Chapter

A Noninvasive, Orally Stable, Mucosa-Penetrating Polyvalent Vaccine Platform Based on Hepatitis E Virus Nanoparticle

Shizuo G. Kamita, Mo A. Baikoghli, Luis M. de la Maza and R. Holland Cheng

Abstract

Hepatitis E virus nanoparticle (HEVNP) is an orally stable, mucosa-penetrating delivery platform for noninvasive, targeted delivery of therapeutic and diagnostic agents. HEVNP does not carry HEV genomic RNA and is incapable of replication. The key characteristics that make HEVNP an ideal and unique vehicle for diagnostic and therapeutic delivery include surface plasticity, resistance to the harsh environment of the gastrointestinal (GI) tract, significant payload capacity, platform sustainability, and safety. Furthermore, HEVNP is easily produced using currently available expression/purification technologies; can be easily formulated as a liquid, powder, or solid; and can be distributed (and stored) without the need for a temperature-controlled supply chain.

Keywords: hepatitis E virus, HEV, nanoparticle, HEVNP, noninvasive vaccine, mucosal delivery, cryo-EM

1. Introduction

1.1 Noninvasive vaccine delivery by nanocarriers

Currently, most vaccines, drugs, and diagnostic/therapeutic agents are administered through invasive routes such as injection. There has been vast interest in the development of noninvasive, targeted, stable, and convenient drug delivery platforms that obviate the drawbacks of invasive delivery methods (reviewed in [1–6]). Systemic drug delivery through noninvasive routes requires that the delivery platform protects the drug compound while it traverses physiological barriers. Noninvasive delivery platforms, as in the case of traditional delivery platforms, should also distribute the drug effectively and selectively so that only the targeted cells receive the therapeutic agent. The rapid evolution of nanotechnology has shed light on the huge potential of nanocarrier platforms for targeting and drug delivery. Recent developments in the optimization of drug nanocarriers in terms of packaging, delivery, and targeting have the potential to revolutionize noninvasive administration and delivery of therapeutics and diagnostics through the mucosa. Several nanocarrier systems have been developed that take advantage of these

developments and additionally show diminished toxicity in nontargeted cells and tissues. Despite these early successes, instability under physiological conditions, inefficient targeting, toxicity, and lack of bioavailability impose serious limitations for the development of an effective mucosal delivery platform.

While there are several routes of mucosal drug delivery, the oral and nasal routes are among the most safe and preferred by patients. The inherent characteristics of a nanocarrier such as structural composition, size, and natural stability play major roles in the potential success of a drug delivery system (reviewed in [1–4, 7–9]). For many nanocarrier platforms, problems with enzymatic degradation, limited penetration of the thick mucosal layer, and subsequently transportation of drugs through transcellular or paracellular routes are major shortcomings. The majority of currently available nanocarriers consist of simple structures that are on the nanometer to micrometer scale. Theranostic delivery vehicles that are currently used or considered for use fall into a handful of categories including polymers, lipids, solid-lipid carriers, gold carriers, nanotubes, immunostimulant complexes, magnetic carriers, and virus-like particles (VLPs)
[3, 8, 9]. The size and exact composition of these nanocarriers are commonly altered and optimized based on their intended application. The key factors required for entry and distribution of theranostics include high degrees of bioavailability, the ability to withstand physiological conditions without degradation or premature exposure of the drug, and efficient distribution by overcoming the physical and enzymatic barriers through noninvasive routes.

1.2 Nanocarrier platform based on viral capsids

Evolutionarily, viruses adapt and coevolve with their host. Genetic engineering techniques and elucidation of viral structures have enabled virologist to generate empty capsids, called VLPs, which retain the physical characteristics of the capsid structure but lack the viral genome. VLPs thus exhibit the structural characteristics of the authentic virus but are incapable of replicating. In addition to being noninfectious, VLPs are generally nontoxic, biodegradable, and highly biocompatible. Structurally, the symmetrical configuration of VLPs allows them to be developed as nanocarrier systems that can entrap not only foreign nucleic acids but also peptides and imaging agents within their internal cavity. The exterior surface of VLPs, in some cases, can be tagged with targeting ligands without disruption of the VLP structure. The VLP assembles spontaneously and forms highly ordered structures following recombinant expression of the capsid protein (CP) in prokaryotic, eukaryotic, and cell-free protein expression systems. Currently, there are numerous ongoing VLP-based clinical trials worldwide [10–13]. From these clinical trials, a handful of VLP-based vaccines have been approved by the US FDA and other governmental regulatory agencies. For example, VLPs of hepatitis B virus (HBV), human papillomavirus (HPV), influenza virus, human parvovirus, and Norwalk virus have shown success in clinical trials or have been commercially developed as vaccines. The effectiveness of the delivery of therapeutic and/or diagnostic payloads using VLPs, as well as VLP surface modulation by attachment of ligands and tracking molecules, has been recently reviewed [10, 11, 14]. Here, a description of the key advantages and application of hepatitis E virus nanoparticles for use in vaccine development will be discussed.

2. Structure of hepatitis E virus capsid and HEVNP

Significant effort has been invested in characterizing the structure of the capsid of HEV by biochemical methods, imaging (X-ray crystallography and

cryogenic electron microscopy (cryo-EM)), and molecular biological techniques [15–22]. These studies have revealed the underlying architecture and biochemical composition of HEV (reviewed in [1, 2, 23, 24]). The authentic HEV is composed of 180 monomers of capsid protein (CP) that are assembled into an icosahedral cage in an RNA-dependent manner with a triangulation number of 3 (T = 3). Native HEV has a virion diameter of approximately 45 nm. When the native CP is truncated (leaving amino acid (aa) residues 112–608), this truncated CP forms a smaller particle with a diameter of approximately 27 nm. This structure, known as the HEV nanoparticle (HEVNP), is composed of 60 monomers (i.e., 30 CP dimers) of the truncated CP and forms a T = 1 icosahedral conformation. The CP is comprised of three domains: S (shell domain, aa 118–317), M (middle domain, aa 318–451), and P (protrusion domain, aa 452–606) (**Figure 1**). The S domain is the most conserved region among HEV genotypes and, along with the M domain, is responsible for the formation of the HEV capsid base [17, 19, 25]. The P domain, as the name suggests, protrudes from the capsid surface and plays a role in CP dimerization [18, 26], HEV capsid antigenicity [19, 27, 28], and recognition by the host cell receptor [29]. The M domain interacts strongly with the P domain through a long proline-rich hinge; however, the biological roles of the S, M, and P domains are independent [19, 22, 25]. This modular functionality allows the P domain to be genetically modified while (i) causing no or minimal effects on capsid formation and (ii) retaining capsid stability and resistance to acidic and proteolytic conditions found in the mammalian GI tract. Additionally, genetic modification of the P domain results in invisibility of the capsid to host immune surveillance as will be discussed below. Since the P domain of HEVNP is repeated

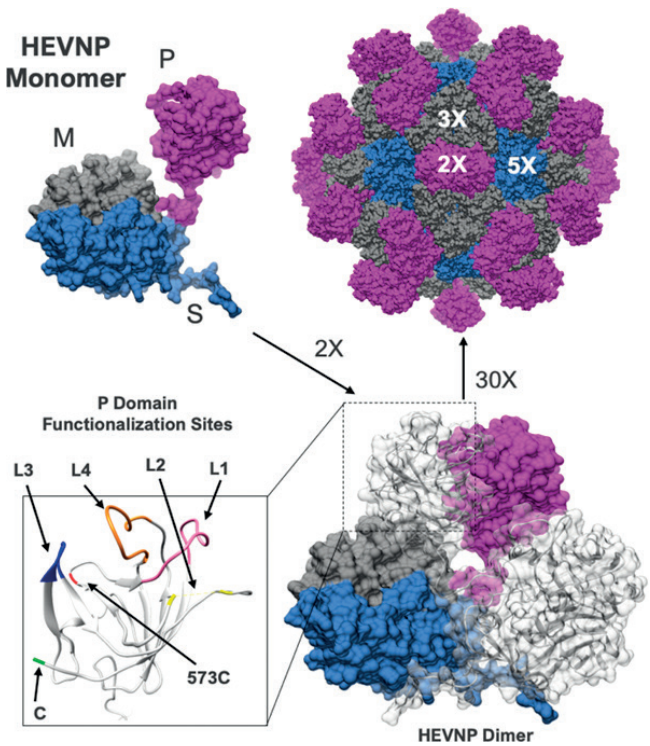

Figure 1.
Modular composition of HEVNP. HEVNP is formed by 30 homodimers of the HEVNP monomer. The dimer is the building block of HEVNP. The HEVNP monomer is composed of three domains: Shell (S), middle (M), and protrusion (P). The P domain has four surface-exposed loops (L1–L4) and a C-terminus that can be genetically or chemically (e.G., at N573C) modified in order to functionalize the HEVNP surface.

60 times on the surface of the capsid, it provides high accessibility for surface modulations that may include targeting ligands, imaging molecules, tracking molecules, and immunogenic peptides.

3. Advantages of waterborne HEVNP for vaccine development

As mentioned above, 30 dimers of the truncated CP of HEV will spontaneously self-assemble into HEVNP following heterologous expression in insect cells or bacteria. Unlike HEV, HEVNP does not encapsulate genomic RNA and is, thus, incapable of replication. HEVNP is, however, capable of encapsulating foreign RNA or DNA. As a vaccine delivery vehicle, HEVNP possesses a combination of advantageous characteristics including surface plasticity, stability within the harsh conditions of the GI tract, significant payload capacity, and platform sustainability.

3.1 Surface plasticity

The utility of the HEVNP as a mucosa-penetrating vaccine delivery platform was successful demonstrated by the development of an orally administered HEVNP-based HIV vaccine [21]. In this groundbreaking study, a 15-amino-acid-long peptide from the V3 loop of HIV-1 gp120 (called P18) was genetically inserted on the surface of HEVNP, generating the HEVNP-P18 construct (also known as 18-VLP). This insertion was successfully made after residue Y485 of the truncated CP, a location that is within the antibody-binding site of HEVNP. Cryo-EM studies revealed that the HEVNP capsid maintained its icosahedral shape and was not disrupted by the P18 insertion. The successful insertion after Y485 resulted in fully formed, stable HEVNP. In contrast, attempts of insertion after aa residues A179, R366, A507, and R542 all failed to achieve the quaternary assembly of HEVNP. Clearly, surface modification of HEVNP via modulation at Y485 by peptide insertion does not interfere with capsid stability or the formation of T = 1 icosahedral organization.

Following the successful insertion of P18 after Y485, four additional aa residues (T489, S533, N573, and T586) have been identified as targets for modulating the surface of HEVNP [30]. These sites are found within four surface-accessible loops (L1, aa 483–491; L2, aa 530–535; L3, aa 554–561; and L4, aa 582–593) that are found on the P domain (**Figure 1**). These sites (as well as Y485) were identified based on (i) their three-dimensional localization on the surface of HEVNP and (ii) the likelihood that mutation would result in minimal or no distortion of the HEVNP structure. In order to test the hypotheses generated by the structural analyses regarding these sites, site-directed mutagenesis was performed in order to replace these residues with a cysteine residue. All of the cysteine mutation constructs successfully assembled into stable icosahedral capsids and were subjected to surface modulation through covalent chemical conjugation. The conjugations were performed via a cysteine acylation reaction with maleimide-linked biotin, and the conjugation efficiency at each site was determined using labeled streptavidin. Of the mutations that were generated (HEVNP-485C, HEVNP-489C, HEVNP-533C, HEVNP-573C, and HEVNP-586C), the HEVNP-573C construct showed the greatest streptavidin signal. This indicated that the N573C mutation of HEVNP-573C is the most surface-visible site for modulation. More recent structural analysis has identified additional aa residues that are found on the P domain (aa residues 510–514 and 520–525) as well as the M domain (residues 342–344 and 402–408) that may be utilized as conjugation sites in a future study.

In order to demonstrate the functionality of the HEVNP-573C construct, a breast cancer cell-targeting ligand LXY30 [31] was chemically conjugated to HEVNP-573C in order to generate HEVNP-573C-LXY30 [30]. The HEVNP-573C-LXY30 construct

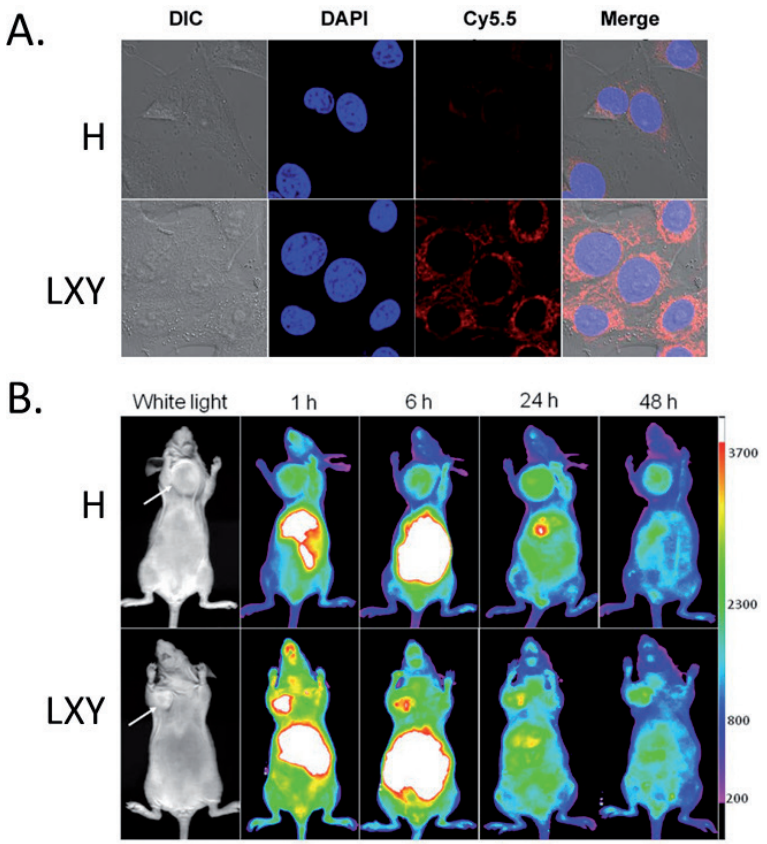

Figure 2.
Selective binding and internalization of LXY30-tagged HEVNP. Breast cancer cells (MDA-MB-231) were inoculated with Cy5.5-labeled HEVNP (row H) or Cy5.5-labeled HEVNP tagged with LXY30 (row LXY). At 1 h post inoculation, the cells were visualized for nuclear dsDNA (DAPI) or Cy5.5. Cy5.5 staining is significantly higher in the cytoplasm of cells inoculated with HEVNP tagged with LXY30 (A). Female SPF BALB/c mice were injected with MDA-MB-231 cells (5 x 10^5). Following the formation of tumors (white arrows), 0.1 nmoles of Cy5.5-labeled HEVNP (row H) or Cy5.5-labeled HEVNP tagged with LXY30 (row LXY) was injected into the tail vein. Optical imaging of live mice at 1, 6, 24, and 48 h post injection (p.i.) showed that LXY30-tagged HEVNP selectively binds to the tumor at 1 h p.i. Staining was also seen in the abdominal regions at 1 and 6 h p.i., likely due to the accumulation of HEVNPs in the liver or other organs prior to degradation (B). Modified from Chen et al., 2016.

selectively binds to cells in the breast cancer cell line MDA-MB-231 (**Figure 2A**). Furthermore, in vivo fluorescence microscopy demonstrates that HEVNP-573C-LYX30, unlike HEVNP-573C, is selectively delivered to breast cancer tumors (formed following the subcutaneous injection of MDA-MB-231 cells in female SPF BLAB/c mice) (**Figure 2B**). These findings demonstrate that HEVNP can be engineered for surface modulation by covalent attachment of a small molecule while maintaining the integrity of the capsid structure. HEVNP thus presents a unique platform for surface functionalization.

3.2 Gastrointestinal tract stability

The human GI tract is divided into the upper tract and lower tract with the upper tract consisting of the mouth, pharynx, esophagus, stomach, and first part of the small intestine (i.e., the duodenum) and the lower tract consisting of the remainder of the small intestine (i.e., jejunum and ileum) and large intestine [32]. Although the duodenum is the shortest portion of the small intestine, it is connected to and/

or associated with the liver, gallbladder, and pancreas through various ducts, veins, and arteries. Following ingestion of HEV, the virus capsid will be exposed to extreme conditions including highly acidic and then alkaline pH, a wide range of digestive enzymes, bile, bacteria and other microorganisms, thick mucosal layers, and mucosal flow throughout the 5-plus-meter length of the human digestive tract. At the cellular level, the inner surface of the intestines is lined with a layer of several types of simple columnar cells including villi and goblet cells that face the lumen. The villi and goblet cells are primarily involved in the absorption of digested nutrients and secretion of a thick (ca. 200 μm) layer of mucosa composed primarily of mucin, respectively. Microfold cells (M cells) are also found in the intestines which play important roles in the initiation of mucosal immunity and the transport of antigens across the epithelial cell layer. HEV has evolved to efficiently overcome these barriers (chemical, enzymatic, mechanical, physical, immunological, etc.) and to eventually initiate a productive infection of cells of the liver and other tissues. Although HEVNP is unable to replicate, it retains the inherent ability of HEV to efficiently target and deliver therapeutic agents through the GI tract with little or no toxicity [1, 2]. This ability of HEVNP to deliver, through oral dosing, a therapeutic payload in a targeted manner using a modular format is currently unavailable through other nanocarrier platforms. Additionally, HEVNP is highly stable to long-term storage at room temperature. Thus, the need for a temperature-controlled supply chain for storage and distribution of HEVNP is minimized or eliminated. This makes the storage and distribution of HEVNP significantly less difficult especially in less developed regions. In addition to being stable at room temperature, HEVNP is water soluble and can be formulated as a liquid (as well as a cream, powder, or solid) which allows HEVNP to be administered noninvasively as a drink or droplet.

3.3 Significant payload capacity

HEVNP functions as an epitope nanocarrier through display of the epitope on its surface. HEVNP also has a large hollow core with a width that ranges from approximately 10 to 12 nm (**Figure 3A**) that can be loaded with a payload such as a nucleic acid chain, peptide, or small molecule. This large hollow core results from the space within HEVNP that in HEV encapsulates genomic RNA. A payload can be encapsulated within the hollow core of HEVNP using a simple process that reversibly disassembles HEVNP and then reassembles it in the presence of the payload molecules. This reversible process occurs through chemical reduction, chelation of Ca^{2+}, and the subsequent return of Ca^{2+} (**Figure 3B**). Specifically, HEVNP disassembles in the presence of DTT and EGTA and reassembles by the slow addition of Ca^{2+}. If peptide molecules such as insulin or inorganic molecules such as ferrite are present during the reassembly process, these molecules are encapsulated within the reassembled HEVNP. Similarly, in the presence of DNA or RNA, the reassembled HEVNP will incorporate the nucleic acid molecule, and HEVNP can function as an orally deliverable DNA vaccine nanocarrier (reviewed in [2]). For example, plasmid DNA-encoding HIV envelope gp120 has been encapsulated by HEV VLP, and this construct has been used to orally deliver the plasmid to the spleen, Peyer's patches, and mesenteric lymph nodes of mice [33]. Cell-mediated immune (specific cytotoxic T-lymphocyte (CTL) response) and specific humoral responses are generated locally and systemically. A payload is not essential for HEVNP capsid formation or capsid stability, but having this capacity offers a way to further increase the epitope signal beyond the 60 epitope copies that can be placed through chemical conjugation or genetic insertion on the HEVNP surface via the P domains.

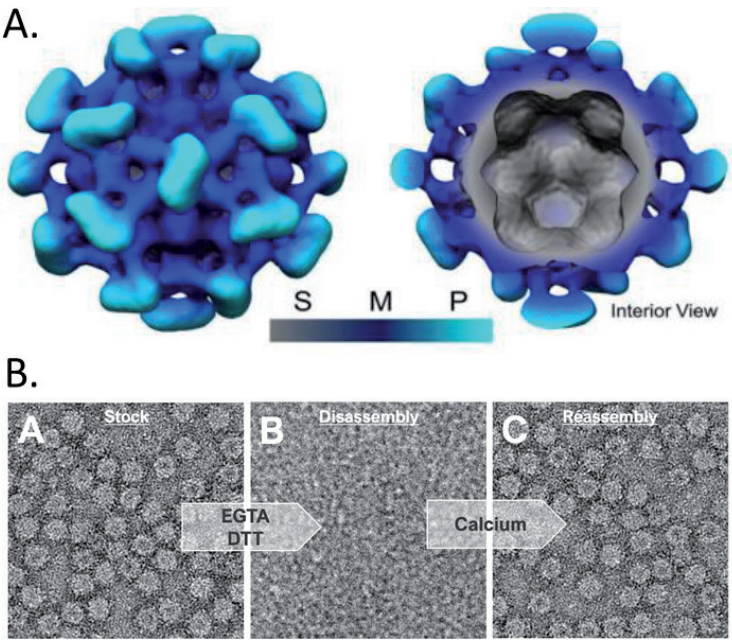

Figure 3.
Structure and disassembly/reassembly of HEVNP. The HEVNP monomer is composed of three domains: Shell (S), middle (M), and protrusion (P). The surface and interior localization of these domains is indicated by the color map (A). A large hollow core is found in the interior of HEVNP (right image in A). The hollow core can encapsulate various payloads such as nucleic acids, peptides, small proteins, or small molecules. Electron microscopic images of the process of HEVNP disassembly (following the addition of EGTA and DTT) and reassembly following the addition of calcium (B).

3.4 Platform sustainability by prevention of self-immunity

While HEVNP exhibits natural tolerance against the harsh enzymatic environment associated with the digestive tract, its repeated use as a drug delivery vehicle will quickly result in self-immunity if a mechanism is not in place to avoid this common problem. As discussed above, insertion of the HIV-1 P18 peptide onto the surface P domain maintains the icosahedral arrangement of P18-HEVNP and indicates that intermolecular forces between the truncated CP of the recombinant nucleocapsid are not disrupted by the insertion of P18. Additionally, since the antigenicity of HEVNP lies specifically within the P domain, the insertion of the P18 peptide significantly lowers immune detection of the HEVNP vehicle [21] (**Figure 4A**). The immune reactivity of P18-HEVNP has been tested by two antibodies, 447-52D and HEP224. Antibody 447-52D specifically targets the V3 loop of HIV-1 gp120, and monoclonal antibody HEP224 targets the conformational epitope (i.e., the three loops around Y485) of the P domain of HEV CP. Based on ELISA experiments, antibody 447-52D shows preferential binding of P18-HEVNP. On the other hand, the binding of antibody HEP224 to the conformational epitope of the P domain of HEV CP is disrupted by the insertion of HIV P18 without altering the structural characteristic of HEVNP. Thus, insertion of specific peptides into the exposed P domain serves as a practical strategy to escape antibody recognition by the immune system (i.e., issues with self-immunity) while triggering the desired humoral and cellular responses against the attached/inserted antigen. Similarly, an HEV-specific monoclonal antibody, Fab230, fails to recognize HEVNP after maleimide-biotin conjugation at position N573C of HEVNP [30] (**Figure 4B**). Additionally, the geometrical constraints provided by the M domain provide a

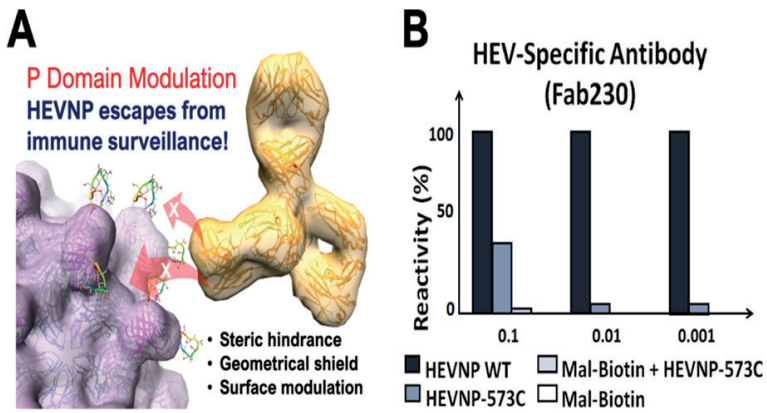

Figure 4.
3-D modeling of HEVNP surface modulation. Surface modulation through chemical conjugation or genetic modification promotes the escape of HEVNP from immune surveillance (A). Surface modulation of HEVNP with maleimide-biotin or mutation of the P domain at residue N573 dramatically reduces cross reactivity with the HEV-specific monoclonal antibody fab230 by ELISA (B).

physical barrier for antibody binding which helps HEVNP avoid immune system surveillance by HEV-specific antibodies. These findings show the sustainability of the HEVNP nanocarrier platform.

3.5 Safety of HEVNP

HEV annually causes acute and self-limiting infection in about 20 million people worldwide [34–36]. The majority of people infected with HEV show clinical symptoms that are relatively mild, and death rates from hepatitis E are low. The disease, however, is more severe in pregnant women, and chronic infection may occur in immunocompromised individuals. Although the exact mechanism of the increased severity of the disease during pregnancy is unknown, there is some evidence that increased viral replication in placental tissues plays a role [34, 35]. Thus, in a large proportion of the population, HEV is naturally a low-virulence pathogen. The low virulence of HEV and the inability of HEVNP to replicate (because it does not carry HEV genomic RNA) suggest that an HEVNP-based nanocarrier will not induce undue virulence in patients.

3.6 Established production and engineering technology

A common eukaryotic cell-based technology for vaccine production utilizes recombinant baculoviruses and insect cells. Baculoviruses are arthropod-specific viruses that are commonly used to produce recombinant proteins for basic research and commercial applications. Baculoviruses have been successfully used to produce human therapeutics and diagnostics since the late 1990s [12]. A recent example of a baculovirus-based vaccine is Flublok (released in 2013 by Protein Sciences Corporation), a vaccine against human influenza virus. The baculovirus expression vector system is also used to express the major capsid protein L1 of human papilloma virus. The recombinant L1 capsid protein forms a VLP-based vaccine (Cervarix™) that protects against cervical cancer [12, 37]. The commercial GMP technology that is currently used to express and purify these vaccines and others can be easily adapted for the production of recombinant truncated CP and the engineering of HEVNP-based vaccine delivery nanocarriers.

4. Single epitope modification of HEVNP: HIV-1 GP120 P18 example

As discussed earlier, insertion of the P18 peptide from the GP120 protein of HIV-1 results in a stable HEVNP that displays P18 on its surface (in the P domain after residue Y485). Additionally, the P18 insertion significantly lowers the immune system response against HEVNP. When the P18-HEVNP construct is orally inoculated into mice, it induces strong and specific cell-mediated and humoral responses in comparison to immunization with HEVNP [21]. After three rounds of oral immunization, the cell-mediated response includes the lysis of cytotoxic T lymphocytes (CTLs) in three immune system-associated organs. Similarly, humoral responses (IgG, IgA, and IgM induction) in the sera and intestinal fluids are detected by ELISA. These responses were generated by P18-HEVNP without the need for an external adjuvant coadministration.

5. Multiple epitope modifications of HEVNP: MOMP example

Over 120 million people are annually infected with *Chlamydia trachomatis*. Because the initial stages of chlamydia are generally asymptomatic, many individuals are unaware that they are infected and do not seek antibiotic treatment. As the infection spreads, chronic abdominal pain, pelvic inflammatory disease, ectopic pregnancy, and infertility can result. The major outer membrane protein (MOMP) of *Chlamydia*, in its native trimeric form (nMOMP), has been demonstrated to impart significant protection against chlamydial infection and disease in a mouse model. MOMP is a structurally rigid, 40 kDa trimer-forming protein that makes up about 60% of the total mass of the outer membrane of *Chlamydia* [38, 39]. MOMP itself is characterized by five constant domains (CDs) and four variable domains (VDs) which help to define the immunogenicity of various serovars of *Chlamydia* [40, 41].

MOMP is the immunodominant antigen of *Chlamydia* and has multiple epitopes for T-cell and B-cell activation; thus, it induces both cell-mediated and humoral immunity [42–46]. Mice that are vaccinated with the nMOMP with Freund's adjuvant are significantly protected from the effects of *Chlamydia* in terms of a shedding assay and infertility [47]. In contrast, denatured MOMP does not offer this protection. The denatured MOMP, however, induces a greater humoral response than nMOMP. Robust protective activity following vaccination with nMOMP and other adjuvants has also been reported [45, 48]; this activity was similar to that of mice that were immunized intranasally with live *Chlamydia* elementary bodies (EBs). These and other studies [47, 49–52] using various readouts (i.e., body weight, lung weight, number of inclusions forming units recovered, length of shedding, etc.) demonstrate the important role of MOMP in inducing protection against *Chlamydia*.

Formulating a vaccine with a properly folded membrane protein such as MOMP remains a genetic engineering challenge. The use of membrane proteins for vaccine applications requires a platform that can be engineered to enable proper folding of the membrane protein, potentially allow for adjuvant incorporation, and be amenable to the display of multiple epitopes employing multiple display strategies. In the past 10 years, incorporating membrane proteins into nanolipoprotein particles for both solubility and stabilization has become increasingly common with varied success.

In our laboratory, HEVNP has been used as a platform to display two MOMP VD epitope sequences (VD1 and VD4). With this construct, HEVNP-VD1/HEVNP-VD4, the VD1 and VD4 peptide sequences were genetically incorporated at S533 and T485, respectively, of the truncated capsid protein, a region corresponding

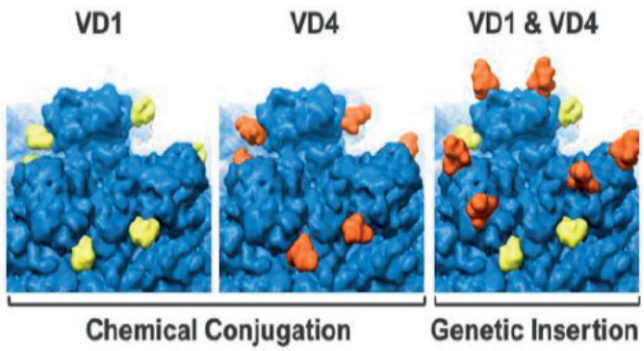

Figure 5.
Chlamydial vaccine design. Peptide sequences from variable domain 1 (VD1, yellow epitopes) and/or VD4 (orange epitopes) from the major outer membrane protein of Chlamydia *were chemically conjugated to amino acid residue N573C (left and center panels) or genetically inserted (after amino acid residues S533 and T485, respectively, right panel) to the P domain of HEVNP.*

to surface-exposed loops L2 and L1, respectively. Analysis of the visibility of these epitopes by structural modeling (**Figure 5**) and by ELISA using VD1- or VD4-specific antibodies (data not shown) indicates that all three of these constructs are highly immunogenic, suggesting that the epitopes are authentically displayed on the surface of HEVNP. Furthermore, in preliminary animal experiments, anti-MOMP IgG levels in the serum of mice that are immunized by HEVNP-VD1/HEVNP-VD4 (prime and two boosts) are like those found following immunization with a whole *Chlamydia* cell vaccine. These findings are highly exciting; and we are currently investigating whether there is also a cell-mediated immune response induced by HEVNP-VD1/HEVNP-VD4 and, if there is, how this response compares with that induced by a whole cell vaccine.

6. Conclusions

As a nanocarrier, HEVNP is a structure that can display multiple epitopes on its surface; and simultaneously it can deliver a payload, for example, an epitope encoding nucleotide sequence, peptide, or small molecule. Unlike nanoparticles generated from polymers, lipids, nanotubes, or other carriers, HEVNP delivers epitopes and payload through the mucosa of the GI tract without the need for any, potentially deleterious, exogenous enhancers such as a mucosal breakdown enzyme, pH regulator, or uptake cofactor. The key characteristics that make HEVNP an ideal and unique vehicle for vaccine delivery include: (i) *Surface plasticity*. Sites on the P domain can be engineered for site-specific attachment or insertion of the epitope(s). Even when the surface of HEVNP is genetically or chemically modified, the core structure of HEVNP remains intact. (ii) *GI tract stability*. Even when surface modified, HEVNP is stable to the harsh conditions of low pH and proteolytic enzymes that are found in the GI tract. This allows HEVNP to deliver epitopes orally. HEVNP has the capability to penetrate the mucosal lining of the entire GI tract and other mucosa-lined cavities or organs and directly target cells of the basement membrane. (iii) *Significant payload capacity*. The large hollow core of HEVNP can package and protect large biological molecules including DNA and RNA. (iv) *Platform sustainability*. Immune recognition of the carrier platform is negated with HEVNP. The surface P domain carries the primary antigenic sites of HEV (and HEVNP). Thus, modification of the P domain by chemical conjugation or genetic insertion of a vaccine epitope completely neutralizes endogenous immunogenicity

against HEVNP carrier platform. In addition, the HEVNP nanocarrier platform can overcome many of the drawbacks of other nanocarrier platforms including issues with (1) formulation, (2) production, (3) safety, (4) cold chain distribution, (5) target selectivity, and (6) signal amplification.

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Conflict of interest

None.

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