Received: 25 May 2023 | Revised: 31 July 2023 | Accepted: 2 August 2023

DOI: 10.1002/rmv.2474

REVIEW

WILEY

Prospects for developing an Hepatitis C virus E1E2-based nanoparticle vaccine

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Funding information
University of Maryland Strategic Partnership: MPowering the State; National Institutes of Health

Abstract

Globally, more than 58 million people are chronically infected with Hepatitis C virus (HCV) with 1.5 million new infections occurring each year. An effective vaccine for HCV is therefore a major unmet medical and public health need. Since HCV rapidly accumulates mutations, vaccines must elicit the production of broadly neutralising antibodies (bnAbs) in a reproducible fashion. Decades of research have generated a number of HCV vaccine candidates. Based on the available data and research through clinical development, a vaccine antigen based on the E1E2 glycoprotein complex appears to be the best choice, but robust induction of humoral and cellular responses leading to virus neutralisation has not yet been achieved. One issue that has arisen in developing an HCV vaccine (and many other vaccines as well) is the platform used for antigen delivery. The majority of viral vaccine trials have employed subunit vaccines. However, subunit vaccines often have limited immunogenicity, as seen for HCV, and thus multiple formats must be examined in order to elicit a robust anti-HCV immune response. Nanoparticle vaccines are gaining prominence in the field due to their ability to facilitate a controlled multivalent presentation and trafficking to lymph nodes, where they can interact with both arms of the immune system. This review discusses the potential for development of a nanoparticle-based HCV E1E2 vaccine, with an emphasis on the potential benefits of such an approach along with the major challenges facing the incorporation of E1E2 into nanoparticulate delivery systems and how those challenges can be addressed.

KEYWORDS
HCV E1E2, nanoparticles, nanoparticulate delivery systems, vaccine

Abbreviations: AR, antigenic region; bnAbs, broadly neutralising antibodies; cryo-EM, cryoelectron microscopy; DAAs, direct-acting antivirals; E2p, dihidrolipoyl acetyltransferase; FDC, follicular dendritic cell; Gts, genotypes; HCV, Hepatitis C virus; HCVcc, HCV cell culture virus; HCVpp, HCV pseudoparticles; HVR-1, hypervariable region 1; ID_{50}, dilution at which 50% inhibition is achieved; LS, lumazine synthase; mAb, monoclonal antibody; mbE1E2, membrane-bound E1E2; NP, nanoparticle; PLG, poly(D,L-lactic-co-glycolic acid); PPZ, polyphosphazene; sE1E2, secreted E1E2; sE2, secreted E2; Th1, Th2, T helper 1, 2; TMDs, transmembrane domains; VLP, virus-like particle.

1 | INTRODUCTION

Hepatitis C virus (HCV) is an RNA virus with highly error-prone replication resulting in high mutation rates, making vaccine development for this pathogen challenging. HCV is a major cause of severe liver diseases and cancer, and the global burden is over 58 million chronically infected individuals with an annual increase of 1.5 million new infections.[1] Moreover, HCV is responsible for more deaths in the USA than all other infectious diseases combined.[2] Nearly 75% of infected individuals progress to chronic HCV infection, which often leads to cirrhosis or hepatocellular carcinoma, a frequently fatal form of liver cancer. The World Health Organization established a goal to reduce new HCV infections by 90% and associated deaths by 65% by 2030.[1] The availability of direct-acting antivirals (DAAs) will play a large part in achieving this goal as they cure existing HCV infections.[3,4] However, most HCV-infected individuals are unaware of their infection status because they are asymptomatic until liver damage is extensive enough to present as liver disease. Moreover, DAAs remain too costly for many in the U.S., particularly for high risk groups such as intravenous drug users[5] and even more so in developing countries with high disease burdens. In addition, DAA-treated individuals can become re-infected which decreases effectiveness in high-risk groups. Therefore, preventive measures such as vaccines are a key priority,[6] and there is a clear and urgent need for a prophylactic HCV vaccine.[7] The high rates of spontaneous clearance and presence of immune memory among individuals who clear their first HCV infection[8–11] point to the feasibility of such a vaccine.[7,12–14]

There is broad agreement that both B and T cell immunity contribute to the control of acute HCV infection.[15–17] Thus, for a successful vaccine, the immunogen will need to robustly elicit both responses. Consistent with this idea, broadly neutralizing antibodies (bnAbs) infused into humanized mice or chimpanzees protect against HCV infection.[18–20] Moreover, viral clearance is associated with a robust induction of bnAbs early in the infection.[21–24] A robust and timely induction of bnAbs is therefore a requirement for a protective HCV vaccine. A major challenge in developing a successful HCV vaccine is its remarkable genetic diversity, which has six major genotypes (gts 1–6), two less common genotypes[25] (gts 7–8), and intra-genotypic diversity resulting in 90 subtypes.[26] The virus actively escapes the B cell and T cell responses in infected individuals.[27] In particular, escape of the B cell response was observed in a clinical trial of a monoclonal antibody (mAb) therapeutic where escape variants accompanied viral rebound.[28] HCV can also escape the immune response via glycan shielding, direct cell-cell transmission, and downregulation of major histocompatibility complex proteins.[29] An ideal vaccine should elicit high antibody titers directed against multiple conserved E1E2 epitopes to ensure broad neutralization,[19,30–33] in conjunction with cytotoxic and tissue-resident memory T cells to achieve immunity and protection against a high diversity of HCV isolates.

1.1 | Virus neutralisation and the E1E2 glycoprotein complex

In developing a prophylactic vaccine for HCV, the choice of antigen has not always been clear due to production issues associated with the E1E2 glycoprotein complex, in part due to its membrane association. The HCV envelope glycoprotein complex, as the target of the protective antibody response, has been the primary focus of most vaccine development to date. The envelope complex of HCV contains two glycoproteins, E1 and E2, which are encoded as part of the HCV polyprotein expressed in infected liver cells. This polyprotein is processed in the ER by signal peptidases and cellular glycosylation machinery to produce the mature E1E2 complex. These glycoproteins are membrane-anchored via their C-terminal transmembrane domains (TMDs), resulting in a membrane-bound E1E2 (mbE1E2) complex. A secreted version of the E2 protein (sE2) lacking the transmembrane domain[34] has been the focus of a number of vaccine studies.[35–41] However, immunological assessment in chimpanzees of an E1E2 vaccine produced superior immune responses as compared to E2 administered alone and resulted in sterilising immunity against homologous virus challenge,[42,43] but with some cross-neutralisation capacity against heterologous isolates.[44] Additional studies in mice[36,45] also showed an E1E2-based vaccine candidate to be superior to E2 alone. Moreover, E1E2 has been tested in humans and is well-tolerated.[46] However, due to the limited neutralisation breadth observed in the human clinical trial,[47,48] ongoing research efforts should likely focus on developing an optimised, modified form of E1E2 that can elicit more robust, cross-neutralising responses against multiple heterologous isolates. This includes stabilising mutations that focus the immune response on conserved epitopes and alternate platforms like nanoparticles (NPs). Alternatively, a multivalent vaccine consisting of E1E2 subunits from multiple genotypes could be a consideration, albeit a more complex vaccine formulation to develop.

To develop a vaccine for HCV, the candidate antigen needs to be produced at large scale, reproducibly, and at a cost that is not prohibitive. Most antibodies with broad virus neutralising activities to diverse HCV isolates recognise conformational epitopes in E2 and in the E1E2 complex.[19,49–55] Previous work identified highly-conserved antigenic regions (ARs) on E2 that elicit bnAbs.[55–57] These regions are called antigenic domains B, D, and E (used for this review). Other groups have designated such regions as epitopes I-III[58] or antigenic regions (which include epitopes that contain residues from both E1 and E2[19,51]). Domains B and D (Figure 1) are conformation-sensitive, whereas domain E contains a linear epitope.[56] However, while not conformation-sensitive in the same sense as domain B and D NAbs, domain E adopts multiple conformations, as observed when bound to different anti-domain E mAbs,[60–63] one of which is associated with resistance to viral escape.[61] Thus, any successful vaccine candidate should elicit robust induction of antibodies similar to AR4A, HEPC74, AR3A, and HC84.26 as those mAbs are most prominently associated with viral clearance.[32] Of these, HEPC74 and AR3A bind to domain B and HC84.26 binds to domain D. Parts of antigenic domains B, D, and E

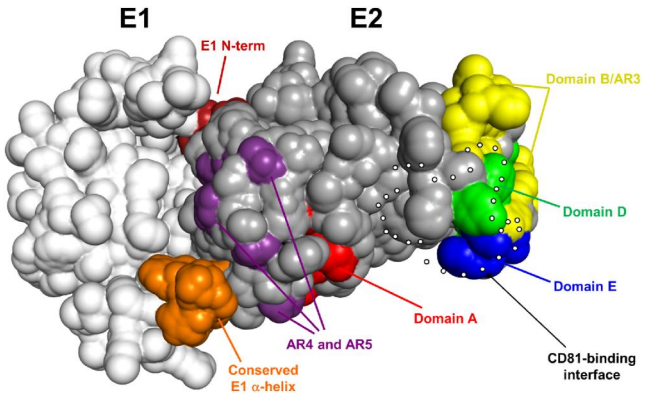

FIGURE 1 Conserved antigenic domains on the E1E2 complex. The E1E2 complex (RCSB ID 8FSJ) is represented as a solvent-excluded surface. The antigenic domains in the E1 ectodomain are the N-terminal residues 192–202 (dark red) and a conserved α-helix (α2, residues 314–327). This helix appears to have a role in viral entry as mutants in this region result in structurally intact but noninfectious virions.[59] For the E2 ectodomain, the conserved antigenic domains are domain A (red, residues 581–584 and 627–634), domain B/AR3 (yellow, residues 431–439 and 529–535), domain D (green, residues 420–428, 441–443, and 616), and domain E (blue, residues 412–423). The AR4/5 region, which requires the presence of both E1 and E2 is coloured dark purple and comprises residues 635, 646, 649, 652, 657, 658, 667, 671, 676, 677, and 696.

comprise the majority of the CD81-binding interface on the E2 ectodomain[64] and thus mAbs that bind to these domains can block receptor binding as part of their role in virus neutralisation. In addition, studies with a panel of E1-specific mAbs showed that that E1-specific responses can both protect against virus challenge and broadly neutralise HCV pseudoparticles (HCVpp) of various genotypes.[65,66] However, a similar correlation between E1-specific mAbs and viral clearance has not yet been identified.

1.2 | Structure-based vaccine design – Past limitations and future prospects

Limited high resolution structural data are available for the E1 glycoprotein. The E1 ectodomain contains 158 residues (residues 192–349 of the H77 sequence; NCBI Refseq ID NC_004102), of which eight are cysteine residues that form four putative disulfide bonds, and five potential N-glycosylation sites. The reported X-ray structure of an N-terminal fragment of E1 (residues 192–270) exhibits domain swapped and disulfide cross-linked oligomers,[67] that are no doubt the result of the C-terminal residue truncation and lack of E2 context. As noted in a recent review,[68] small helical fragments of E1, one a crystal structure comprising the IGH526 binding motif in complex with the IGH526 Fab,[69] and the other and NMR structure of the transmembrane helix[70] have also been reported.

In contrast to E1 and E1E2, the E2 ectodomain has been subjected to a number of structural studies. The initially reported structures of the core E2 ectodomain structure[71,72] revealed a folded domain with significant unstructured regions, with the overall fold stabilised by numerous disulfide bonds between conserved cysteines. The two core structures unexpectedly exhibited different disulfide bonding patterns. This is likely due to different truncations of the E2 sequences used in the two structures,[73] but does highlight the possibility that disulfide bond exchange can occur within the E1E2 complex or among multiple E1E2 complexes. Additional structures of E2 in complex with various monoclonal antibodies from humans,[74–77] and recently macaques[78] have been reported. These structures are primarily complexes with neutralising antibodies, and as such show engagement mostly with domains B and D (Figure 1) which make up the majority of the interface that binds to the co-receptor CD81. Common features of these structures are a biased VH1-69 germline gene usage and a CDRH3 loop disulfide motif.[74,75] Additional computational and experimental studies[79–81] have suggested that E2 (and by extension E1E2) exists in putative "closed" and "open" conformations that dictate co-receptor binding and antibody neutralisation. In addition, subsequent studies highlighted the potential role of hypervariable region 1 (HVR-1), which resides at the N-terminus of E2 as a region on E1E2 that can control such activity.[82] The authors proposed that HVR-1 acts as an entropic "safety catch" that modulates E1E2 receptor binding activity and thereby antibody sensitivity. Removal of HVR-1 induces an E1E2 "hyperactive" state that is both highly susceptible to antibody neutralisation and prone to spontaneous inactivation.[82] Other viral envelope glycoproteins, including HIV Env[83] and the SARS-CoV-2 spike[84] have exhibited similar metastable behaviour and distinct conformations.

For quite some time, a lack of structural information regarding the E1E2 glycoprotein complex hindered vaccine design efforts, including design and production of E1E2-based NPs. Recently, a structure of the mbE1E2 complex bound to the antibodies AR4A, AT1209, and IGH505 was determined by cryoelectron microscopy (cryo-EM),[85] revealing details about the interface between E1 and E2 and the AR4A interface with the complex that will greatly aid in rational design of a vaccine against HCV. In addition, the recent development of a soluble, secreted E1E2 complex (sE1E2)[36,45] and determination of its structure[86] further enables the potential assembly of E1E2-based NPs as vaccine candidates. In particular, the sE1E2 complex adopts a native structure, with an RMSD for Cα atoms between the two structures of 1.34 Å. This structure demonstrates that a native E1E2 complex that is untethered from the membrane can be made with the appropriate scaffold, which opens up the possibility of developing native E1E2 NP vaccine candidates. Moreover, these structures afford opportunities to stabilise the E1E2 interface and modify the E1 and E2 surfaces to make the complex more amenable to NP incorporation. This review will focus on the important considerations relevant to the potential and feasibility of making an E1E2-based NP vaccine.

2 | ANTIGEN SIZE AND THE IMMUNE RESPONSE

Subunit vaccines, which comprise a limited portion of the infectious virus known to be immunologically important, are typically safer to use than the live attenuated viruses used for most of the history of

vaccination. This enhanced safety stems in part from the ability to produce highly purified preparations of the subunit vaccine. However, the ease of production and high level of purity comes at the expense of immunogenicity. One factor is that intact live or attenuated viruses often contain immunopotentiating components that facilitate a robust immune response. Another factor is size, which affects antigen uptake and clearance/degradation and also determines the presentation mode of the vaccine antigen. Subunit vaccines are most often either monomeric or low-order oligomers (e.g. HIV env trimers[87]), whereas live and attenuated viruses present the vaccine antigen in a polyvalent manner in a confined volume on the virion itself. For example, the SARS-CoV-2 virus, an inactivated form of which is used as a vaccine,[88] contains on average 26+/−15 spike trimers on the surface of the virus,[89] a valency that does not exist in the purified spike subunit ectodomain and likely aids in eliciting a robust immune response. A similar effect has been observed for inactivated HCV produced in Huh7.5 hepatoma cells,[90] where the antibody titre achieved after immunisation of mice with inactivated HCV was five orders of magnitude larger than that for the E2 ectodomain alone. However, it is unclear based on that study and subsequent studies[91,92] whether the enhanced antibody titre translated to enhanced neutralisation potency or protective efficacy. It is possible that an effective HCV vaccine will require precise immunopotentiation of the principal neutralising determinants in order to facilitate a protective response. The varied and variable host cell components contained in an inactivated HCV vaccine[93] might amplify the overall immune response at the expense of HCV-specific targeted Toll-like receptor activation and a balanced T helper 1/2 (Th1/Th2) response. Moreover, an issue that plagues inactivated HCV as a vaccine candidate is the inability to produce the virus in the amounts needed for larger scale studies. However, the recent development of high titre cell culture virus for use as a vaccine candidate[91,92] has made proof-of-concept studies using inactivated HCV more feasible.

3 | COMMON MICRO- AND NANOPARTICULATE SYSTEMS

3.1 | Dispersions, emulsions and supramolecular assemblies

In order to recapture some of the immunogenic potency of the virus vaccine system, a variety of particulate and emulsion-based formulations have been explored as detailed in recent reviews.[94–96] One of the most common methods of making a particulate subunit vaccine is to formulate the subunit antigen with a heterogeneous Alum adjuvant, however it typically forms aggregated microparticles ranging from 0.5 to 10 μm in size.[97] Additional platforms for particulate and emulsion formulations, which have been explored with HCV have been recently reviewed[98] (Figure 2). These include polymer-based nanoparticulates, such as those based on bioerodible poly(D,L-lactic-co-glycolic acid) or PLG[99,100] and micelle-forming pluronics,[101] emulsions, such as MF59[102] and Montanide,[103] and nanovesicles, which include liposomes[104] - a basis for the advanced AS01[105] and CAF01[106] systems. In addition, for some membrane-associated glycoprotein vaccine candidates, the formulation process creates rosettes of antigen protruding from a hydrophobic micelle core, thereby creating nanoparticulate vaccines without the use of additional protein scaffolds.

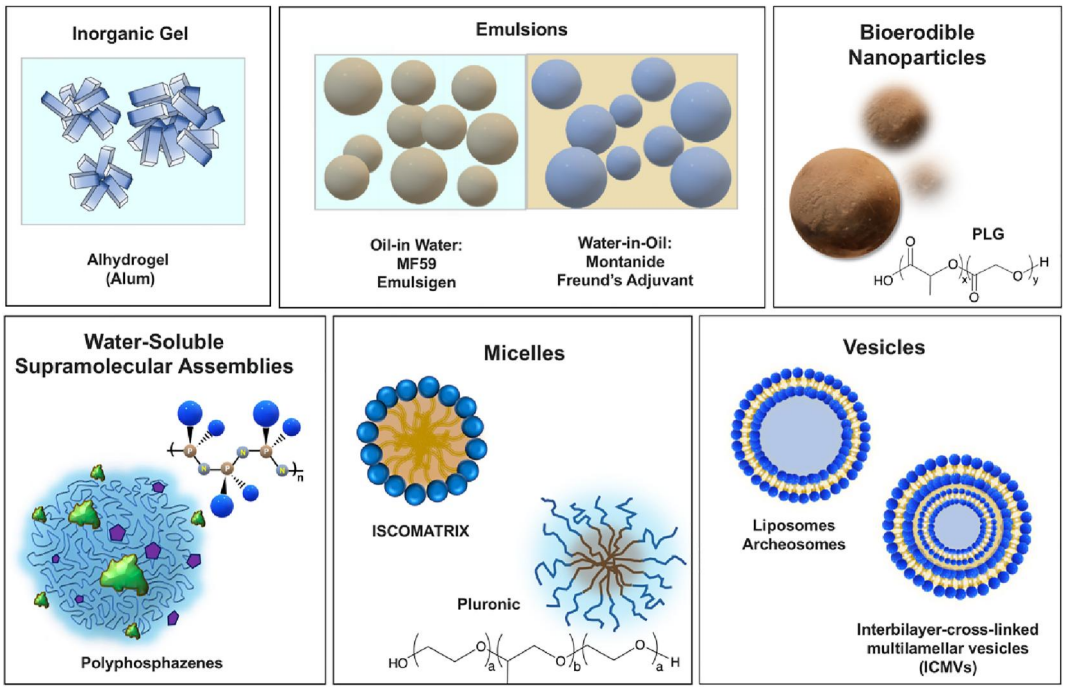

FIGURE 2 Overview of polymer, emulsion and vesicle-based delivery systems (Reprinted with permission from Andrianov AK, Fuerst TR. Immunopotentiating and Delivery Systems for Hepatitis C virus (HCV) Vaccines. Viruses. 2021; 13(6)).

The most notable examples are Flublok[107,108] and the protein-based SARS-CoV-2 vaccine that uses full-length spike.[109] Biodegradable polyphosphazene (PPZ) adjuvants, such as PCPP and PCEP, are synthetic nano-sized macromolecules, which are completely water-soluble and not biphasic.[98,110–112] However, when mixed with HCV and other antigens in aqueous solutions, they spontaneously assemble into supramolecular nanoassemblies with virus-mimicking dimensions (~60–120 nm).[113,114] Thus, PPZ adjuvants and delivery systems act as immune-potentiating nanoassemblies that enhance antigen uptake, provide multivalent antigen presentation, and stimulate innate immunity.[110–112] We and others have demonstrated through animal studies that PPZ adjuvants increase vaccine immunogenicity, enable antigen dose-sparing, and achieve long-lived protection in challenge studies.[115–119]

3.2 | Virus-like particles

Another platform that is particulate in nature is virus-like particles (VLPs). VLPs are often highly immunogenic and exhibit a marked increase in immunogenicity relative to a subunit vaccine containing a single component.[120] Well-known examples of VLP-based vaccines are Energix (hepatitis B virus) and Gardasil (human papilloma virus). However, VLPs can be difficult to produce, and making a VLP for HCV has been particularly challenging. In particular, the baculovirus Sf9 cell-based HCV VLP production system exhibited less efficient polyprotein cleavage than observed in mammalian cells and as a result two different forms of E2 are present in those VLPs.[121] There is also the challenge of producing uniform HCV VLPs in high yields. The Sf9-based HCV VLPs have been used in non-human primate studies, but immunisation with the VLPs failed to produce a robust antibody response in chimpanzees.[122] However, a T cell response was observed and the chimpanzees were protected from a homologous virus challenge. Mammalian systems have also been developed for HCV VLPs.[123,124] The VLPs produced in mammalian cells are morphologically similar to native virions and also incorporate apolipoproteins C and E, which are present in native virions.[125–128] The mammalian VLPs have not yet progressed to non-human primate studies,[123,129] but a recent study in pigs[129] suggests that this system can be produced at a scale amenable to assembling pre-clinical data required for eventual human trials. A third type of particulate assembly that has been developed more recently for use in vaccines is protein-based NPs. NP platforms for the multivalent display of vaccine antigens have proven effective in boosting immunogenicity for antigens such as Ebola GP,[130] HIV env,[131–133] SARS CoV-2 spike,[134] RSV F,[135] and influenza haemagglutinin.[136]

3.3 | Common protein-based NP systems

The most common NP platforms currently in use contain either 24 potential attachment sites as in the case of ferritin or 60 attachment sites in a variety of other potential scaffolds. Ferritin is a naturally occurring iron storage protein and for NP delivery systems, the Helicobacter pylori ferritin is typically used,[137] although other bacterial ferritins have been used as well.[138] For platforms containing 60 attachment sites, there are naturally occurring single component options such as *Bacillus stearothermophilus* dihydrolipoyl acetyltransferase (E2p)[139] or *Aquifex aeolicus* lumazine synthase (LS).[140] There are also single component variants of KDPG aldolase from *Thermotoga maritima* called I3[141] and mi3[142] which contain 60 potential attachment sites. There is an additional system that allows 60 potential attachment sites in a two-component platform, I53 (Icosahedral assembly from 5mers and 3mers). This platform takes a pentameric Lumazine synthase RibH2 from *Mesorhizobium loti* and a trimeric wild-type KDPG aldolase from *Thermotoga maritima* modified such that the two proteins interact to form an icosahedral shell[143] with the antigen of interest displayed on the KDPG aldolase trimer components. The above platforms all have been used as "cis" genetic fusions with their target antigens. Another trend in NP delivery has been to generate NPs than can be produced separately from their associated antigens and then coupled after purification in what is called a "plug and display" modular assembly.[144] This system, which uses components called SpyTag and SpyCatcher, takes advantage of the propensity of some bacterial cell surface proteins to autocatalyse isopeptide bond formation[145,146] to create a covalent tethering system incorporated on the surface of NPs. The original platform used the AP205 phage coat protein, but it has since been expanded to other formats, including ferritin,[138,147] mi3,[147] and I53-50.[147] The plug-and-display format is particularly useful for antigens that are difficult to produce in NP form as genetic fusions and/or for producing mosaic NPs in which a number of variants of a particular antigen are coupled simultaneously to the same NP in order to increase the breadth of neutralisation of the vaccine.[136,148]

3.4 | NPs, antigen retention, and the immune response

Of the differently-sized particulate platforms used for vaccination, NPs appear to have an advantage over larger particles such as microparticles at priming cytotoxic T cells.[149,150] Over the past ten to 15 years, taking advantage of this observation with protein-based NPs has received a lot of attention. Recent research on antigen retention and the immune response[151] sheds light on the origins of the potential utility of NPs as vaccine platforms. It has been known for quite some time that particles of this size effectively drain to the lymph nodes[152] but that is only part of the story. In a recent paper by Aung, et al.[151] analysis of the fate of different antigens within the lymph nodes and how that affects the immune response revealed that intact antigen retention is a key determinant of a robust immune response. In particular, antigens that localised to the subcapsular sinus or extrcellular regions of the lymph nodes encountered a host of metalloproteases and were thus rapidly degraded. In contrast, antigens that were localised to follicular dendritic cells (FDCs), which exhibit limited expression of extracellular proteases, remained intact

and spurred the creation of large germinal centres. Keeping the antigen (in this case the HIV env protein) intact, thereby preserving conformational epitopes, facilitated a more robust immune response. The authors accomplished this in one of two ways, either through an extended dosing schedule or via using different NP versions of HIV env. Similar results also were observed using HIV env in a different study by Zhang et al.[133] The authors in the Zhang et al. showed that two different NP versions of HIV env were retained for 420 times longer in FDCs and thereby elicited much stronger germinal centre reactions, a key component of protective immunity. The fact that two separate groups showed that protein-based NPs facilitate targeting to FDCs where antigen retention is enhanced and leads to robust B cell recognition of the intact antigen is an intriguing finding that highlights the potential of protein NP-based vaccines as a platform to increase the potency of subunit vaccines.

4 | PROOF-OF-PRINCIPLE NP STUDIES FOR HCV

The first HCV NP study[153] was conducted using E2 residues 384–661 from genotype 1b (Con1) covalently linked to *H. pylori* ferritin. The NP was expressed in Drosophila S2 cells and purified by sequential sucrose gradient centrifugation. The purified ferritin-E2 NP was antigenically intact as evidenced by binding to the antibodies AR3A and AP33 and the cellular receptor CD81. Ferritin-E2 exhibited enhanced binding to the antibodies and CD81 most likely due to an avidity effect. The NP also exhibited enhanced binding to patient sera relative to sE2 alone. However, relative to sE2 alone, E2-ferritin did not produce higher serum endpoint titres at any of the doses used in the study. Despite a lack of enhanced antibody response, at the medium dosage used E2-ferritin exhibited enhanced neutralisation of cell culture virus (HCVcc) strains Con1(GT1b) and JFH1(GT2a). Moreover, when tested against a panel of HCVcc, sera from E2-ferritin inoculated mice exhibited greater neutralisation breadth, with 10 out of 13 strains neutralised with a dilution at which 50% inhibition was observed (ID_{50}) > 100, relative to sE2 alone, which only neutralised 6 out of 13 strains with an ID_{50} > 100. One observation from this study that is both cautionary and applicable to all nanoparticle-based vaccine studies is that immunisation of mice elicited a noticeable dose-dependent response to the ferritin nanoparticle. These kinds of responses can divert the immune system and negatively impact efficacy. Moreover, nanoparticle scaffolds should have as little sequence homology to human proteins as possible to avoid immune responses that break tolerance to self-antigens.

The second NP study[154] tested multiple NP platforms and an optimised version of sE2 from two different genotypes (GT1a and GT6a). The sE2 versions used incorporated optimizations that truncated HVR1 (residues 384–411) and both the variable region 2 (450–494) and β-sandwich loops (540–550) in an effort to focus the immune response on only the most highly-conserved antigenic epitopes. The authors called the designs E2mc3. Moreover, these constructs are truncated at residue 645, so the E2mc3-v1 core used in these studies excludes a number of residues at the N- and C-termini and the two loops that are present in the Li et al. study. The authors initiated the study using three NP platforms: ferritin, E2p, and I3-01. When the E2 is modelled as displayed on the surface of the ferritin 24mer, the overall particle diameter is estimated to be 24.5 nm, and for the 60mer NPs E2p and I3-01 the estimated diameters are 34.5 and 37.5 nm, respectively. As with the previous study, the NPs exhibited enhanced binding to a panel of E2-specific mAbs, with the 60mer E2p NPs exhibiting a larger increase in general than the 24mer ferritin. This aligns with what one would expect for fully occupied 60mers versus 24mers. The authors were unable to produce NPs for the I3-01 platform using the GT6a E2mc3-v1 antigen, so I3-01 was not used for antigenicity or immunisation experiments.

The authors immunised mice with E2mc3-v1 and the corresponding ferritin and E2p NPs using E2mc3-v1 from GT1a and GT6a. At early time points, there was a clear trend towards higher antibody titres in the NP groups relative to E2 alone. However, the titres converged and in some cases titres were higher for E2 alone groups at the last time point (week 11). Due to the fact that the NP comprises up to 49% (ferritin) to 58% (E2p) of the mass of the particle, the NP groups got less antigen as the dosage was based on the total protein amount, not the E2 amount. When looking at the ability of mouse sera to neutralise HCV, the E2mc3-v1-E2p NP-inoculated mouse sera exhibited better homologous and heterologous neutralisation activity than sera from mice inoculated with E2mc3-v1 itself. This neutralisation trend did not correlate with antibody titre, which converges at later time points, indicating a shift in the quality of the immune response in favour of the E2p-E2mc3-v1 group. However, the ferritin NP group did not show better homologous or heterologous neutralisation than E2mc3-v1 alone, indicating that the ferritin platform is not a productive system for this antigen. The authors further examined the antibody response to the E2mc3-v1 and corresponding E2p NP groups. Compared to E2mc3-v1 alone, the E2p-E2mc3-v1 group showed a greater frequency of E2-specific B cells and used significantly more Vh groups. In addition, the E2mc3-v1 alone group exhibited highly skewed germline gene usage relative to the E2p-E2mc3-v1 group based on HCDR3 length distribution. In general, the E2p-E2mc3-v1 group engaged more naïve B cells, and activated diverse germline genes, likely accounting for its enhanced neutralisation potency.

The third NP study[155] utilised a permuted version of E1E2 in which the order of the antigens in the open reading frame was reversed (i.e. E2E1) and the I53-50 platform for NP display. This permutation allowed fusing E1 to the NP scaffold I53-50A subunit, which is trimeric. This arrangement mimicked the putative trimeric E1 configuration on the virion, for which there exists biochemical[156] but not structural evidence thus far. The authors used the GT1a resistant strain AMS0232 for the E2 and E1 ectodomains, and in the NP construct fused E2 residues 384–698 to the E1 ectodomain (residues 192–325) separated by a Gly-Ser rich linker and a hexaarginine furin cleavage site. As expected, the construct exhibited binding to E2- and E1-specific mAbs but not E1E2-specific mAbs. Interestingly, the E2E1 NP exhibited better binding to E2-specific neutralising antibodies, but sE2 alone exhibited better binding to

AT1211, which targets domain C and non-neutralising antibodies that target domain A. This behaviour is consistent with that observed previously for both mbE1E2 and sE1E2[36] and with the E1E2 structures that show antigenic domain A in the E1E2 complex abutting the base and stem regions of E2, which are present in both E1E2 and E2E1 complexes but absent in sE2. The authors immunised rabbits with sE2, E2E1-I53-50A trimers, and E2E1-I53-50 NPs. To avoid skewing endpoint titres with antibodies to the NP scaffold, the authors produced an E2E1-foldon trimer for analysis. Serum antigen binding titres for the E2E1-I53-50 NP group were higher to E2E1-foldon immobilised on the plate than sE2, but not significantly higher than the sera from rabbits immunised with E2E1-I53-50A trimers. When tested against the homologous pseudovirus, sera from all six rabbits immunised with E2E1-I53-50 NPs neutralised the pseudovirus with a median ID_{50} of 457, whereas only one sample in the E2-immunised group was able to neutralise the homologous HCVpp, and sera from five of six rabbits immunised with E2E1-I53050A trimers were able to neutralise the homologous HCVpp (median ID_{50} ~ 100). Heterologous neutralisation was observed, but it was weak for the three single-species groups. Therefore, the authors leveraged the flexibility of the I53-50 NP system to produce mosaic NPs and a NP cocktail with E2E1s from multiple strains. Incorporating E2E1s from multiple genotypes increased the neutralisation breadth relative to the group immunised with a single strain of E2E1.

5 | FUTURE PROSPECTS FOR A NP-BASED HCV VACCINE

From the aggregated data for other viruses and the three HCV NP immunisation studies discussed above, it is likely that a NP-based vaccine containing E1E2 will facilitate an enhanced immune response with consistent and controllable production and solution properties. It will also preserve not only the E2 regions that are strongly associated with viral clearance but also the AR4A epitope that is associated with viral clearance,[32] thereby making a more potent immunogen. The recently-determined cryo-EM structures of mbE1E2[85] and a soluble secreted form of E1E2[86] will allow modifications of the E1E2 complex that both enhance its immunogenicity and enable its efficient incorporation into NP platforms. However, the above studies also contain some data that serve as cautionary tales. In the He et al. study, the I3-01 system was eliminated from consideration because they were unable to produce a NP form for GT6a E2mc3-v1. This observation underscores that, while these systems are designed as modular and adaptable, a fair amount of optimization might be required for some antigens, particularly those containing multiple post-translational modifications or multiple disulfide bonds like E2 and E1E2. E2 is generally well-behaved in solution and can be isolated from a variety of eukaryotic expression systems in monomeric form[40] and yet from the data in this study it is clear that not all NP systems are compatible with this antigen. E1E2, in both membrane-bound and secreted native-like forms, is not as well behaved in solution as E2[36,157] and, thus, the bar for getting native E1E2 NPs will undoubtedly be much higher. Perhaps the SpyTag-SpyCatcher plug-and-display systems[144] will mitigate this issue (Figure 3), but such a study has not yet been completed. Those systems are flexible, but for antigens that are not well-behaved, there is no guarantee of high occupancy on the NP after coupling and post-purification assembly, and as such the preparations could include contamination with empty NPs that dilute the immune response to the antigen.

Moreover, re-purification can be disadvantageous as significant recovery losses can occur. Thus, the presence of unreacted SpyTag-SpyCatcher nanoparticles can potentially complicate manufacturing as the unreacted components might be difficult to remove. In addition, the plug-and-display NPs and two component NPs are often expressed either entirely or in part in bacteria. From a vaccine manufacturing standpoint, it would be advantageous to express the components together in eukaryotic cells. However, efficient secretion to the media can be an issue for NPs.[158] A recent study found that this issue can stem from cryptic transmembrane domains[158] which can then be rectified in some cases via computational "degreasing" which alleviates the propensity of some hydrophobic interfaces in designed NPs to impede secretion. Analysing the secretion efficiency for a given NP system is therefore a critical step that needs to be taken early in the process to avoid potential roadblocks after the preclinical stage. An additional cautionary piece of data from this study is the inferiority of neutralisation for E2mc3-v1-ferritin relative to E2mc3-v1 alone. These data are inconsistent with the data from Li et al. and perhaps result from the modifications to E2 in the context of the ferritin NP system. That is, not all modifications that optimise the antigen on its own will necessarily be directly transferrable to a given NP platform. This complicates the issue considerably, but considering the aggregate data in the HCV vaccine development field, it is not surprizing. E1E2, and even E2, are glycoproteins with unusual structures that are likely highly dynamic and thus any changes to the protein can have unexpected effects on antigenic epitope presentation. Tethering the glycoproteins to a NP adds another variable to the interplay between structure and dynamics that must be tested on a case-by-case basis.

The consistent positive theme from the three HCV NP studies is that successful NPs outperform subunit vaccines, making an E1E2-based NP in any one of the major kinds of platforms (Figure 4) an attractive option. This observation could be related to the retention of the NPs in FDCs, and therefore tailoring the size of the NPs to favour FDC retention is a potential avenue of optimization. For example, the unsuccessful E2mc3-v1-ferritin NP from the He et al. study is somewhat smaller than the successful E2(384–661)-ferritin NP from the Li et al. study. Comparing FDC retention of these two NPs and the E2p-E2mc3-v1 NP could yield useful information that directs future NP studies. Ferritin has been used successfully in this context in other systems, most notably for SARS-CoV-2 spike,[159–162] so it appears to be a matter of matching the antigen to its most appropriate NP platform. For HCV E1E2, this represents an experimental challenge waiting to be met.

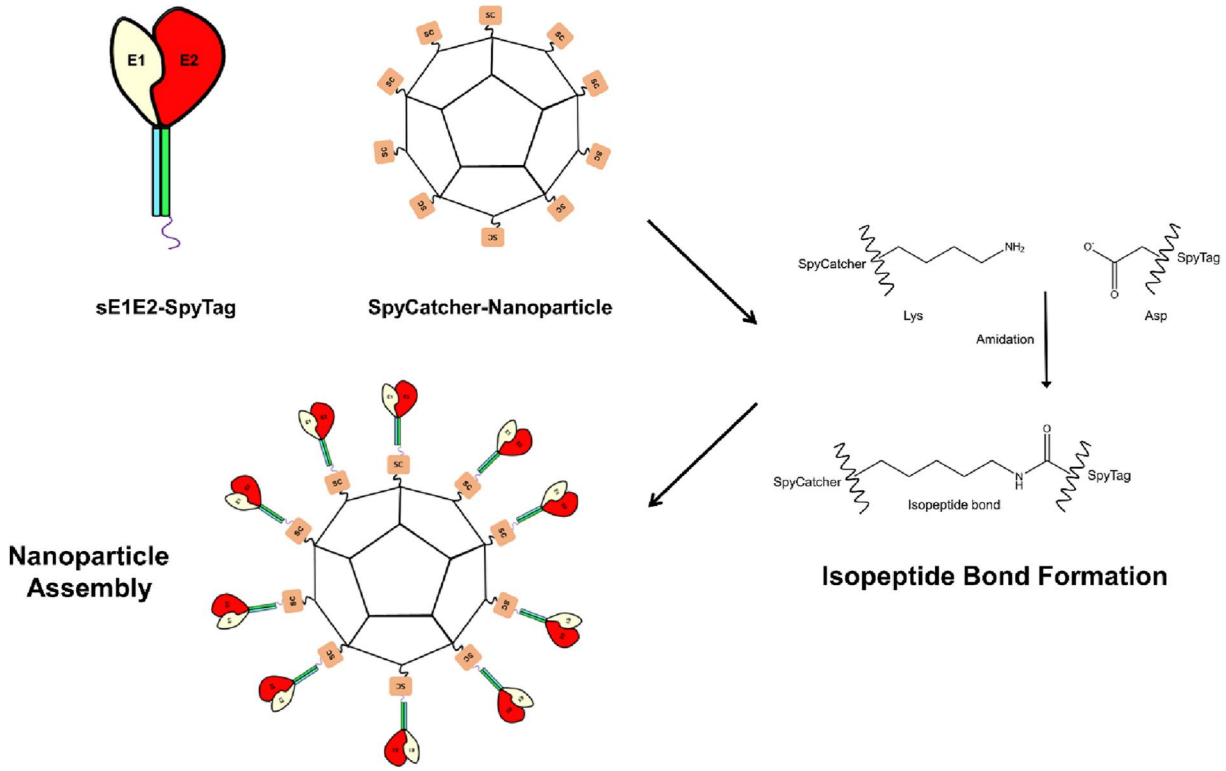

FIGURE 3 The SpyCatcher-SpyTag system as a potential platform for making an E1E2-based nanoparticle. The sE1E2 construct with the SpyTag at the C-terminus of one of the subunits (SpyTag attachment to the E2 subunit is shown) and the Spycatcher nanoparticle can be purified separately. The two components can then be coupled by leveraging the spontaneous formation of an isopeptide bond upon the interaction of SpyCatcher and SpyTag, creating an E1E2 nanoparticle assembly.

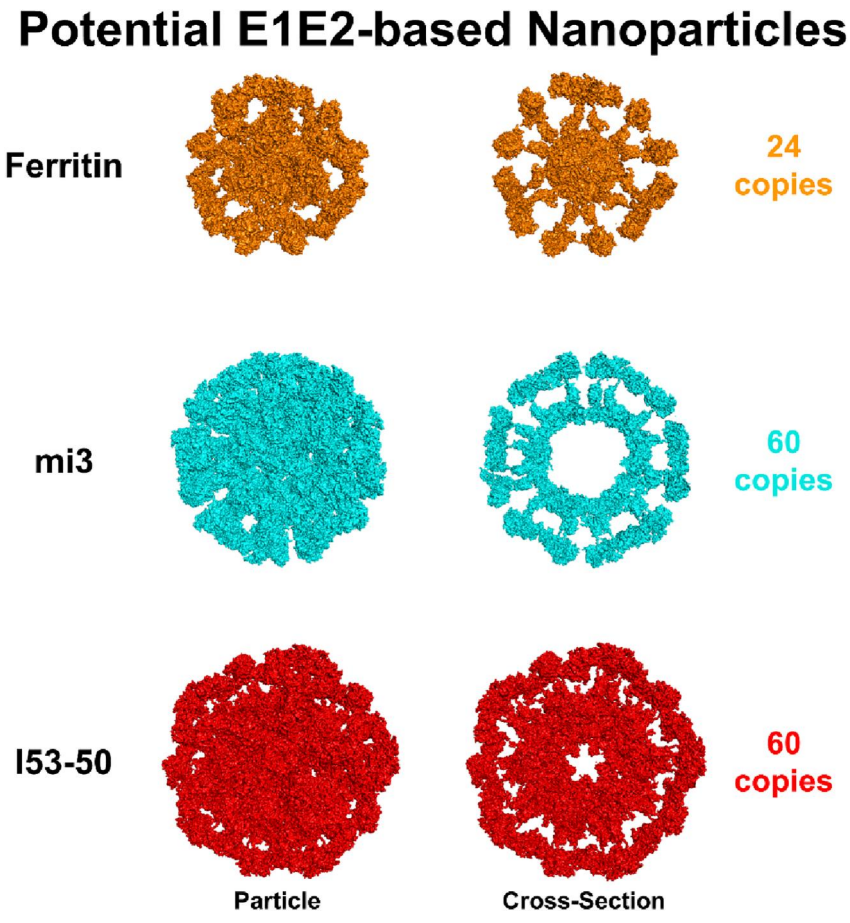

FIGURE 4 E1E2-based nanoparticles modelled for three common platforms. A model of sE1E2 derived from a recent cryo-EM structure was superposed onto the attachment points of each monomer for the associated model platforms. Both the full particle and cross-section are shown to provide a view of the approximate size and antigen density on each type of nanoparticle.

Another potential avenue of exploration with E1E2 NPs is the potential to create mosaic and cocktail mixtures as a means to enhance neutralisation breadth as in the E2E1 NP study.[155] It is not clear, nor has it been systematically investigated, whether the best choice for an HCV vaccine will be a single patient-derived strain derived from an elite neutraliser or a mixture of representative strains from multiple genotypes, perhaps varying based on the geographical distribution of genotypes and population to be vaccinated. The successful development of mosaic NP vaccine candidates against SARS-CoV-2[148] suggests that once a NP-based E1E2 vaccine system is established, the mosaic versus single-strain immunogen design can be more thoroughly investigated.

AUTHOR CONTRIBUTIONS

Eric A. Toth conceptualised and wrote the original draft of the manuscript. Alexander Andrianov and Thomas R. Fuerst edited and revised the manuscript. All authors approved the final version of the manuscript.

ACKNOWLEDGEMENTS

This work was supported by NIH R01s AI132213 and AI168048 (to Alexander K. Andrianov and Thomas R. Fuerst), R21 AI154100 (to Eric A. Toth and Thomas R. Fuerst), and the University of Maryland Strategic Partnership: MPowering the State (Thomas R. Fuerst). The authors would like to thank Gild Ofek and Brian Pierce for helpful discussions and critical reading of the manuscript prior to publication.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

ETHICS STATEMENT

Ethics approval was not needed.

PERMISSION TO REPRODUCE MATERIAL FROM OTHER SOURCES

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How to cite this article: Toth EA, Andrianov AK, Fuerst TR. Prospects for developing an Hepatitis C virus E1E2-based nanoparticle vaccine. *Rev Med Virol*. 2023;33(5):e2474. https://doi.org/10.1002/rmv.2474