Hepatitis E virus capsid as a carrier of exogenous antigens for the development of chimeric virus-like particles

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Abstract

Virus-like particle (VLP), a self-assembled multiprotein structure, can stimulate robust immune responses due to its structure similar to native virions that curries multiple copies of the target epitopes. Utilizing VLPs as vaccine platforms to present exogenous antigens is a promising and challenging approach in the vaccine development field. This study aims to investigate the potential of hepatitis E virus (HEV) truncated capsid as a VLP platform to present foreign antigens. The S and M domains of HEV capsid protein were selected as the optimal carrier (CaSM). The exogenous antigen Seq8 containing three neutralizing epitopes from three different foot-and-mouth disease virus (FMDV) strains was linked to the C-terminal of CaSM to construct a chimeric VLP (CaSM-Seq8). The construct was successfully expressed and purified. Morphological analysis showed that CaSM-Seq8 self-assembled into VLPs similar to CaSM VLP (˜26 nm in diameter) but smaller than native HEV virions. Further, the thermal stability and the resistance to enzymatic proteolysis of Seq8 were enhanced when it was attached to CaSM carrier. The antigenicity analysis revealed a more robust reactivity against anti-FMDV antibodies when Seq8 was presented on the CaSM particles. Upon injection into mice, FMDV-specific IgGs induced by CaSM-Seq8 appeared earlier, increased faster, and maintained higher levels for a longer time than those induced by Seq8 alone or the inactivated FMDV vaccine. This study demonstrated the potential of utilizing HEV truncated capsid as an antigen-presenting platform for the development of chimeric VLP vaccines.

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Keywords: Hepatitis E virus; exogenous antigen carrier; Virus-like particle; Immunogenicity;

Introduction

A virus-like particle (VLP) is a vacant self-assembled multiprotein structure that does not contain any viral genome [1,2]. VLPs can stimulate robust humoral and cellular immune responses because of their relatively large size similar to native virions and the presence of multiple copies of the target epitopes[3-5]. Given these unique characteristics, utilizing VLPs as platforms for the presentation of exogenous antigens becomes a promising approach for the development of effective recombinant vaccines[4,6].

Hepatitis E virus (HEV) is an icosahedral spherical virus, with a diameter of 27-32nm, belonging to the *Orthohepevirus* genus of the*Hepeviridae* family [7,8]. The HEV capsid protein (encoded by the ORF2) contains three domains named: S domain (residues 129-319), M domain (residues 320-455), and P domain (residues 456-606). These three domains play different structural and functional roles in the HEV capsid [9]. The S and M domains form an internal scaffold shell connected to the P domain through a long proline-rich hinge. This latter allows the P domain to protrude from the surface of the HEV particles with a certain flexibility for a better exposition of the dominant neutralization epitopes and the motifs critical for the interaction with the host cell receptors. Therefore, such a structural assembly allows an optimal exposure of the functional P domains as protruding spikes [9,10]. Previously, it has been reported that a truncated HEV ORF2 protein (aa112-660) expressed in insects cells could form VLPs similar to the native HEV virions[11]. Further, the truncated HEV capsid protein also self-assembled into VLPs even with the N-terminal truncation up to residue 125 and the C-terminal truncation up to residue 602, indicating that the essential elements for the self-assembly were located between residues 125-602 [12] and the S domain was indispensable for the formation of T=1 partciles [13]. However, later on, an N-terminal truncation up to aa367 has been reported as the largest truncation that maintained the self-assembly ability as it has been shown with the recombinant vaccine p239 [13,14]. Considering the structural features and the ability of truncated HEV capsid proteins to maintain the self-assembly property, we investigated the use of the S and M domains as an antigen-presenting carrier (CaSM) by replacing the protruding P domain with an exogenous antigens.

Several antigen-presenting platforms, based on the HEV capsid protein, were previously designed by inserting foreign antigens at different positions: (1) inserting an 18 amino acids peptide of HIV-1 gp120 into the HEV capsid between residue 485 and 486 [6]; (2) inserting a myc-tag/FLAG-tag/HA-tag between residue 488 and residue 489 to present multiple foreign antigens [15]; (3) adding a B cell epitope tag consisting 11 amino acids to the C-terminal[16]; (4) linking hepatitis A virus VP1 (aa24–171) to the C-terminal of the P domain to make a bivalent vaccine[17]; (5) fusing HEV ORF3 p70-123 to ORF2 p112-608[18]. However, in such designs, the inserted fragments were mere linear epitopes or too short which could not evoke potent immune responses, and some epitopes were attached to the C-terminal of the P domain, which may restrict the flexibility and exposure of the exogenous antigens and/or the P domain [19]. Therefore, in this study, we aimed to overcome these drawbacks by selecting a large exogenous antigen, substituting the entire P domain, and linking it to the M domain through the HEV intrinsic proline-rich hinge.

Foot-and-mouth disease virus (FMDV), belonging to the genus*Aphthovirus* of the family *Picornaviridae* , is the causative pathogen of foot-and mouth disease (FMD), an acute and contagious disease of clove-hoofed animals with devastating economic repercussions[20,21]. The genome of FMDV encodes a large polyprotein that can be cleaved into four structural proteins (VP1-4) and several non-structural proteins (NSP) [21]. Seven FMDV serotypes have been identified: O, A, C, Asia 1, SAT 1, SAT 2, and SAT 3, with the Serotype O as the predominant type in Asia; this Serotype O can be further classified into 11 topotypes according to the characteristics of the VP1 protein [21-23]. Previous studies have revealed that two immunogenic regions of VP1 aa140-160 (G-H loop) and aa200-213 (C-terminal fragment), can elicit neutralizing antibodies[24,25]. Further studies revealed that although the G-H loop could elicit neutralizing antibodies, it had low immunogenicity because of its short length and the lack of T-cell epitopes[25]. The immunogenicity of the G-H loop could be enhanced drastically when it was incorporated with glutaraldehyde, liposome, polyinosinic cytidylic acid, or when linked with hepatitis B core protein, and such formulations also conferred complete protection in the immunized animals [26-28].

In the present work, we aimed to assess the truncated HEV capsid protein as a carrier of exogenous antigens. Such carrier (named CaSM) contains the S and M domain of the HEV capsid, and the foreign antigen would substitute the protruding HEV P domain, yielding a construct 'S domain-M domain-exogenous antigens' (CaSM-EAg). As a foreign antigen, we constructed the FMDV Seq8 antigen by combining three FMDV G-H loops (aa140-160) obtained from the three most prevalent FMDV/O topotype strains: O/HN/CHA/09, O/IRN/2010, and O/Mya/98. Then, we investigated whether the chimeric CaSM-Seq8 could self-assemble into VLPs presenting the Seq8 antigen on the surface, and whether this combination would enhance the physical, biological, and immunological characteristics of the inserted antigen Seq8.

1. **Materials and methods**
2. **Construction of the target clones**

The plasmid pET28-W2-1 containing the gene coding for the ORF2 protein of HEV W2-1 isolate (GenBank: JQ655734.1) has been synthesized (Gene Create Co., Wuhan, China). The pET-28-Seq8 plasmid has been previously designed, synthesized and stored in our lab. The 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 the *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, the ligated gene was further amplified by PCR.

Next, all 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: pET28a-$_{NcoI}$ CaSM$_{XhoI}$, pET28a-$_{NcoI}$ Seq8$_{XhoI}$, and pET28a-$_{NcoI}$ CaSM-Seq8$_{XhoI}$. Then, the expression constructs were used to transform competent E. coli BL21 cells (Vazyme Co., Nanjing, China). For each target protein, several clones were selected and tested by PCR and DNA sequencing to verify the correct insertions of the target genes.

Protein expression and purification

The clones containing the genes that encode for CaSM, CaSM-Seq8, and Seq8 were cultured at 37 overnight in Luria–Bertani broth (LB) containing 1mM Kanamycin. Then, the overnight grown bacteria were diluted in 1000mL of LB (1:100 dilution) and incubated at 37 for 2-4h, until the OD_{600} reached 0.6. Then, isopropyl β-D-1-thiogalactopyranoside (IPTG) was injected into the medium (final concentration: 1mM), and the protein expression was induced for another 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 lysozyme and 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 analyzed by SDS-PAGE.

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. Briefly, the columns were equilibrated with the lysis buffer, loaded with the prepared supernatants; then, washed with the washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 30 mM imidazole, pH=8.0); the target proteins were eluted with the elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole, pH=8.0). The eluted proteins were analyzed by SDS-PAGE, and their concentrations were determined by Quick Start Bradford Protein Assay Kit (Bio-Rad Laboratories, Inc. USA). The purity of the target proteins was evaluated by analyzing the SDS-PAGE gels using the Image J software.

Transmission electron microscopy (TEM)

CaSM, CaSM-Seq8, and Seq8 were diluted to 0.1mg/ml and loaded on the carbon-coated grids for 10min and negatively stained with 2% uranyl acetate for another 10min. The grids were dehydrated and observed by Transmission electron microscope F30 (Philips, The Netherlands). Further, the shape and diameter of the particles on the TEM micrographs were analyzed using the Image-Pro Plus 6.0 software.

Computational analysis of the oligomerization states of the target proteins

The Phyre2 server [29] was used to predict the 3D structures of CaSM, CaSM-Seq8, and Seq8; and the models were refined using GalaxyWeb Refine server [30] as previously described [31]. Then, GalaxyWeb Homomer server[32] was used to predict the oligomerization state and assembly pattern based on the monomer's 3D structures. The predicted models with the highest TM score (structure-based method) or the highest Docking score (ab initio docking method) were selected as the optimal models.

Immunomagnetic separation (IMS) of native HEV virions

Feces samples containing native HEV virus (genotype 4, strain NJ703 [GenBank: AY789228]) were stored at -80 in our lab[33]. Feces supernatant was prepared using phosphate-buffered saline (PBS). Then, HEV virions were purified from the feces supernatant by IMS using Dynabeads(r) Protein A (Nanoeast Bio-Technique Co. Ltd, China). The HEV 5G5 monoclonal antibody was used to coat the IMS beads[34], and 1.25μL Tween-20 and 500μL feces supernatant were added into the coated IMS beads. After the HEV virions were captured by 5G5 antibody, the IMS beads were magnetized and washed using 0.05% Tween-20-PBS. Then, the beads were eluted three times using 50μL of 100 mM glycine buffer (pH=2.5). The eluent was centrifuged and supplemented with 50μL Tris buffer (pH=8.0).

Analysis of the thermal stability and the enzymatic proteolysis of the target proteins

To study the thermal stability of CaSM, CaSM-Seq8, and Seq8, the samples were diluted to 0.1mg/ml, aliquoted, and stored at different temperatures (-80, 4, and 37). After 1, 3, 5, 7 days post-incubation, the samples were examined by SDS-PAGE to determine the degradation rate.

To explore their resistance to protease digestion, 0.1mg/ml of 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.

Antigenicity analysis

First, the antigenicity of CaSM, CaSM-Seq8, and Seq8 was evaluated by a computational approach. Since the antigen Seq8 contains three neutralizing epitopes (three different VP1 G-H loops), the exposure of these epitopes in CaSM-Seq8 and Seq8 was evaluated by Ellipro server[35]. This server calculates the Protrusion Index (PI) of each amino acid in a given protein's structural model. The PI values of these epitopes in both CaSM-Seq8 and Seq8 were compared by a paired parametric t-test. Moreover, the area under the PI curve (AUC) of each epitope was calculated as an index evaluating the overall protrusion of each G-H loop as a whole.

Next, to experimentally evaluate the antigenicity of these proteins, the immunoreactivity of CaSM, CaSM-Seq8, and Seq8 against anti-FMDV specific antibodies was tested by an indirect enzyme-linked immunosorbent assay (ELISA) and Western Blotting. Since the G-H loops in the Seq8 antigen were obtained from O/HN/CHA/09, O/IRN/2010, and O/Mya/98 strains, the following anti-FMDV specific antibodies were used: (1) sera of pigs infected with O/Mya/98+O/HN/CHA/09 strains (sharing the same G-H loops with Seq8; (2) sera of pigs infected with O/JMS/00+O/GX/09-7 strains to test the cross-reactivity with different topotypes of FMDV/O serotype; (3) negative sera obtained from FMDV-free pigs; (4) purified anti-FMDV/O polyclonal antibodies (pAbs), commercially available for the treatment of FMD. The reactivity difference between the target proteins was statistically assessed by a two-way ANOVA, followed by Tukey post-hoc test.

Mice immunization and IgG detection

All animal experiments were performed according to ARRIVE guidelines (Table. S1) [36] and in strict accordance with 'Care and Use of Laboratory Animals of Southeast University'. Welfare evaluations and interventions were performed before, during, and after the experiments.

A total of forty 6-week-old female BALB/c mice were purchased from Comparative Medicine Center of Yangzhou University. The mice were randomly divided into 4 groups and kept in a pathogen-free envi-

ronment. CaSM, CaSM-Seq8, and Seq8 (final amount 10μg) were mixed with ISA206 adjuvant (SEPPIC, France), and all the mice were immunized by intramuscular injections (group1: CaSM-Seq8+ISA206, group2: Seq8+ISA206, group3: 150μl/mouse of the commercial FMDV inactivated vaccine as a positive control group, group4: 150μl/mouse of saline+ISA206 as a negative control group). The commercial FMDV inactivated vaccine (O/Mya98/XJ/2010+O/GX/09-7) was bought from Jinyu Baoling Biological Medicine Company (Huhehaote, China). Blood samples were collected from the internal iliac vein before immunization and at weeks 2, 4, 6, 8, and 10 post-immunization; and centrifuged at 12000rpm for 20min. Then, the sera were pooled and stored at -80. The induced anti-FMDV-specific IgGs in the mice sera were detected by an indirect ELISA. Seq8 was used as a coating antigen and HRP-conjugated goat anti-mouse IgG (Thermo Fisher Scientific Inc, USA) was used as a secondary antibody. The difference in antibody levels was assessed by two-way ANOVA followed by Tukey post-hoc test.

By the end of the study, the mice were euthanized by inhaling a 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), heated to 100 for 5min, and then, loaded into 10%-15% gradient polyacrylamide gel. After the completion of electrophoresis, the gel was stained in Coomassie staining solution (300ml methanol, 100ml acetic acid, 1.25g of Coomassie R-250, adjust with ddH_2O to 1000ml).

For Western blotting, the SDS-PAGE gel without staining was washed with the transfer buffer (25mM Tris base, 190mM glycine, 20% v/v of methanol, pH 8.3). Then, the proteins in the gel were transferred onto the NC membrane at 200mA for 2h in a transferring cassette (Bio-Rad Laboratories, Inc. USA). 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. 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 reactive bands were developed by the addition of 3,3'-Diaminobenzidine solution.

Indirect enzyme-linked immunosorbent assays

The assays were performed as previously described [37]: (1) 96-well flat-bottom plates were coated with the target antigens (200ng/well) in 1M Urea-phosphate buffered saline (PBS) at 4 overnight; (2) after washing with PBS supplemented with 0.5% Tween 20 (PBST), 100μl of the primary 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; (3) after washing with PBST, the HRP-conjugated secondary antibody diluted 1: 5000 in 100μl 1% Casein-PBS was added, and the plates were incubated at 37 for 1h; (4) after washing with PBST, the plates were developed by the addition of 100μl of 3,3',5,5'-Tetramethylbenzidine chromogenic substrate. (5) The reaction was stopped by adding 50μl 2M H_2SO_4 and after a 20 min incubation at 37, the absorbance of each well was read at 450/630 nm.

Software and statistical analysis

SnapGene 2.3.2 (GSL Biotech) was used for the design, editing, and manipulations of the primers and DNA sequences. PyMol 2.0.4 (Version 2.0 Schrödinger, LLC.) was used for the visualization of the protein structure models. 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.). Statistical analysis was performed using IBM SPSS V.24 (International Business Machines Corp.). p[?]0.05: no significance (ns); 0.01[?]p¡0.05: *; 0.001[?]p¡0.01: **; 0.0001[?]p¡0.001: ***; p¡0.0001: ****.

1. **Results**
2. **Design and preparation of the target recombinant proteins**

The target genes (*NcoI* -CaSM-*XhoI* ,*NcoI* -CaSM-Seq8-*XhoI,* and *NcoI* -Seq8-*XhoI*) were successfully amplified and inserted into the pET28a vector. Then, the constructed plasmids were successfully transformed into competent E. coli BL21 cells. After the selection of the target clones 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. 1A and B).

Assessment of the thermal stability and the proteolysis resistance

Physical properties, like thermal stability and resistance to proteolysis, are crucial in vaccine design and development. Therefore, we sought to study whether these properties could be optimized when the Seq8 antigen was presented on CaSM particles. According to the thermal stability results (Fig. 1C), CaSM was stable at 4 and 37, and only a slight degradation occurred after 5 and 7 days of storage at 37. Likewise, CaSM-Seq8 was also very stable at both 4 and 37. By contrast, Seq8 was extremely thermo-labile with a complete degradation after 1 day at 37 and a gradual degradation at 4 starting from day 1 to day 7 (Fig. 1D). These results indicated that CaSM could indeed enhance the thermal stability of the presented foreign antigen.

Next, we studied whether the carrier CaSM could enhance the resistance of the exogenous antigen to enzymatic digestion (pepsin and trypsin). As shown in Fig. 1E, CaSM and CaSM-Seq8 were resistant to pepsin but vulnerable to trypsin. However, Seq8 was vulnerable to both pepsin and trypsin and was totally digested. This indicated that CaSM carrier could enhance the resistance of the presented Seq8 antigen to pepsin.

Morphological assessment of the particles formed by the target proteins

The target proteins were visualized under a transmission electron microscope. Both CaSM and CaSM-Seq8 formed spherically-shaped VLPs. According to the radii calculation and frequency analysis, the radius of CaSM was 13.03+-1.739nm (Fig. 2A), and the radius of CaSM-Seq8 particles was 12.91+-1.823nm (Fig. 2B). However, on the Seq8 micrographs, abundant smaller aggregates (radius: 6.68+-1.438nm) and fewer irregular large particles were observed (Fig. 2C), without the classical VLP shape observed with CaSM and CaSM-Seq8. As a control, native HEV virions were also observed, and displayed a radius of 21.36+-2.575nm (Fig. 2D).

To further investigate the reasons for these morphological differences, GalaxyWeb Homomer server was used to compute the assembly patterns of the studied proteins [32]. The results indicated that CaSM had the ability to form pentamers that assembled into decamers, like the native HEV T=1/3 VLPs (Fig. 2E). Furthermore, CaSM-Seq8 showed the same assembly pattern as CaSM and the native HEV T=1/3 VLPs (Fig. 2E). However, Seq8 was predicted to form only dimers (Fig. 2F), which could explain the observations on the electron micrographs.

Antigenicity analysis

First, we sought to assess the antigenicity of Seq8 and CaSM-Seq8 computationally based on the predicted 3D structure models. According to the protrusion index (PI) calculation and comparison, most amino acid residues were more exposed in CaSM-Seq8 than in Seq8 (Fig. 3A&B). Since the Seq8 antigen contains three different neutralizing epitopes (3 G-H loops), the AUC of each epitopes was calculated to evaluate the exposure of each epitope as a whole. The result revealed that the AUC of epitope I and III were significantly higher in CaSM-Seq8 than in Seq8 (Fig. 3C). All these results suggested that the epitopes were more protruding in CaSM-Seq8 than in Seq8, and thus, the CaSM-Seq8 might have a higher antigenicity than Seq8.

Next, we assessed and compared the antigenicity of CaSM-Seq8 and Seq8 experimentally. The results of the indirect ELISA revealed that at almost all dilutions, the reactivity of CaSM-Seq8 against O/Mya/98-

or O/HN/CHA/09-induced antibodies (Fig. 4A), O/JMS/00- or O/GX/09-7-induced antibodies (Fig. 4B), and purified anti-FMDV/O pAbs (Fig. 4C) was stronger than that of Seq8. Similarly, the Western blotting results revealed that the reactive bands of CaSM-Seq8 had higher grayscales than those of Seq8 (Fig. 4E). Altogether, these results were in accordance with the computational analysis and indicated an enhanced antigenicity of Seq8 when it was attached to the CaSM protein.

Immunogenicity analysis

After the injection of CaSM-Seq8, Seq8, and FMDV inactivated vaccine into mice, the induced FMDV-specific IgG levels were monitored up to 10 weeks post-injection using an indirect ELISA (Fig. 5A). 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 (at week 2) than in the inactivated vaccine group or Seq8-immunized mice (at week 4). Additionally, the humoral response induced by the inactivated vaccine and Seq8 began to decrease approximately at week 6 and week 8, respectively. By contrast, the CaSM-Seq8 induced antibodies maintained the highest levels until the end of the experiment. These results indicated that CaSM platform improved the immunogenicity of Seq8, which is in accordance with results of the computational analysis of the antigenicity, where the exposure of the G-H loops was found enhanced by attaching the Seq8 antigen to CaSM.

Discussion

Hepatitis E virus, an icosahedral virus with a diameter of approximately 27-32nm, is an oral-transmitted virus causing acute viral hepatitis[7]. Its capsid protein is encoded by ORF2 and contains three structural domains: S domain (residues 129-319), M domain (residues 320-455), and P domain (residues 456-606) which forms protruding spikes outside the VLP (Fig. 5B) [10,38].

In the present study, the HEV truncated capsid (aa112-455) named CaSM, comprising the S and M domains as well as the flexible proline-rich hinge linking the P domain, was selected to present the exogenous antigen Seq8. The selection of this truncated protein was based on the structural features of these domains reported after the elucidation of high-resolution structures of the HEV capsid [9,10,38]. The S domain, which is the most conserved region among HEV genotypes[33], formed an icosahedral shell serving as the base for the arrangement of the subsequent domains (M and P)[38]. Although HEV capsid seemed to share similar folding and morphology with other viruses such as caliciviruses[39,40], the arrangement of the M domain seemed to be unique to the HEV capsid, because its strong interaction with the S domain allows the enhancement of the VLPs stability[38]. Furthermore, unlike the P2 domain of caliciviruses that is inserted into the P1 domain (M domain) [39], the HEV capsid P domain is independent from the SM-formed shell because of the long proline-rich hinge that links it to the C-terminal of the M domain [9,10,38].

As an exogenous antigen, we designed the Seq8 chimeric protein (Fig. 5C) by combining three neutralization epitopes (VP1 G-H loops) derived from 3 different FMDV/O strains as previously reported[26,27]. According to previous studies, these G-H loops elicited neutralizing antibodies and protective immune responses against virulent FMDV strains [24,26,28]. However, the VP1 G-H loop had low immunogenicity [24,28] and accordingly, many approaches were adopted to improve the immunogenicity of this peptide, including the incorporation of other T and B cell epitopes[25-28]. Therefore, in the present work, we explored the use of HEV CaSM to present Seq8 and the improvement of the immunogenicity of the G-H loops in the chimeric VLPs. In this chimeric construct, the Seq8 antigen was linked to the C-terminal of the CaSM as a substitute of the P domain in the HEV capsid (Fig. 5D).

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[10,38,41]. This suggested that the substitution of the P domain would not interfere with the proper aggregation of the S and M domains of the CaSM-Seq8 and the formation of the desired chimeric VLPs.

To experimentally confirm these predictions, 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 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[12,13]. More interestingly, the CaSM-Seq8 chimeric protein also formed VLPs with approximately the same diameter and morphology than CaSM particles. It is to note that the VLPs formed by CaSM and CaSM-Seq8 were smaller than the native HEV particles, which is very likely due to the difference in the proteins' size (38.3 KDa and 47.9 KDa for CaSM and CaSM-Seq8 respectively, and 70.9 KDa for the native HEV capsid protein). This size parameter could also explain why Seq8 antigen alone (10.6 KDa) could not assemble into spherically-shaped VLPs similar to the CaSM and CaSM-Seq8 ones. These results along with the computational predictions point 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. However, further determination of the high-resolution structure of CaSM-Seq8 chimeric VLPs is needed to confirm these observations.

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 three G-H loops were more exposed when attached to CaSM than when presented on Seq8 alone, suggesting thus, that the antigenicity of Seq8 would be enhanced in CaSM-Seq8 chimeric VLPs. Indeed, the Western blotting and ELISA results showed that the immunoreactivity of CaSM-Seq8 against FMDV-specific antibodies was more robust than that of Seq8 alone. Likewise, after injection into mice, as chimeric VLPs, CaSM-Seq8 induced higher anti-FMDV IgG levels that appeared earlier, increased faster, and lasted longer compared to the humoral responses induced by Seq8 or the FMDV inactivated vaccine. These results indicated clearly that on one hand, the formed chimeric VLPs did not interfere with the presentation of the Seq8 epitopes; and the optimal exposure of these epitopes enhanced the overall antigenicity/immunogenicity of Seq8 epitopes, on the other hand. The improved antigenicity and immunogenicity of the Seq8 moiety in the CaSM-Seq8 can be explained by: 1) 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 host immune system (immunogenicity) and the FMDV-specific antibodies (antigenicity); 2) the self-assembly of CaSM-Seq8 into relatively large VLPs (compared to Seq8 alone) permitted carrying repetitive copies of the target epitopes, which can stimulate more robust immune reactions [42]; 3) numerous T cell epitopes have been located in S and M domains [43], which participate in activating a stronger cellular immune response.

Furthermore, it has been previously reported that HEV VLPs could be used as an oral delivery system [6,16]. Therefore, we investigated the thermal stability and proteolysis resistance the CaSM-Seq8 chimeric VLPs. As a result, both properties were enhanced when Seq8 was carried by CaSM particles, suggesting thus, the potential of CaSM antigen-presenting platform in the design of oral vaccines.

However, further investigation is needed to overcome some limitations of this work: 1) only FMDV Seq8 was used as the exogenous antigen, and whether CaSM was an excellent VLP platform for other antigens needed further research; 2) high-resolution structures of the CaSM VLPs, CaSM-Seq8 VLPs, and Seq8 should be determined to study their structural and immunological characteristics at the molecular level; 3) since many other VLP platforms (HBV, HPV, HIV, etc.) were reported previously[42,44], a comparative study would be of great interest.

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 was 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 that is worth further investigation to evaluate the neutralization activity of CaSM-Seq8- induced antibodies and their in vivo protection against virulent FMDV strains, and to select the best adjuvant, the optimal dose,

and the best administration route.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Figure legends:

Figure. 1. Preparation and proteolysis analysis of CaSM, CaSM-Seq8, and Seq8 proteins. A) SDS-PAGE analysis of two dilutions (0.5x and 0.1x) of the purified recombinant proteins; B) Determination of the target proteins purity by the analysis of the SDS-PAGE bands using Image J; C) SDS-PAGE gel showing the thermal stability of the purified proteins at 37 and 4, followed by the grayscale quantification of the target bands (D); E) SDS-PAGE gel showing the proteolytic action of pepsin (Pep) trypsin (Try) on CaSM, CaSM-Seq8, and Seq8; followed by a grayscale analysis of the target bands (NC: negative control).

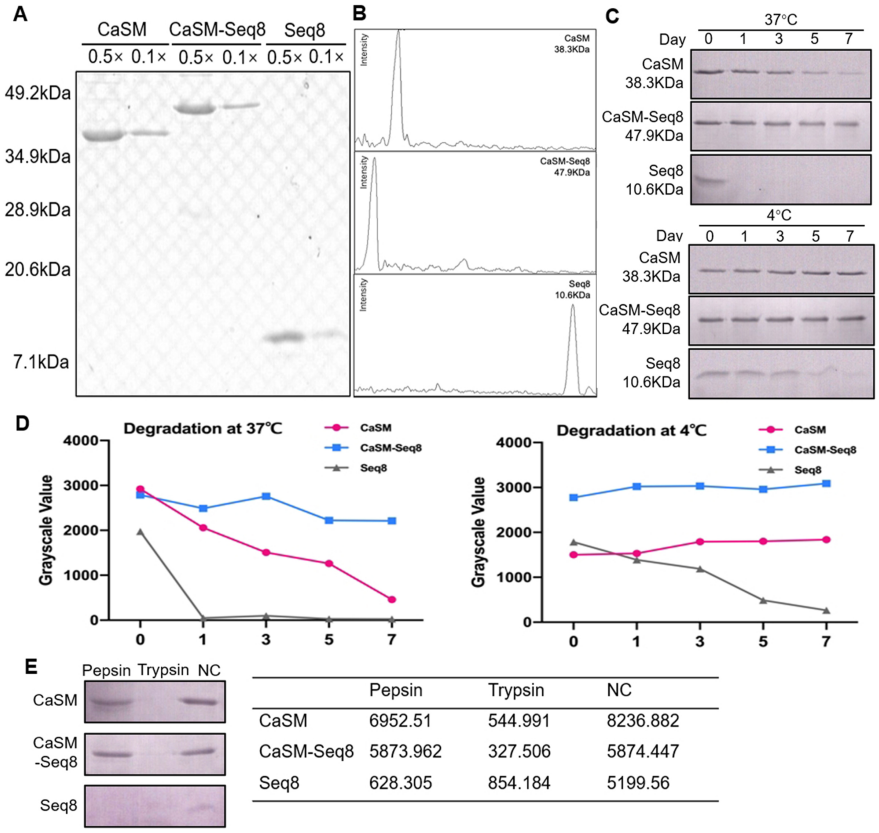
Figure. 2. Morphological analysis of target proteins. A, B, C, and D) Electron micrographs (top) and the radius/frequency analysis (bottom) of CaSM, CaSM-Seq8, Seq8, and native HEV virions, respectively. E) The self-assembly patterns of CaSM, CaSM-Seq8 and HEV capsid protein (PDB ID: 3HAG) as predicted by the GalaxyWeb server. F) The dimerization of Seq8 as predicted by the GalaxyWeb server.

Figure. 3. Computational analysis of the antigenicity of the target proteins. A) Protrusion index (PI) of the Seq8 residues in both CaSM-Seq8 and Seq8 alone; B) Comparison of the fluctuation of PI of the three G-H loops between Seq8 and CaSM-Seq8; C) Column bar representation of the area under the PI curve of the three G-H loops in Seq8 and CaSM-Seq8 proteins.

Figure. 4. Experimental evaluation of the antigenicity of the target proteins. A, B, C, and D) Comparison of the immunoreactivity of CaSM, CaSM-Seq8, Seq8 with FMDV-specific antibodies using indirect ELISA. The anti-FMDV antibodies used in the experiment were: A) O/Mya/98+O/HN/CHA/09-induced antibodies in pigs; B) O/JMS/00+O/GX/09-7-induced antibodies in pigs; C) purified anti-FMDV/O pAbs; D), negative sera collected from FMDV-free pigs; E) Western blotting analysis with the corresponding grayscale values. ****$p<0.0001$, ***$p<0.001$, **$p<0.01$, *$p<0.05$, and ns: $p>0.05$.

Figure. 5. A) Immunogenicity analysis by monitoring the induced FMDV-specific antibody titers in the mice immunized with CaSM-Seq8, Seq8, and the FMDV inactivated vaccine; **$p<0.01$, *$p<0.05$, ns: $p>0.05$. *$p<0.05$, and ns: $p>0.05$.

B) Structure of the HEV capsid protein (S domain in blue, M domain in purple, and P domain in green) and its self-assembly into T=1 VLP (PDB ID: 2ZTN). C) Structure of the FMDV capsid protein (PDB ID: 1FOD) with the VP1 G-H loop shown in green (red box) and a schematic of the design of the recombinant antigen Seq8 containing three different VP1 G-H loops. D) Predicted structure of the constructed CaSM-Seq8.



	Pepsin	Trypsin	NC
CaSM	6952.51	544.991	8236.882
CaSM-Seq8	5873.962	327.506	5874.447
Seq8	628.305	854.184	5199.56