by substituting only the P domain by a foreign antigen, this latter is also presented as a protruding domain in the chimeric VLPs, enhancing therefore its antigenicity and immunogenicity. This makes the CaSM particles a potential presentation platform of exogenous immunogens. Furthermore, the CaSM-Seq8 investigated here, showed promising preliminary results as chimeric vaccine against FMDV. However, further investigation is needed to overcome some limitations of this work: (1) Only Seq8 was used as a foreign antigen, and whether CaSM is an optimal VLP carrier for a broad range of exogenous antigens need further research; (2) High-resolution structures of the chimeric VLPs should be determined to study in detail the structural behavior of both CaSM particles and the exogenous antigens upon linking them together; (3) Since many other VLP carriers (HBV, HPV, HIV etc.) have been reported previously [31, 33], a comparative study would be of great interest; (4) Studies on the neutralization activity of CaSM-Seq8 and its in vivo protection against virulent FMDV strains are crucial to determine the real potential of this chimeric VLP as an FMDV vaccine.

Methods

- **Construction of target clones**

For HEV, isolate W2-1 strain of genotype I was used (GenBank: JQ655734.1). HEV ORF2 W2-1 plasmid was synthesized (Gene Create Co., Wuhan, China), and Seq8 plasmid was previously designed and stored in our lab. Polymerase chain reactions (PCR) were performed using 2×Taq Master Mix (Vazyme Co., Nanjing, China) to amplify the target genes (*NcoI*-CaSM-*BamhI*, *BamhI*-Seq8-*XhoI*, *NcoI*-CaSM-*XhoI*, and *NcoI*-Seq8-*XhoI*). The products *NcoI*-CaSM-*BamhI* and *BamhI*-Seq8-*XhoI* were digested by *BamhI*

restriction enzyme (Thermo Fisher Scientific Inc, USA) and linearly ligated by T4 DNA Ligase (Thermo Fisher Scientific Inc, USA) to construct *NcoI*-CaSM-Seq8-*XhoI*. Then, ligated gene was also amplified by PCR.

Next, the target genes (*NcoI*-CaSM-*XhoI*, *NcoI*-Seq8-*XhoI*, and *NcoI*-CaSM-Seq8-*XhoI*) were digested by *NcoI* and *XhoI* endonucleases (Thermo Fisher Scientific Inc, USA), and inserted into a linearized plasmid (*NcoI*-pET28a-*XhoI*) to construct the recombinant plasmids below: pET28a-$_{NcoI}$CaSM$_{XhoI}$, pET28a-$_{NcoI}$Seq8$_{XhoI}$, and pET28a-$_{NcoI}$CaSM-Seq8$_{XhoI}$. Then, the recombinant plasmids were transformed into competent cell E. coli BL21 (Vazyme Co., Nanjing, China) and several clones were selected and tested by endonuclease digestion and DNA sequencing to verify the target genes.

- **Protein expression and purification**

The clones containing target genes were cultured overnight in Luria–Bertani broth (LB) with 1mM Kanamycin at 37°C. The next day, the overnight grown bacteria were diluted in 1L of LB containing Kanamycin (1:100 dilution) and incubated for 2-4h at 37°C, until the OD_{600} grew to 0.6, then Isopropyl β-D-1-thiogalactopyranoside (IPTG) was injected into the medium to a final concentration of 1mM and the protein expression was induced for another for 3-4h. After centrifugation, the pellets were suspended in a lysis buffer (50mM NaH_2PO_4, 300mM NaCl, 10mM Imidazole, pH 8.0). The cell lysis was enhanced by the addiction of lysozyme to a final concentration of 0.5mg/ml and an incubation on ice for 2h. The viscosity of the sample was then reduced by the addition of Deoxyribonuclease (DNase) (Sigma-Aldrich, USA) and the samples were centrifuged at 120000×g for 20 min at 4°C. Finally, the supernatants and pellets were aliquoted and tested by SDS-PAGE for protein expression and solubility.

The soluble fractions of the proteins were purified using the Ni-NTA affinity chromatography column (QIAGEN Sciences, MD, USA) according to the manufacturer's instructions. The combination of His Tag on C-terminal of proteins and Ni-NTA. Then the columns equilibrated with the lysis buffer, washed with washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 30 mM imidazole, pH=8.0) and the proteins were eluted with an elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole, pH=8.0). The eluted proteins were tested by SDS-PAGE and their concentrations were determined by Quick Start™ Bradford Protein Assay Kit (Bio-Rad Laboratories, Inc. USA).

- **Computational analysis of the oligomerization state of the target proteins**

The 3D structures of the target proteins were predicted using phyre2 server [34], and refined by GalaxyWeb Refine server [35] as previously described [36]. Then, GalaxyWeb Homomer server [26] was used to predict the oligomerization state from the monomer 3D structures. The predicted models with a highest TM score in structure-based method or a highest Docking score in the Ab initio docking method were selected as the optimal models.

- **Transmission electron microscopy**

The purified proteins were diluted to 0.1mg/ml, then loaded on the carbon-coated grids for 10min and negatively stained with 2% uranyl acetate for 10min. The grids were dehydrated under native condition and observed by Transmission electron microscope F30 (Philips, The Netherlands). Further, the shapes and diameters of the particle on the electron micrographs were analyzed using the Image-Pro Plus 6.0 software.

- **Physical properties evaluation**

To study the thermal stability of recombinant proteins, all the proteins were diluted to 0.1mg/ml, aliquoted and stored at different temperature -80℃, 4℃ and 37℃. After 1, 3, 5, 7 post incubation, the samples were examined by SDS-PAGE to determine the degradation rate.

To explore their resistance to protease digestion, 0.1mg/ml CaSM, CaSM-Seq8, and Seq8 were mixed with an equal volume of simulated gastric fluid (diluted HCl 1.64ml, ddH_2O 80ml, pepsin 1g, adjust with ddH_2O to 100ml) or simulated intestinal fluid (KH_2PO_4 0.68g, trypsin 1g, adjust with NaOH to pH=6.8, total volume 100ml). Then, the samples were incubated at 37℃ for 2h, and analyzed by SDS-PAGE after the incubation period.

- **Antigenicity of the target proteins**

First, the antigenicity of the target proteins was evaluated by a computational approach. Since the Seq8 antigen contains three immunogenic domains (three different VP1 G-H loops) the exposure of these domains in CaSM-Seq8 and Seq8 predicted 3D models was evaluated by Ellipro server [37]. This server calculates the Protrusion Index (PI) of each amino acid in a given protein structure model. The PI values obtained for the three immunogenic domains in both CaSM-Seq8 and Seq8 proteins were compared by a paired parametric t test. Moreover, the area under the PI curve (AUC) of each domain was calculated as an index of the accumulated protrusion, representing the exposure of each domain as a whole. These AUCs were also compared by paired parametric t test.

Next, to experimentally evaluate the antigenicity of the purified proteins, the immunoreactivity of CaSM, CaSM-Seq8, and Seq8 against anti-FMDV polyclonal antibodies (pAbs) and against sera of mice and pigs immunized with different FMDV stains (JMS+AsiaI strains and Mya98+Cathy strains respectively), were tested by an indirect enzyme-linked immunosorbent assay (ELISA) and Western Blotting. The reactivity difference between the different proteins were tested by two-way ANOVA followed by Tukey test.

- **Mice immunization and IgG detection**

All animal experiments were performed according to ARRIVE guidelines (Table. S1) [38], and in strict accordance with the guidelines for the Care and Use of Laboratory Animals of Southeast University, and were approved by the ethics committee of the Institutional Animal Care and Use Committee of Southeast University.

A total of 40 6-week-old female BALB/c mice were purchased form Comparative Medicine Center of Yangzhou University. The mice were randomly divided into 4 groups and kept in a pathogen-free environment. The proteins (final amount 10μg) were mixed with adjuvant ISA206 (SEPPIC, France), and then all the mice were immunized by intramuscular injection (group1: CaSM-Seq8+ISA206, group2: Seq8+ISA206, group3: 150μl/mouse commercial FMDV inactivated vaccine as a positive control group, group4: 150μl/mouse Saline+ISA206 as a negative control group). Blood samples were collected from the internal iliac vein before immunization and at week 2, 4, 6, 8 and 10 post immunization and centrifugated at 12000rpm for 20min. Then, the sera were pooled and stored at -80℃. The induced anti-FMDV IgGs in the mice sera were detected by an indirect ELISA using Seq8 as the coating antigen, and HRP-conjugated goat anti-mouse IgG (Thermo Fisher Scientific Inc, USA) as a secondary antibody. The difference in antibody levels were assessed by two-way ANOVA followed by Tukey post-hoc test. Welfare evaluations and interventions were performed before, during, and after the experiments.

By the end of the study, the mice were euthanized by inhaling lethal dose of carbon dioxide (CO_2) in the mice asphyxiation chamber (CO_2 flowing rate: 2.5L/min). The mice euthanasia protocol was approved by Institutional Animal Care and Use Committee of Southeast University.

- **SDS-PAGE and Western Blotting**

All samples were mixed with an equal volume of 2×loading buffer (5% SDS, 20% Glycerol, and 0.002% Bromophenol Blue; 1.4% v/v of β-mercaptoethanol) and heated to 100℃ for 5min; then loaded into 15% SDS-PAGE gel. After the completion of electrophoresis, the gel was stained in Coomassie staining solution (300ml methanol, 100ml acetic acid, adjust with ddH_2O to 1000ml with 1.25g of Coomassie R-250).

For Western blotting, the SDS-PAGE gel was not stained but washed with transfer buffer (25mM Tris base, 190mM glycine, 20% v/v of methanol, pH 8.3) and then the proteins in the gel were transferred onto the NC membrane in a transferring cassette (Bio-Rad Laboratories, Inc. USA) at 200mA for 2h. Next, the membrane was incubated in a blocking buffer (20mM Tris, 137mM NaCl, 0.1ml Tween 20, pH=7.6, supplemented with 5% w/v of skim milk powder) at 37℃ for 2h. Then, the primary antibody was added to the blocking buffer to a final dilution of 1:500 followed by overnight incubation with shaking at 4℃. The next day, after washing, the membrane was incubated in the blocking buffer containing 1:2000 of the HRP-conjugated secondary antibody for 2h at room temperature. Finally, the signal was developed by the addition of 3,3'-Diaminobenzidine solution.

- **Indirect enzyme-linked immunosorbent assays**

The assays were performed as previously described [39]. Briefly, 96-well flat-bottom plates were coated with target antigens (200ng/well) in 1M Urea-phosphate buffered saline (PBS) at 4℃ overnight; after 3 washings with PBS supplemented with 0.5% Tween20 (PBST), 100μl of the first antibody, serially diluted (1:100, 1: 200, 1: 400, 1: 800, 1: 1600, 1: 3200, 1: 6400, 1:12800 and 1:25600) in 1% Casein-PBS was added into the wells and incubated at 37℃ for 1h; after 3 washings with PBST, the HRP-conjugated

secondary antibody diluted 1: 5000 in 100μl 1% Casein-PBS was added and the plates were then incubated at 37℃ for 1h; after 3 washings with PBST, the plates were developed by the addition of 100μl of 3,3',5,5'-Tetramethylbenzidine chromogenic substrate and the reaction were stopped by 50μl 2M H_2SO_4 after 20 min incubation at 37℃; finally, the absorbance of each well was read at 450 nm.

- **Software and statistical analysis**

SnapGene 2.3.2 (GSL Biotech) was used for the design of the primers DNA sequences editing and manipulations. PyMol 2.0.4 (Version 2.0 Schrödinger, LLC.) was used for the visualization of the structure models and the preparation of all figures containing protein structures. All the figures were prepared using Adobe Photoshop CC 2019 (Adobe Systems Incorporated), Image J 1.52K (NIH, USA), GraphPad Prism 8 (Graphpad Software, Inc.), and Image Pro Plus 6.0 (Media Cybernetics, Inc.). All Statistical analyses were performed using IBM SPSS V.24 (International Business Machines Corp.). $p \geq 0.05$: no significance (ns); $0.01 \leq p \leq 0.05$: *; $0.001 \leq p \leq 0.01$: **; $0.0001 \leq p \leq 0.001$: ***; $p \leq 0.0001$: ****.

Conclusions

In this study, the potential and value of utilizing HEV truncated capsid protein (CaSM) as a VLP carrier to present exogenous antigens was verified by us. Firstly, we found out that S and M domains of HEV capsid protein were the essential elements for the formation of T=1 VLP, and P domain, which is a protrusion outside the VLP linked by a flexible hinge could be substituted by exogenous antigens (Seq8). Moreover, these chimeric protein could also assemble into a VLP. Via bioinformatic analysis, we found out assembly mechanism of CaSM-Seq8 is same as native T=1 or T=3 VLPs. Secondly, we also demonstrated that some physical properties of Seq8 like thermal stability and proteolysis resistance were enhanced when carried by CaSM. Thirdly, though computational analysis, we proved that the immunogenic domains of Seq8 were more exposed when carried by CaSM, which indicated a higher antigenicity. Further, the higher antigenicity was also proved by immunology experiments. Moreover, via mice immunization, we demonstrated that CaSM-Seq8 had higher immunogenicity than Seq8. All these conclusions supported that CaSM is an optimal and promising presenting-platform of exogenous antigens in vaccine design.

Declarations

Ethics approval and consent to participate

All the animal experiments were approved by the ethics committee of Institutional Animal Care and Use Committee of Southeast University, and implemented in accordance with the animal experimentation guideline of Institutional Animal Care and Use Committee of Southeast University and ARRIVE guidelines (Table. S1).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analyzed in the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JHM, TYL, NB, and RHS conceived and planned the study. TYL, NB, and YZ performed the analyses and summarized the results. TYL, NB, YZ, SA, WJW, and ZZL contributed to the interpretation of the results. TYL and NB took the lead in writing the manuscript. All authors provided critical feedback and helped shape the final version of the manuscript.

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Not applicable.

Abbreviations

aa	Amino acid
APS	Ammonium persulfate
AUC	Area under the curve
bp	Base pair
BSA	Bovine serum albumin
CaSM	A carrier containing S and M domains
CaSM-EAg	S domain-M domain-exogenous antigens
CO_2	Carbon dioxide
DAB	Diaminobenzidine
ddH2O	Double distilled water
DNase	Deoxyribonuclease
E. coli	Escherichia coli
ELISA	Enzyme-linked immunosorbent assays
FMDV	Foot-and-mouth disease virus
HEV	Hepatitis E virus
HRP	Horseradish peroxidase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	Kilo Dalton
LB	Luria–Bertani broth
NC	Negative control
NC membrane	Nitrocellulose membrane
ns	No significance
OD	Optical density
ORF(s)	Open reading frame(s)
pAbs	Polyclonal antibodies
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with 0.5% Tween20
PCR	Polymerase chain reactions
Pep	Pepsin
PI	Protrusion index
pORF1(2/3)	Protein encoded by open reading frame 1(2/3)
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Trishydroxymethylaminomethane
Try	Trypsin
VLP(s)	virus like particle(s)

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Figures

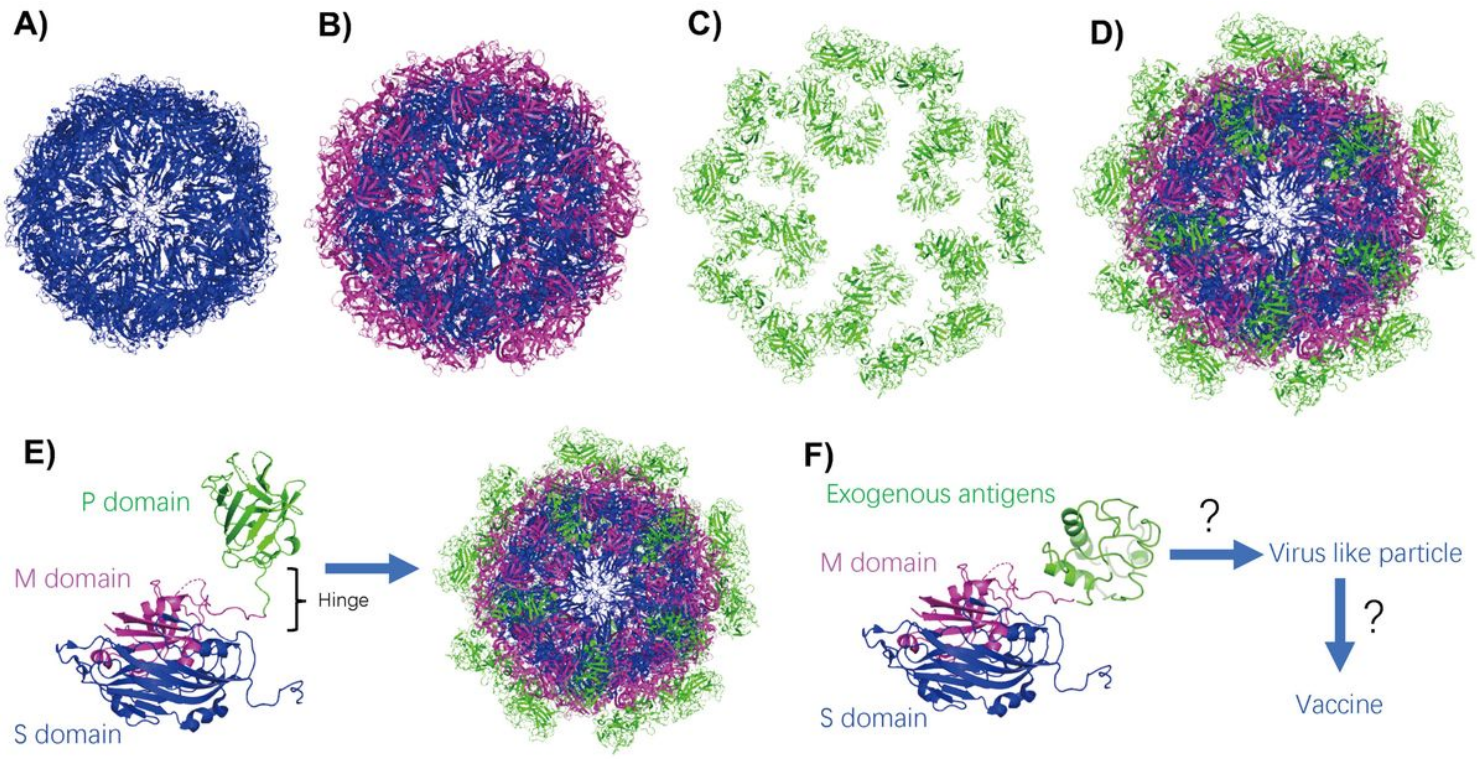

Figure 1

Theory-based approach for the design ORF2 chimeric VLPs. A) 60 copies of S domain in T=1 HEV VLP forming a continuous internal scaffold shell of the VLP; B) T=1 ORF2 VLP displaying S (blue) and M

(purple) domains only; C) Exposure of the protruding P domains; D) A complete T=1 VLP is displayed with S (blue), M (purple), and P (green) domains; E) Asymmetric unit of the ORF2 protein with the different structural domains; F) The hypothesis tested in the present study: substituting the P domain with a foreign chimeric antigen could still allow the S and M domains to form VLPs with exogenous antigen as a protruding spike? Would this chimeric VLPs enhance the antigenicity and immunogenicity of the inserted antigen? A, B, C, D, and E, figures were prepared using the HEV ORF2 structure 2ZTN retrieved from the Protein Data Bank.

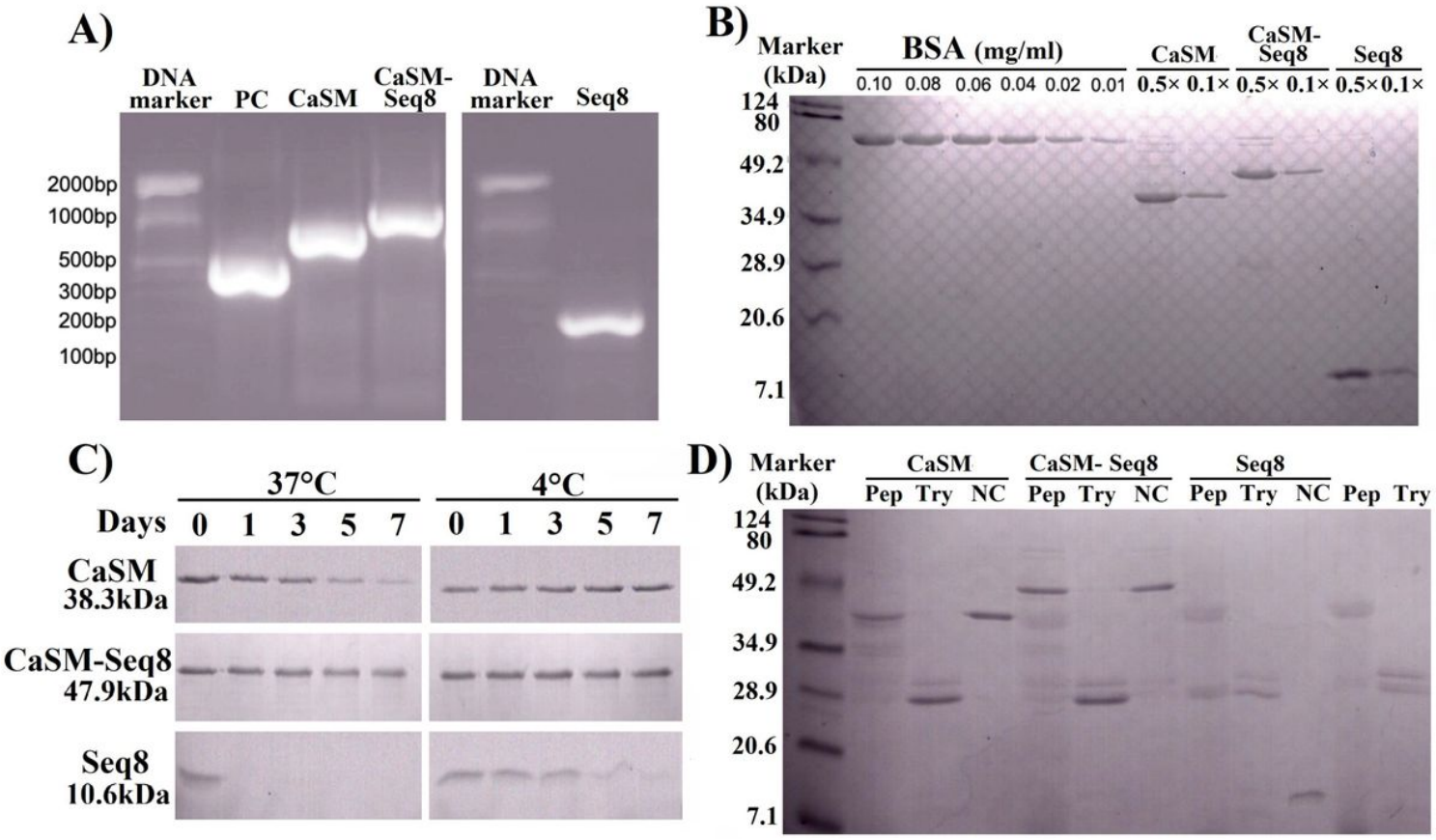

Figure 2

Preparation of the target recombinant proteins and physical properties assessment. A) Target genes amplified by PCR and tested by agarose gel electrophoresis (PC: positive control); B) SDS-PAGE gel of two dilution of the purified recombinant proteins (0.5× and 0.1×) with a serial dilution of bovine serum albumin. C) SDS-PAGE gel showing the thermal stability of CaSM, CaSM-Seq8, and Seq8 at 37°C and 4°C. D) SDS-PAGE showing proteolytic action of pepsin (Pep) trypsin (Try) and on CaSM, CaSM-Seq8, and Seq8 (NC: negative control).

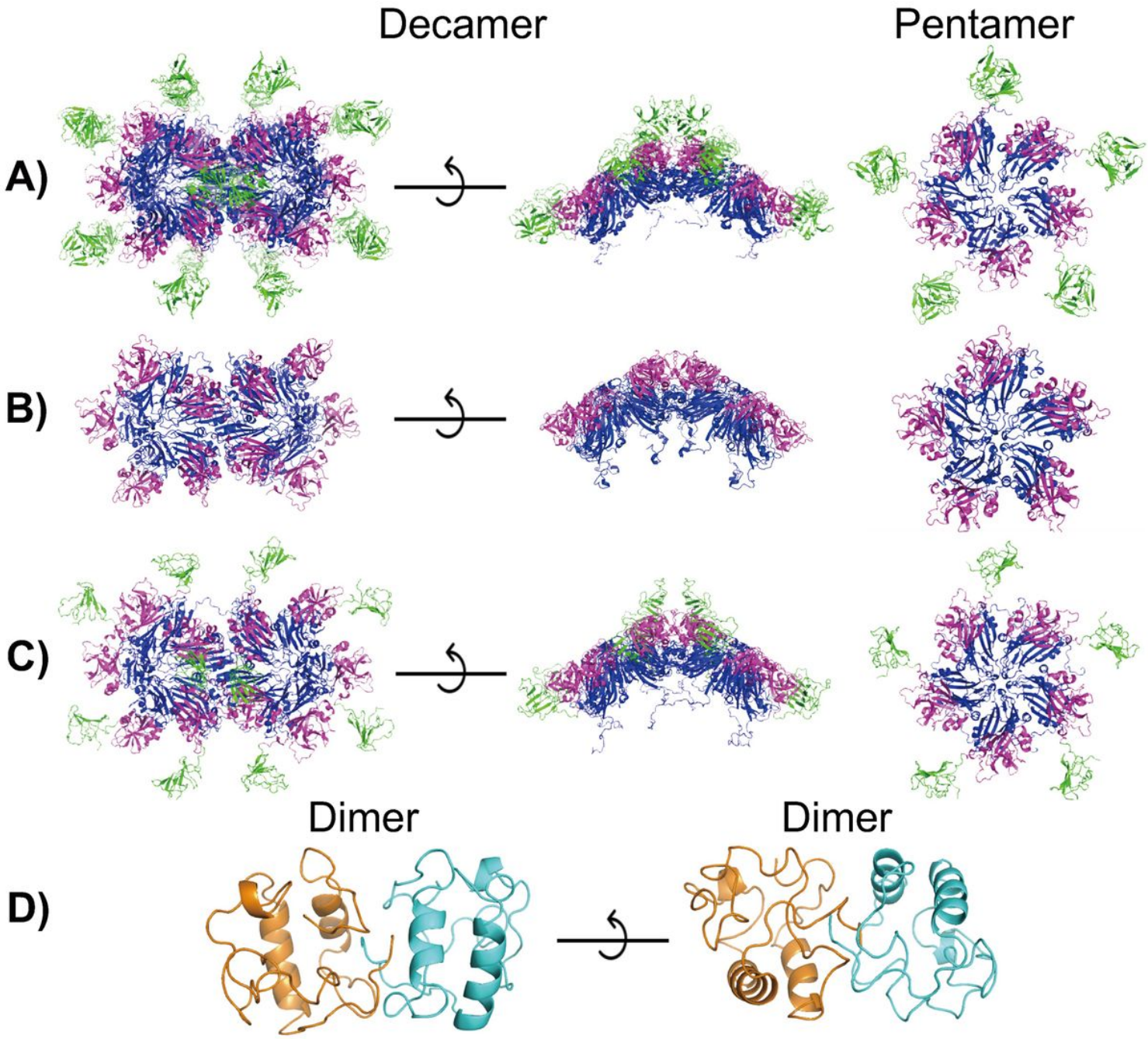

Figure 3

Prediction of the oligomerization states of the target proteins. A) Decamer and pentamer intermediates of HEV T=1 VLP assembly process; B) Structure of the CaSM decamers and pentamers; C) Structure of the CaSM-Seq8 decamers and pentamers; D) Structure of the Seq8 dimer. The oligomers in (A) were obtained by analyzing HEV ORF2 structure 3HAG retrieved from the Protein Data Bank; the oligomers in (B, C and D) were predicted by GalaxyWeb Homomer web-server.

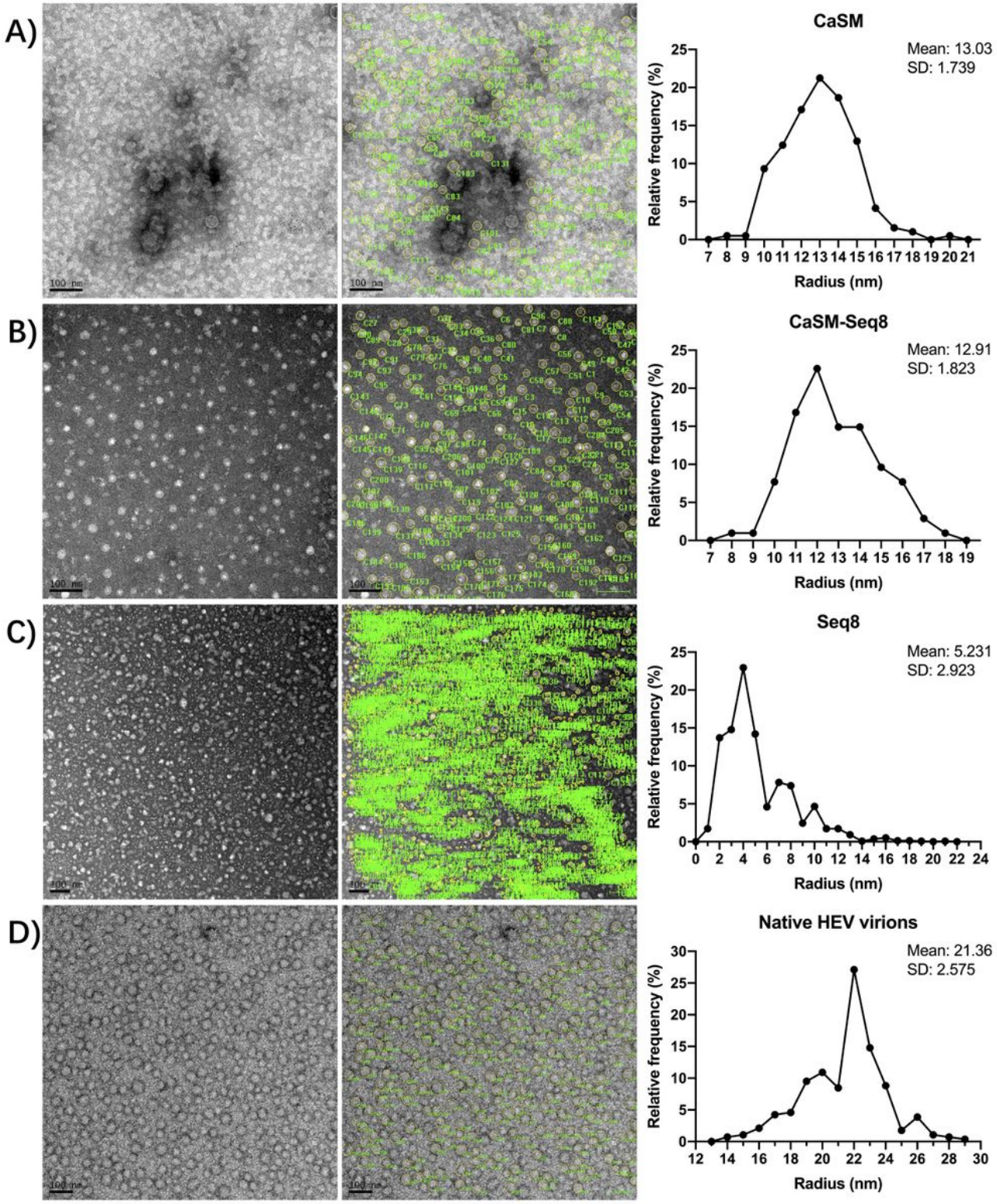

Figure 4

Electron micrographs and radius analysis of the purified proteins and native HEV virions. The results are presented in A, B, C, and D for CaSM, CaSM-Seq8, Seq8 and native HEV virions, respectively; The obtained micrographs for each sample are reported on the left; the representative particles selected for radius frequency distribution analysis are depicted in the middle; and the result of radius frequency distribution analysis with Mean and SD are summarized on the right.

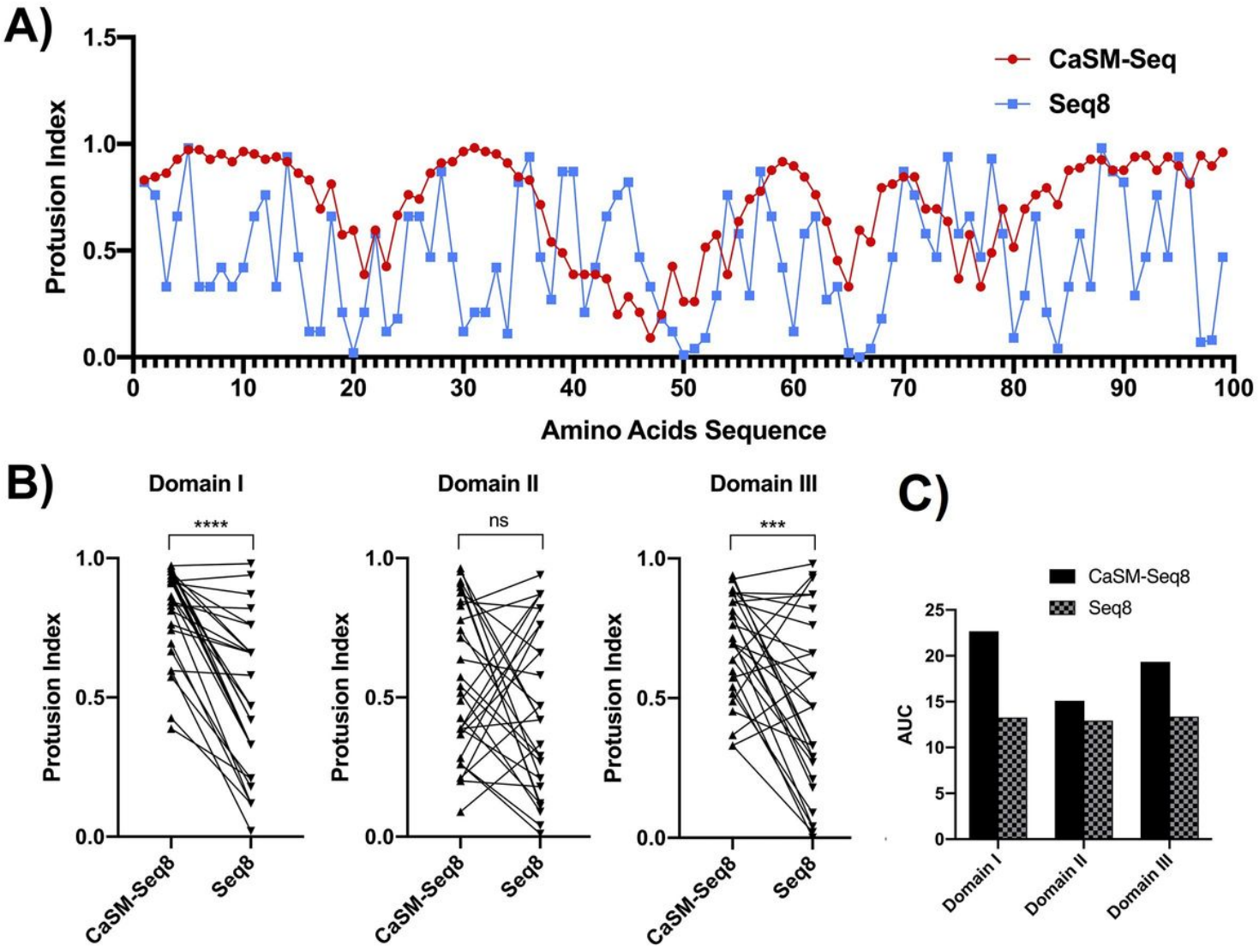

Figure 5

Computational analysis of the antigenicity of CaSM-Seq8 and Seq8. A) Protrusion index (PI) curve of Seq8 amino acids in both CaSM-Seq8 and Seq8 alone; B) Fluctuation of PI of the three immunogenic domains of Seq8 alone and when linked to the CaSM protein; C) Column bar representation of the area under the PI curve of the three immunogenic domains of Seq8 alone and when linked to the CaSM protein. ****p<0.001, ***p<0.01, and ns: p>0.05 represent the significance levels of the paired parametric t test.

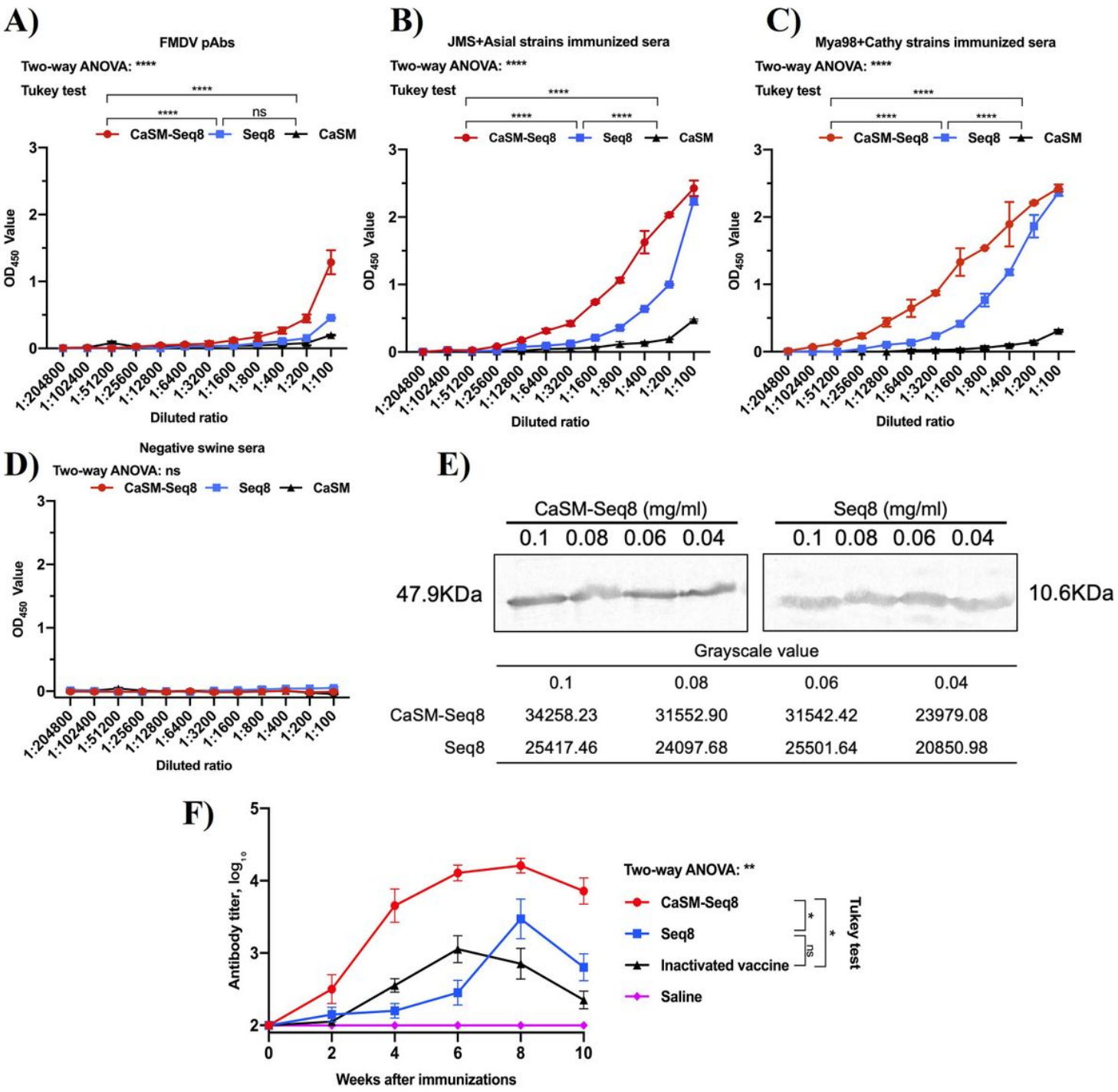

Figure 6

Antigenicity/Immunogenicity analysis. A, B, C and D) Comparison of the immunoreactivity of the target proteins against FMDV-specific antibodies using indirect ELISA; E) Western blot analysis with calculated grayscale values, using FMDV polyclonal antibodies as the primary antibody; F) immunogenicity analysis by monitoring the induced FMDV-specific antibody titers in mice immunized with CaSM-Seq8 Seq8 and an FMDV inactivated vaccine. **p<0.01, *p<0.05, ns: p>0.05.

Supplementary Files

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