Structural Determinants and Membrane Functionalization of Enveloped Protein Nanocages

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Abstract

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There is an unmet need for better intracellular drug and therapeutic molecule delivery vehicles. As a potential avenue to combine the targeting capabilities of viruses, the modularity and tractability of artificial systems, and the biological compatibility of Extracellular Vesicles (EV), our lab worked to adapt our self-assembling protein nanocages into Enveloped Protein Nanocages (EPN). Expression of these EPN in mammalian producer cells results in the exocytosis of vesicles containing multiple self-assembling protein nanoparticles. Here I describe my efforts to gain additional control over our EPN platform and endow it with new capabilities by functionalizing the EPN membrane via specific recruitment of different transmembrane proteins (TMP). Incorporation of these designed TMP has allowed us to target EPN to specific cells in a mixed population and complete a successful preliminary immunogenicity study using mRNA-delivery of EPN constructs. Furthermore, continued characterization of different

EPN constructs has led to new insights about the basic biology behind protein-protein and protein-lipid interactions within membrane-bound nanoparticles.

Acknowledgements

As with any scientific endeavor, it's important to recognize that I have stood on the shoulders of giants and benefited greatly from the time, effort, and energy of the people who came before me. I owe a great deal to those who pioneered the protein design space, and created the tools from which I do my work–where would I be without a self-assembling protein nanocage? As a direct beneficiary (or perhaps dependent) of modern medicine I am supremely thankful for all of the scientists over the centuries who have helped humanity march forward technologically, and am grateful to have my chance to contribute to this progress. Leonard E. Read succinctly puts forth in his essay *I, Pencil*: "These legions are among my antecedents."

With flowery and abstract appreciations out of the way, I do need to specifically recognize some of my "direct antecedents" on the EPN project: Joerg Votteler, Marc Lajoie, Cassie Ogohara, Kate Dapron, Betsy Gray, and of course Neil King and Wes Sundquist. The prior work of these former Sundquist, Baker, King, and Stetson lab members made possible what I have accomplished thus far. Special thanks to Ben Schmitz, a Sundquist lab member currently ~~suffering through~~ working tirelessly to contribute our EPN images.

I have had the pleasure of watching my mentor, Dr. Neil King, develop his mentoring style and lab culture throughout my graduate student tenure. Neil has worked tirelessly to try to create an open, productive, and innovative environment, and I am appreciative of the opportunity he gave to a rotation student who said "I'm just sticking around for a quarter before joining a different lab, but this seems kind of interesting."

and then promptly broke his leg and was absent for the next few months. I have genuinely enjoyed the interactions with my fellow lab members past and present.

My committee members Alex Merz, Dan Stetson, Kelly Lee, and Jeff Chamberlain have consistently been supportive and instructive, and their added perspective has helped me focus on the goals and tasks at hand, rather than the tasks I wanted to accomplish. On a few occasions problems I had with my project were solved by committee members.

My research training began during my teen years, and the mentorship of James Amonette at the Pacific Northwest National Laboratory helped guide and inform me that, while geochemistry wasn't my passion, research most definitely was. Later on I received mentorship from Ernesto Nakayasu and many others while learning about proteomics (an internship I landed through playing soccer with many of the scientists), where I was first exposed to two staples of my PhD: western blots and cell culture. Mary Stewart, a post-doc in the Cookson Lab at UW, was influential in getting me to rely upon myself rather than running to the nearest authority figure for guidance. My first full-time research position in the labs of Hans-Peter Kiem and Jennifer Adair, with direct mentorship by Olivier Humbert were crucial in exposing me to what I had been interested in for many years–gene therapy. This formative exposure to gene therapy research was critical to my continued motivation to work on this challenging project, and I can't imagine having success in my PhD without the training I received in their labs.

Throughout my PhD journey, I've relied heavily upon my friends, in particular Jordan Pettibon and Destiny McCoy. My family has also been supportive for over two

decades (despite my mother's insistence I should be an engineer) in my explicit desire to pursue a career in research and I greatly appreciate what they've done for me.

Lastly, and most importantly, I have to thank my wife Maggie both for her support, and for her pushes to help get me across the finish line. I could not ask for a better partner.

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1. Background and Motivation

There is an unmet need for better intracellular drug and therapeutic molecule delivery vehicles. A large motivation for the work described herein was to establish better methods for delivering gene therapies to target cells. Gene therapy is the treatment of cells with nucleic acids to help repair or replace a defective genetic element; according to the FDA “Human gene therapy seeks to modify or manipulate the expression of a gene or to alter the biological properties of living cells for therapeutic use.” (Center for Biologics Evaluation and Research n.d.). Often, gene therapy is also thought of as a singular and permanent or durable method to treat a chronic condition, as once the patient has the repaired gene, they would be cured indefinitely. Many different diseases, both chronic and acute, have gene therapy strategies FDA-approved (Center for Biologics Evaluation and Research n.d.). From this list, both Juno Therapeutic’s BREYANZI and Novartis’ KYMRIAH are CD19-directed autologous T-cell therapies directed against different lymphomas, while treatments for chronic disease include products such as Novartis’ ZOLGENSMA against spinal muscular atrophy or bluebird bio’s ZYNTEGLO against ß-thalassemia.

A common problem in the therapeutic space is that there is a constraint on the size and charge for a drug, based on the ability of a molecule to diffuse across the hydrophobic cell membrane. As a fundamental pillar of biology, the cell membrane is meant to be (mostly) impermeable, regardless of the best intentions of the treating clinician. Many small molecule drugs are designed to be relatively small and hydrophobic, meaning that they can diffuse relatively efficiently and have their intended

effect on their intercellular target (or simply work outside the cells). In contrast, all gene therapies (and many protein-based therapies) are unable to diffuse into a cell. The large size and high charge of nucleic acids means that the overwhelming majority gene therapies require a vehicle for transporting the therapeutic from outside the patient cells to inside the patient cells.

Looking to nature, viruses have adapted over billions of years to transport their nucleic acid genomes into host cells, and then hijack host cellular machinery to replicate their genomes and make new viral protein products, a process which is very similar to what is required in a gene therapy. Early gene therapies used engineered Moloney Murine Leukemia Virus (MMLV), including in the first treatment of human cells (Rosenberg et al. 1990). While these gamma retroviruses (including MMLV) are efficient in transducing cells, issues arose around safety, particularly when delivered *in vivo*. In 1999, the death of Jesse Gelsinger ("The Death of Jesse Gelsinger, 20 Years Later" 2019), an otherwise healthy patient with a controllable disease, revealed some of the ongoing problems with the boom in gene therapy clinical trials. Gene therapy clinical trials were effectively halted in the United States after the especially publicized trial, in light of regular failures to inform patients about risks. In Jesse's specific case, neither he nor his parents were informed that high Adenovirus (AV) doses had led to the deaths of two monkeys and adverse reactions in other patients.

Gene therapies are currently imperfect, and their delivery vehicles (historically viral) have enormous effects on safety and efficacy of the therapies. As an example, early gene therapies utilized Gammaretroviruses (GV) as they are efficient in transducing human cells. Unfortunately GV have a tendency to integrate into oncogenic

spaces in the human genome and induce cancers as the inserted viral genome (or in this case, the inserted therapeutic transgene) causes readthrough into neighboring genes thereby disregulating cellular function in a process termed “insertional mutagenesis” (Uren et al. 2005). Because of the possibility of oncogenesis from an inserted transgene, efforts should be made to reduce the chances not only of insertions into oncogenic areas of the human genome, but also in reducing which cells are transduced in the first place. As an example, ß-thalassemia is an inherited genetic condition in which there is not enough hemoglobin produced, resulting in reduced ability to capture and transport sufficient oxygen. In this case a gene therapy would ideally transduce only red blood cell progenitors, and would minimize the chances of transducing a non-relevant cell type, such as a muscle cell. This targeting of relevant cells reduces the number of transductions which could potentially turn into cancers, and also greatly reduces the amount of therapeutic vector which is required.

In an effort to reduce the possibility of off-target transductions, *ex vivo* gene editing of autologous cells has become a desired approach. This method of gene editing involves withdrawing cells from the patient, purifying the target cells (usually stem cells), transducing the cells with viral vector, expanding the cells to a larger number, and then reinfusing the patient with these cells (which are their own edited cells). While *ex vivo* gene editing has helped to limit off-target genome insertions resulting from non-target cell transductions, it is by no means a perfect strategy. Nevertheless, *ex vivo* gene therapies have been used to treat a number of diseases, notably including X-linked Severe Combined Immunodeficiency (X-SCID), a disease which manifests as a failure to form an immune system. This absent immune system led to the extreme

circumstances of David Vetter, who lived his 12 years in a literal plastic bubble (American Experience 2019) to avoid possible infections, and resulted in the colloquialism of the “Bubble Boy" disease. In a cohort of 10 boys with X-SCID, 9 were successfully treated with *ex vivo* retroviral gene therapy, but 4 developed leukemia as a result of the insertional oncogenesis from the *ex vivo* treatment (Hacein-Bey-Abina et al. 2008), meaning that despite reducing the off-target cells, the smaller number of cells transduced still resulted in cancers within a few years. Additionally, the extensive handling of stem cells and length of time they’re kept out of the patient’s body, as well as the various stimulations and induced expansions, can result in less-fit stem cells, a key disease factor which may not manifest for decades in patients. The switch from GV and AV to replication-incompetent HIV-based lentiviral vectors (LV) has resulted in efficient transduction with lower rates of oncogenic insertions, but now necessitates *ex vivo* handling, as human sera is capable of inactivating the VSV-G pseudotyping present on these recombinant lentiviral vectors (see Chapter 2 “Pseudotyping with VSV-G). This *ex vivo* handling of patient cells is also a severe bottleneck in large-scale adoption of any gene therapy. While work is proceeding on automated methods to expose cells to therapies without intense individual technical interaction (Adair et al. 2016), ideally any therapy would be able to be directly injected *in vivo* and bypass the removal, manipulation, and reinfusion of patient cells entirely. An additional factor leading towards the increased adoption of LV in gene therapies is the ability of the LV to infect, and then the integrase to transduce quiescent cells. This ability to target quiescent cells is in contrast to GV and AV which only act on actively dividing cells (Cooray, Howe, and Thrasher 2012). While technically possible, many of the fears about

lentiviral recombination towards an HIV-like pathogenesis have fortunately never been borne out in the clinic, but remain a concern especially when informing patients that they'll be treated with an HIV-based virus. This current standard of care using LV to *ex vivo* gene edit a patient's own cells leaves much to be desired, especially with respects to scalability of the process and viability of the manipulated cells.

A newer route of viral delivery of nucleic acids has been the use of Adeno-Associated Virus (AAV), which are non-integrating and carry less danger of insertional mutagenesis, while also being much better tolerated *in vivo* than the preceding AV or GV vectors. AAV are however substantially less efficient per viral capsid in both gene delivery and expression than their integrating viral counterparts. This lower efficiency of AAV has led to much larger doses required to get a positive clinical outcome, and the large dose of viral particles can lead to adverse reactions, not unlike what was seen with the Gelsinger case in his reaction to a large bolus of adenoviral particles. Recent deaths in clinical trials have put holds on trials, including two deaths in a recent trial by Audentes Therapeutics for their therapy to treat X-linked myotubular myopathy ("High-Dose AAV Gene Therapy Deaths" 2020). The relative fragility and lower expression levels of enveloped retroviruses is a large challenge in the gene therapy field, and being able to produce suitable amounts of retrovirus for whole animal studies is a large task, one which is currently being worked on (Sheu et al. 2015). An advantage of the AAV vector is that its "naked" viral capsid is easier (although not trivial) to manufacture than the enveloped viruses. The gene therapy field currently weighs the less-efficient *in vivo* (or less common *ex vivo*) gene delivery via AAV against the more-efficient (yet more difficult to produce) lentiviral *ex vivo* modes. Current trends

point towards increased adoption of the AAV methodology, although this is target and tissue dependent. From the perspective of insertional mutagenesis, much effort has been spent on making the relatively-efficient lentiviral vectors safer, and much effort is now being spent on making relatively-safer AAV vectors more efficient.

Engineering viruses to include new motifs is difficult, with much effort and capital being directed towards retargeting AAV to different tissues. As AAV is a naked capsid, retargeting the capsid involves changes in protein sequence which may have effects on the capsid structure itself, complicating redesign efforts. In contrast to naked AAV, enveloped viruses have transmembrane Envelope Proteins (Env) which are physically separated from their capsids, and lie within their membrane envelope. These Env can function to direct the virus to the correct cellular target (such as T-cells for HIV), and can also aid in fusion of the viral envelope with the target cell envelope. Env proteins from different viruses can be incorporated into a recombinant viral particle, which allows for pseudotyping of viruses with proteins from other viruses, however incorporation of non-native Env proteins proteins (i.e. an Ebola Env onto an HIV membrane) can be difficult and inefficient. Progress on increasing the efficient production or relative safety of these viral vectors has been slow.

In contrast to viral vectors, synthetic liposomes and lipid nanoparticles (LNP) are more readily produced and uniformly purified, but LNP lack the biological specificity of the targeting proteins inherent in viruses. Another distinct disadvantage of LNP is their synthetic nature; while these particles can be synthesized in a lab, they cannot be readily synthesized by a living cell. Given the strength of mRNA-based encoding of protein or protein-lipid hybrid nanomaterials (which leveraged rapid production of the

mRNA-launched SARS-CoV-2 vaccines), the lack of genetic encodability of LNP is a distinct advantage. Lipid nanoparticles are also generally more toxic to the recipient cell, due to a combination of their reliance on endosomal rupture to escape the endosome (leading to release of low pH endosomal contents into the cytosol), the general havoc that cationic lipids can cause within a cell, TLR4 activation, and liver toxicities (which is also seen in other nanoparticle therapeutics)(Zolnik et al. 2010; Kedmi, Ben-Arie, and Peer 2010; Hou et al. 2021). Viruses have been able to leverage rapid selection and high mutation rates to achieve the specificity and efficiency of their fusogenic proteins, a capability which is not readily matched by the synthesis of LNP from lipid precursors.

Extracellular Vesicles (EV) are nanoparticles released by cells as part of their fundamental and routine biological processes. The term “Extracellular Vesicles” is a catch-all for any lipid bilayer bound particle released from a cell, and encompasses microvesicles, exosomes, and (depending on who you ask) apoptotic bodies. Microvesicles are directly released from a cell’s plasma membrane, and typically are larger, anywhere from 100nm to 10μm. Exosomes are formed by budding into the endosomal lumen forming Multivesicular Bodies (MVB), and if these MVB fuse with the plasma membrane the contained exosomes are released into the extracellular environment(Raposo and Stoorvogel 2013; Hessvik and Llorente 2018). These membrane-bound nanoparticles are released from diverse organisms across eukaryotes, prokaryotes, and even archaea (Liu et al. 2021). Once thought to consist simply of cells’ jettisoned garbage, EV are now understood to be integral to intra- and inter-organismal communication and homeostasis (Stahl and Raposo 2019). Significant

recent advances in characterizing the RNA contents of these EV has led to better (if rather incomplete) understanding of the role that EV play in biology (Veziroglu and Mias 2020). The realization that EV are not simply bags of cellular junk, and in fact play a critical role in transporting cargoes between cells (Mathieu et al. 2019) has resulted in an explosion of research interest. Detection and analysis of EV contents from patient samples is also being investigated as a method to screen for cancers or other diseases (von Felden et al. 2021; Gaglani et al. 2021), and benefits greatly from better RNA sequencing and analysis. An internet search will pull up countless articles outlining the use of EV for delivery of therapeutics, but suffice it to say that there's an enormous interest in using particles which routinely deliver cargoes between cells to deliver (therapeutic) cargoes between cells.

There are many difficulties in studying small, membrane bound particles, and the expression, capture, and purification of EV has been exceedingly difficult (Théry et al. 2006). Furthermore, the difficulty of characterization has resulted in poor standardization within the field, with overall impressions (including sentiments at conferences) being that the EV field has been plagued by a large amount of junk science over the years (Bazzan et al. 2021). With the goal of standardizing EV capture and analysis, the International Society of Extracellular Vesicles (ISEV) developed the Minimal Information for Studies of Extracellular Vesicles (MISEV), with the latest guidelines being MISEV2018, and a new version being expected with the upcoming conference in 2023. While there has been increased scientific rigor and standardization within the EV field, it is still a very young (and exciting) field with much of the fundamental biology still unknown. Extracellular vesicles are a class of exquisitely

suitable biologics delivery vectors, but as of now their therapeutic potential remains largely unrealized.

As a potential avenue to combine the targeting capabilities of viruses, the modularity and tractability of synthetic systems, and the biological compatibility of EVs, the King group, in collaboration with the the group of Wes Sundquist at the University of Utah, modified existing self-assembling protein nanocages to create Enveloped Protein Nanocages (EPN) (Votteler et al. 2016). A self-assembling protein has been designed by taking a naturally occurring trimer and adding inter-trimer interfaces to promote the assembly of 20 trimers into a 60-subunit complex with icosahedral symmetry(Hsia et al. 2016). From this self-assembling nanocage scaffold, EPN are formed by the addition of membrane binding and Endosomal Sorting Complexes Required for Transport (ESCRT) recruitment motifs, which allow for budding and membrane scission of the nanocages from producer cells. Expression of these EPN constructs in mammalian producer cells results in the exocytosis of multi-cage vesicles. These vesicles contain multiple distinct nanocages, and are often likened to “a bag of soccer balls” in which many nanocages (soccer balls) are contained within a single vesicle (bag).

During development of this EPN project, it has come to light that canonically “naked” viruses (such as AAV) can be released within a membrane (Santiana et al. 2018; van der Grein et al. 2018). While the release rates of these enveloped-non-enveloped viruses is very low, they seem to be able to more efficiently infect and transduce their target cells on a per-virion basis. Furthermore, the membrane envelope may mask the immunogenic motifs present on the AAV capsid, allowing for increased doses and/or reduced adverse events. There is commercial interest in these

enveloped AAV for more efficient and less immunogenic gene therapy vectors. Clearly, our EPN system has parallels to enveloped AAV, however we also believe that the greater toleration for added motifs and functions on the I3-01 nanocage (Fig. 1A) gives it an advantage over an AAV capsid. It is our hope that we can efficiently engineer released EPN by modifying the protein sequence of the nanocage to encode varied motifs and functions. The tractable “handle” of the nanocage could allow for efficient, specific loading of cargoes, inclusion of motifs to enhance overall production and release of the EPN from the producer cell, or cargo release and delivery within the recipient cell.

In efforts to gain additional control and leverage our EPN platform, I worked primarily to functionalize the membrane of our EPN via specific recruitment of different Transmembrane Proteins (TMP) and developed methods to increase the display of particular TMP.

2. Prior Development of the EPN Platform

Protein Design

Protein engineering used to rely exclusively on modifications of existing natural proteins. Because the protein sequence space is so large (number of residues to the power of 20), it becomes exceedingly difficult or impossible to systematically engineer proteins to have novel biological functions via stepwise mutations. The advent of cheap and powerful computing power coupled with advances in predicting protein structure has allowed for computational design of novel protein sequences, either from natural building blocks/symmetries, or totally *de novo*. Crucially, advances in cloning techniques and the introduction of quick and affordable gene synthesis has allowed for sets of designed protein sequences to be efficiently ordered, tested, and iterated upon. The power of computational protein design to deliver new protein-based structures and functions has generated several classes of novel biomaterials. One notable class of protein biomaterials is self-assembling protein nanocages. Other nanoassembly technologies such as DNA origami (Ke et al. 2009) surpass the current capabilities of protein structure assembly. However, proteins are the workhorses of cells and in nature the protein molecular machinery far surpasses DNA machinery in sophistication and specificity. Focusing on the “protein” platform is a more straightforward path towards clinical relevance and range of material characteristics and capabilities.

The Rosetta program developed in the Baker Lab at the University of Washington was originally designed to better understand the dynamics of protein folding and fold prediction (Simons et al. 1999; Rohl et al. 2004). Later on, researchers realized that the Rosetta program could be used to not only predict protein structure, but also to design

novel proteins with atomic-level accuracy (Kuhlman et al. 2003; Kaufmann, Lemmon, and DeLuca 2010). This breakthrough in protein engineering has allowed for the design of new materials which exactly match structures designed on a computer, and has also fed back into new insights about protein folding itself and has become a staple of the fledgling protein engineering field (Baker 2019; Leman et al. 2020). This newfound ability to design proteins with completely novel (and intended) functions has opened up the gates for developing nanomaterials which were previously unobtainable via stepwise mutation of existing proteins.

Designed Protein Nanocages

Atomic-level accuracy in protein design has allowed for precise engineering of protein-protein interactions, largely driven by design of novel hydrophobic interfaces which drive association and assembly in the aqueous environment. An important early nanocage which was used extensively in this work was I3-01 (Fig. 1A) (Hsia et al. 2016). The I3-01 nomenclature refers to the Icosahedral (**I**) structure made of trimers (**3**) and the first design that was successful (**-01**). Starting with a naturally trimeric protein from a hyperthermophile (which confers increased stability from the high temperature environment), designed interfaces drive the assembly of the trimers to form a higher-order structure of twenty trimers arranged in icosahedral symmetry. Current successful therapeutic applications of protein nanocages revolve around vaccine design, such as a designed RSV vaccine (Marcandalli et al. 2019), a two-component protein nanocage bearing SARS-CoV-2 antigens (Brouwer et al. 2021), including the SKYCovione™ vaccine currently used in South Korea, and even a nanocage which incorporates antibodies into its structure (Divine et al. 2021). Other nanocages have

been constructed, including those with octahedral or tetrahedral symmetries, with dimers, trimers, octamers, or pentamers as their subunits. Work continues on different nanocage structures, notably in pseudosymmetric assemblies which could allow for more fine-tuned display in ordered assemblies. As a specific example, pseudosymmetry could allow for a trimer made of three different (but structurally equivalent) components, meaning an I3-01-like nanocage could actually display three different motifs but still assemble into the same overall cage structure. These examples of functionalized protein nanocages help to demonstrate that as a whole, the platform is engineerable, a property by and large absent from natural viral capsids.

EPN Design

Starting from a self-assembling nanocage scaffold (usually I3-01), addition of membrane-binding and Endosomal Sorting Required for Transport (ESCRT) recruitment motifs allow for the production of Enveloped Protein Nanocages (EPN) (Votteler et al. 2016) (Fig. 1B, C). The ESCRT machinery (which actually consists of several distinct complexes) is a complicated, interwoven set of pathways involved in membrane budding and scission that's present in all eukarya and some archaea (Votteler and Sundquist 2013; Alonso Y Adell, Migliano, and Teis 2016). Many diverse cellular processes such as MVB formation, daughter cell cleavage, and even viral budding all require the ESCRT machinery, with a single constant being that the ESCRT-III complex is involved in every ESCRT-mediated function (McDonald and Martin-Serrano 2009). Our EPN system relies on ESCRT recruitment motifs taken from HIV-Gag (which itself acts to hijack the host cellular ESCRT machinery); this HIV-Gag-derived motif recruits

ESCRT-I and an accessory protein ALIX, which in turn recruits the ESCRT-III complex. Once in place, ESCRT-III subunits work to form spirals around the neck of the budding membrane, ultimately severing the membrane, releasing the two distinct membrane surfaces and the ESCRT complexes.

Transfection of plasmids encoding the three distinct (but genetically fused) elements of self-assembly (the nanocage), membrane binding, and ESCRT recruitment into mammalian producer cells result in vesicles containing multiple cages being released from the producer cells (Fig. 1E). These EPN are relatively modular and tractable, and are able to produce membrane-encapsulated cages using a variety of different membrane binding or ESCRT recruitment motifs. Different nanocage structures have also been tested (notably two-component I32's made from trimeric and dimeric components), and while successful, there has not been much investigation into the comparative differences.

We are able to assay the level of membrane encapsulation of our EPN preps by exposing the supernatant-recovered fraction (SN) to trypsin (T) or triton and trypsin (TT). If an EPN is fully encapsulated, then exposure to trypsin will only cleave any extravesicular motifs, whereas membrane permeabilization by triton alongside trypsin will result in degraded cage protein (as visualized by a smaller molecular weight band) (Fig. 1D).

These EPN allow for utilization of the membrane of the producer cells, which is in stark contrast to "naked" cages. Many comparisons of naked vs enveloped viruses apply to the comparison of nanocages vs EPN. Inclusion of the host membrane places a severe limit on the capability to produce these EPN in mammalian systems (so far solely via

transient transfection), in contrast to the ability to produce and purify nanocages in bacterial systems, or to produce individual components of a multi-component nanocage and then purify and assemble the cages *in vitro*. On the other hand, leveraging the host membrane and including Transmembrane Proteins (TMP) is a huge advantage for the EPN platform, as naked nanocages cannot be made to utilize the greater efficiency in endosomal escape (and ultimately cargo delivery) of naturally derived TMP. Additionally, the membrane of an EPN may aid in masking potentially immunogenic sequences, although we have not tested this idea. Simply put, while EPN are at a comparative production disadvantage compared to nanocages, the potential for efficient delivery is much higher given their ability to fuse to membranes.

Pseudotyping with VSV-G

Vesicular Stomatitis Virus (VSV) is a member of *Rhabdoviridae* with broad tropism that's largely due to the targeting of the VSV G protein (VSV-G) to the LDL receptor. Upon receptor binding VSV-G mediates rapid endocytosis of the viral particle by the target cell. In response to acidification of the maturing endosome, VSV-G facilitates endosomal escape by mediating the fusion of the viral and host cell membranes, resulting in the delivery of capsid into the cytosol. The VSV-G protein is commonly used to pseudotype recombinant viruses due to its combination of targeting and fusion activity in one protein, broad tropism, and ease of use/expression in different viral systems(Lee et al. 2001; Hwang and Schaffer 2013). It is important to emphasize that the ubiquitous use of VSV-G can be largely attributed simply due to it both expressing better and being more efficient in mediating delivery than the other viral (or non-viral) fusogenic alternatives. In fact, pseudotyping with VSV-G can result in

increased viral titers compared to native TMP. Pseudotyping our EPN system with VSV-G was a natural extension of the technology.

In the work presented by Votteler et al. (2016), VSV-G pseudotyped EPN were loaded with Beta-lactamase (BlaM). Upon endosomal escape of the EPN and delivery of the BlaM into the recipient cells, a fluorescent dye was cleaved, resulting in a change in fluorescence. This experiment showed that EPN were able to be successfully pseudotyped with VSV-G and that they were able to deliver functional protein cargo into the recipient cells.

Early EPN Delivery Work

In unpublished work, Dr. Marc Lajoie and Dr. Betsy Gray, previous postdocs in the Baker and Stetson labs at UW, showed that EPN were able to deliver a Cas9 complexed with a guide RNA. The resulting editing efficiency in recipient cells was qualitatively high by western blot. A perplexing result at the time was that the non-assembling 1wa3 trimer variant (which trimerizes but does not form the nanocage structure) was just as successful in gene editing (presumed to mean equivalent delivery) as the EPN-11 (nanocage-forming) construct. This result could not be explained at the time, as it was thought that protein nanocage assembly was required for the formation of functional EPN.

In a line of experiments distinct from the Cas9 delivery outlined above, there were attempts to deliver mRNA encoding for GFP. Initial GFP signal in recipient cells were presumed to be the product of successfully-delivered mRNA. After radio-labeling experiments however, it was determined that the GFP signal was the result of GFP protein carried over from the producer cells, and not new GFP being synthesized from

the deliver mRNA. In the EPN-producing cells, the GFP-encoding mRNA cargo was also expressed to facilitate cargo loading; expression of this mRNA in the producer cells resulted in GFP protein being loaded into the EPN, rather than solely the mRNA itself.

With these two delivery-oriented setbacks, EPN-based cell delivery work was largely abandoned. In Chapter 5 I provide a possible explanation as to why Cas9 delivery via 1wa3 might have been just as efficient as via EPN-11.

3. Recruitment of TMP into EPN

Pseudotyping

Pseudotyping is the process by which a virus can be made to express a transmembrane protein which is not their own. One such example would be producing a LV core with a Foamy Virus Env protein. Pseudotyping is a versatile, if somewhat finicky method which allows us to move beyond the natural transmembrane proteins of a virus, potentially retargeting them or conferring increased infection and transduction rates. Recombinant viruses are most often pseudotyped with VSV-G, and EPN were pseudotyped with VSV-G in the original 2016 EPN paper. Here, we refer to pseudotyping as “passive” recruitment of TMP into membranes. The incorporation of the TMP into particles (i.e. EPN, lentivirus, or exosome) is thought to be a passive process where the budding particles simply take what is present on the membrane surface from which they bud. It is also important to note that many other TMP of native origins (i.e. any cell receptors or proteins found on cell surfaces) would also be included in these membrane-containing particles; such inclusions may be disadvantageous when trying to create defined, identical, and reproducible therapeutics from mammalian producer cells.

Why specific over passive recruitment

While pseudotyping is great, we sought to increase the efficiency of transmembrane protein incorporation into our EPN via engineering the nanocage component. Increasing the amount or variety of TMP in the membrane of our EPN might help to more-efficiently decorate our particles, which could lead to better results for downstream applications such as antigen display, fusogen efficiency, or increased targeting avidity of the EPN. While speculative, decorating our EPN with more of our

target TMP might also lead to fewer non-target (native) TMP being included in the EPN. We coined this strategy of attracting/capturing target TMP to the EPN as “specific recruitment” in contrast to the “passive recruitment” which happens during simple pseudotyping. Additionally, more-strongly driving the cage protein to the inner side of the plasma membrane via interactions with a TMP could lead to more efficient EPN release due to driving up the local concentration of the cage (and the associated ESCRT recruitment/budding). The underlying nanocage structure of EPNs is unique to this class of particles, and leveraging the ability to include functional motifs that increase decoration efficiency was the primary goal of my thesis work.

Minimal Transmembrane Protein

I designed a minimal transmembrane protein, consisting of the signal peptide of CD8 (CD8SP) followed by the Transmembrane Domain (TMD) of PDGFRβ. This small protein was designed to be a single-pass Type I transmembrane protein which would tolerate additions on the extracellular and intracellular domains. Our minimal TMP could therefore include interactions with the cage on the intracellular side and display of a motif on the extracellular side. At first, a superfolder GFP (Pédelacq et al. 2006) (itself an optimized form of the original GFP (Prasher et al. 1992)) was included on the extracellular side of the protein, but this led to poor overall expression. Much better expression of the TMP was achieved by utilization of moxGFP (Costantini et al. 2015), a monomeric version of the oxidative-resistant GFP. It has been widely reported that fluorescent proteins are difficult to secrete largely due to formation of unintended disulfide bonds within and between different fluorescent protein monomers, which is exacerbated by the oxidizing environment of the secretory pathway. With a

relatively-expressible minimal TMP, we next sought ways to induce interactions between the EPN cage and this TMP.

Ns3a and D5.6.1 chemically inducible

Our first attempts at recruitment of TMP to the EPN was leveraging a transient interaction meant to bring two different proteins together. The idea was to drive the interaction of the EPN cage protein to the cytosolic side of a target transmembrane protein. We reasoned that a transient interaction would allow for efficient loading of the TMP onto the EPN without causing aggregation and resulting protein quality control (QC) problems in the producer cells, as well as potentially sidestepping any delivery problems stemming from a permanent cage-TMP interaction in the recipient cell. Our plan was to incorporate a designed protein which binds to a natural protein only upon addition of a drug; thus we would have a transient driven interaction between two different proteins. To this end, we used a *de-novo* protein (D5.6.1) designed to bind a Hepatitis C Virus protease, Non-Structured Protein 3 (NS3a) in the presence of an HCV inhibitor Danoprevir. We genetically fused D5.6.1 to our EPN cage protein, and NS3a to the cytosolic side of the minimal TMP; we also did the reverse, putting D5.6.1 on the TMP and NS3a on the EPN. This was not a very successful avenue, for many reasons. We were reliant on Western blots as the primary readout, and the sensitivity of this blot process is not very discerning for teasing out small differences in TMP levels between samples. We used a biologically-relevant Danoprevir concentration (Jiang et al. 2014) but it was hard to confirm if this system was working due to the aforementioned lack of sensitivity in Western blot. Fusing the viral protease NS3a also negatively impacted the expression level of both the cage and the TMP, and is not the most straightforward path

towards driving interactions between two different proteins. Expression of all of the components was low, the complication of Danoprevir addition during EPN production was technically challenging and complicated, the endpoint readouts were ambiguous, and it was unclear how to more-directly probe if the interaction was having a positive (or any) effect on overall EPN release and decoration efficiency. This method of TMP to EPN interaction was abandoned.

SpyCatcher+Tag

Due to the problems of the NS3a/D5.6.1 transient interaction system, we looked for a system which would be more straightforward in its application and approach. We settled on trying the SpyCatcher/SpyTag (SpyC/T) system as a way to introduce a covalent bond between two different protein partners (Zakeri et al. 2012) (Fig. 2). The SpyC/T system was derived from a Streptococcus pyogenes protein (thus Spy) which was broken into two domains: a larger domain of SpyCatcher, and a smaller domain of SpyTag. These two domains spontaneously fuse and form a covalent linkage. In our EPN-producing cells we are able to leverage this technology to spontaneously and specifically form covalent bonds *in vivo*. In our case, we genetically fused the SpyCatcher motif to exposed termini of our cages, (which varied depending on the EPN construct used) and the SpyTag motif to the intravesicular portion of our TMP proteins. This resulted in spontaneous and specific formation of a covalent bond between cage and TMP, and a single polypeptide chain from inner structural motif to outer TMP (Fig. 3A).

“Final” minimal TMP Design–p#65

With this information in hand, I designed a minimal TMP capable of conjugating

with the EPN cage protein, unceremoniously named "p#65" (to denote that it was plasmid #65 in my archives). The p#65 TMP protein consists of the N-terminal CD8 signal peptide to direct the nascent polypeptide to the Sec translocon, an extracellular moxGFP capable of efficient expression in the secretion environment, an extracellular myc tag, the minimal TMD of PDGFRβ, an intracellular FLAG tag, and a C-terminal SpyTag capable of conjugation to the EPN cage.

Broadly usable

We were able to show efficient conjugation *in vivo* between our minimal designed TMP and the interior EPN cage protein (Fig. 3B). We further reasoned that the specific recruitment of SpyC/T would apply beyond just the "model" EPN-1-SpyC and moxGFP-PDFGR-SpyT system. We believe that most TMP with addressable cytosolic tails will be amenable to the addition of the small SpyTag motif. We can swap the motifs, placing the larger SpyC motif on the TMP and the smaller SpyT motif on the cage component, however this seems to lead to a reduction in overall efficiency of forming the conjugated species. Such a reduction in overall efficiency in our system may be completely different for displaying alternate TMP, and effort should be put into engineering and optimizing different systems. The SpyC/T system is apparently amenable to spontaneous reaction at physiological conditions in cell culture, and we have successfully used it to increase the display efficiency of a DARPin-TMP construct, a CoV-2 construct, a range of minimally-modified natural TMP (Fig. 6 A, B), and malaria CSP constructs (Fig. 6 C,D).

Recruitment of CoV-2

In the interest of investigating potential vaccine applications for EPN, we looked

into testing and producing CoV-2-conjugated-EPN. The idea was to display stabilized CoV-2 spike antigens in a more-native context of the membrane of an EPN, juxtaposed to a solubilized trimer, fusion to a naked nanocage, or display on the surface of a cell (as in the case of the mRNA vaccines). We tested several different CoV-2 antigen constructs: the native CoV-2 Spike, a full-length furin mutant trimer, a stabilized trimer mutant termed “HexaPro” for its 6 proline mutations, as well as minimal RNA Binding Domain (RBD) constructs, and stabilizing “repacked” mutants of the RBD, meant to minimize the size of the overall construct to be expressed (Fig. 4A, B). All of these constructs used the signal peptide sequence unique to the SARS-CoV-2, which has a unique “double start codon.” We also tested both the minimal PDGFRβ TMD and the native CoV-2 TMD. Overall, our rationale was that we were engineering new proteins and therefore we should try different combinations (including the minimal TMD), but a counterbalancing force was that we wanted to display more-native-like antigens with our membranous system compared to the other common vaccine technologies. We found that the HexaPro stabilized full-length trimer, using the native CoV-2 TMD was the best-expressing candidate, despite the large size of the full-length construct (Fig. 4C). EM imaging of the conjugating CoV-2-EPN particles revealed striking trimers covering the surface of our EPN particles (Fig. 4D), and encouraged us to pursue an immunization study in mice, covered in the next chapter.

I3-01 Genetic Fusions

The most straightforward way of ensuring a particular TMP decorates the surface of an EPN would seem to be to genetically fuse the TMP onto the cage construct (Fig. 7A), but early attempts at this “straightforward” approach were unsuccessful. Given the

successes of using the PDGFRβ TMD to conjugate to the EPN cage protein, I thought to retry creating genetically fused Transmembrane EPN (TMP-EPN). I attempted to fuse the PDGFRβ TMD onto different EPN constructs, with and without the membrane binding motifs. While there appeared to be a very small amount of protected release, we deemed the experiments largely unsuccessful in creating any usable amount of EPN (Fig. 7B), and attributed the failure to the dynamics of protein assembly. In this TMP-EPN system, we reasoned that the failure was happening somewhere between the membrane integration of the polypeptide chain and production of the trimerizing cage motif, and then those membrane-bound trimers trying to form a larger cage structure at the plasma membrane or even as they're being transported to the membrane. We could envision many different steps between polypeptide assembly and EPN budding where the overall process could result in an aggregation of unformed cages/EPN and be targeted for degradation. Given the many different ways this system could fail, we reasoned that the most reasonable path towards success was with continuing to use TMP-C-EPN.

While I was unsuccessful in using I3-01 genetic fusions to the PDGFRβ TMD, other scientists in the King Lab were also finding secretion of (non enveloped) I3-01 nanocages from mammalian cells to be very inefficient (relative to secretion of other cages). The I3-01 cage was designed from a naturally trimerizing protein found in a hyperthermophile, called 1wa3, with redesigned interfaces to make the trimers associate into the larger icosahedral nanocage structure. Secretion of the non-assembling 1wa3 was much better than the closely-related I3-01, indicating that the introduction of the designed interfaces was responsible for the decrease in secretion.

These designed interfaces are largely driven by hydrophobic interactions, which could look very similar to natural transmembrane patches within proteins, and could result in targeting these cage sequences to the Sec translocon. In order to evaluate this problem, John Wang, a graduate student within the King Lab, worked to identify and redesign these “cryptic transmembrane” sequences within our cages. This sequence optimizer was termed the “degreaser” (Wang et al. 2022), and allowed for a reduction of the propensity for cryptic transmembrane sequences to be made in unintended places of the gene (Fig. 7C).

With a redesigned I3-01 sequence, we were able to genetically fuse the p#65 model TMP directly to I3-01 and get successful expression and release of protected EPN (Fig. 7D). In addition to a negative interior mutant (to reduce transmembrane insertion potential) John Wang designed improved secretion mutants, and combined these into a well-secreted variant of the I3-01 nanocage. At the time of the testing, EPN were being produced from expi293F cells and the combination of the EPN production characteristics of this cell line (see Chapter 6, section 1 for more details) and the “messiness” of the I3-01 fusions resulted in a high amount of non-enveloped EPN protein, which could be effectively treated with trypsin to result in a large amount of enveloped EPN (Fig. 7E).

The success of one-component TMP-EPN which uses the redesigned I3-01 nanocage implies that the main failure of previous TMP-EPN attempts was the presence of the cryptic transmembrane sequences, and not problems inherent with the transmembrane proteins themselves; there are likely more interesting TMP-EPN which could be designed with this system. TMP-EPN are only recently successful, and this is

an exciting avenue for ensuring display of specific TMP on the surface of the EPN, and ensure that a high amount of the TMP is displayed (one for each of the cage monomers, sixty for each cage). Furthermore, being able to produce a TMP-EPN from a single plasmid may have advantages in the production side due to the increased simplicity of the transfection conditions. There remains a lot of work that could be done to optimize this system, including adding more functional elements to the extracellular side of the EPN.

4. Functionalization of TMP-C-EPN

Broadly usable

We want to make EPNs into an easily-modifiable platform for display of different functional TMP. We have been able to show pseudotyped EPN with different TMP, including native VSV-G and CD4. We routinely add myc and FLAG tags to native TMP, which have been well-tolerated. We suspect that alternative tags or small protein modifications would likely also be tolerated, giving other options for detection of TMP in EPN (although this is largely TMP-dependent rather than an inherent property of EPNs). Heavier modifications including linkers or even newly-designed TMP have also been successfully expressed on EPN, further opening the possibilities for inclusion of useful TMP onto this platform.

While we have successfully expressed and incorporated interesting and varied TMP into EPN, we sought to next test whether some of these TMP would be functional. We had previously incorporated VSV-G and used them to successfully deliver BLaM into recipient cells. Beyond the use of VSV-G, very little had been done to test the incorporation of varying TMP onto EPN.

mRNA EPN vaccines

After showing successful incorporation of designed TMP into EPN, we looked for new ways to leverage TMP on EPN to do biologically interesting and useful things. We thought that EPN might have a place in the vaccine field as often the target antigen in vaccine design is a TMP, with the extracellular portion being targeted by the immune system. The extracellular portion of viral proteins are often redesigned to be soluble and secreted in order to produce large amounts of antigen for characterization or vaccines

themselves. There has been increasing interest in displaying these antigens in a more-native state, including remaining in the plasma membrane as a true TMP rather than a solubilized, stabilized format. With the EPN platform, we are able to engineer a TMP to be recruited specifically to the cage scaffold and budded off, thereby increasing the efficiency of displaying the TMP of interest. Furthermore, a budded EPN displaying a regular array of conjugated TMP may look more similar to the native virus (and thus be more immunologically relevant and protective) than free-floating viral proteins, or even those same proteins stuck in the cell membrane. To reiterate, if we want to recapitulate a native virus via more-native-like structure of displayed antigen, then presenting antigen on the surface of a budded EPN is likely more efficacious than that same protein floating solubly or stuck in the plasma membrane of a cell.

Our first attempt to produce an EPN vaccine leveraged designed/stabilized SARS-CoV-2 sequences. After looking at several different constructs, we found that EPN-1-SpyC and the HexaPro-SpyT pair showed the best ability to decorate EPN (mentioned in more detail in Section 3). The HexaPro CoV-2 construct is a stabilized trimer construct which introduces six different proline residues to help with stability, expression, and immunogenicity of the CoV-2 Spike protein (Hsieh et al. 2020).

With this construct in hand, we also decided to test the route of mRNA delivery for EPN production/inoculation (as opposed to producing, purifying, and delivering the EPN in cell culture). The reason for this change in EPN production/delivery was that production and purification of EPN has been challenging, but more importantly the increased interest in mRNA vaccines towards SARS-CoV-2 made this line of interrogation particularly interesting. Scientifically, we reasoned that genetic delivery of the EPN cage

alongside the CoV-2 TMP would allow for the decorated EPN to bud out of the inoculee's cells, and may provide a better immunological response than the same CoV-2 TMP either being exported solubly, or retained in the plasma membrane.

We then worked with the Fuller Lab to design mRNA-based constructs, with the goal of delivering mRNA into mice and looking for anti-CoV-2 titers. Jesse Erasmus designed single-mRNA constructs which included either an IRES or 2A skip between the EPN and the CoV-2 TMP.

Vaccination of mice with a prime and 4-week boost of ~1ug mRNA resulted in immunological responses, for both the 2A and IRES linkers (Fig. 4E,F). While we do not have data directly comparing HexaPro EPN-CoV-2 to the same HexaPro CoV-2 construct (i.e. we do not have the EPN+TMP vs TMP alone), this data is a great positive result and has encouraged us to further investigate nucleic-acid based approaches for EPN vaccines.

DARPin-EGFR targeting Design and Rationale

In parallel with our EPN vaccine work, we also sought to use designed TMP to direct EPN towards specific cells as a step towards cell-specific delivery of biomolecules. We utilized Designed Ankyrin Repeat Proteins (DARPins)(Mosavi et al. 2004; Li, Mahajan, and Tsai 2006), to design a TMP to bind the Epidermal Growth Factor Receptor (EGFR) present on the surface of some cells. EGFR is commonly associated with poor cancer prognosis and is a common target for anti-cancer therapeutics (Seshacharyulu et al. 2012; Lieser, Chen, and Sullivan 2019; Santos et al. 2021). We reasoned that engineering our EPN to specifically bind these cells would be an appropriate step towards clinical relevance for the platform. We took our designed

minimal TMP “p#65” (chapter #3) and added a DARPin sequence targeting the EGFR N-terminally directly after the CD8SP (such that it is the most distal motif on the TMP). As an additional negative control, we also designed a TMP which was identical except that a Her2-targeting DARPin sequence was used in place of the EGFR-targeting DARPin sequence (Fig. 5A, B). The organization and designed morphologies of the different EPN and TMP combinations (Fig. 5C) meant that we covered a breadth of EPN morphology and display of interior or exterior GFP in order to find constructs which were both expressible and functional in our later flow cytometry assay.

DARPin-EGFR experimental design

With our EGFR-targeting TMP incorporated into EPN, we next needed to demonstrate that these EPN were directed specifically towards EGFR-expressing cells. We used WT K562 cells which do not express EGFR, and K562 cells with an introduced iRFP and EGFR construct (Fig. 5D, E). To reiterate, WT K562 cells had no EGFR or iRFP expression ($EGFR^-/iRFP^-$) while the EGFR-K562 cells expressed both ($EGFR^+/iRFP^+$). With a mixture of the two cell populations, addition of the EGFR-targeting EPN would mean that the GFP on the EPN TMP would colocalize with only iRFP cells (the $EGFR^+/iRFP^+$ cells also become GFP^+ due to the EPN colocalizing) while there would be no colocalization of GFP with the WT K562 cells (and the $EGFR^-/iRFP^-$ cells would remain GFP^-). We found the predicted results to generally hold true, although there was some low level of background colocalization. Additionally, the control Her2-targeted EPN would also fail to colocalize with the $EGFR^+/iRFP^+$ cells, which shows that the DARPin sequences were not broadly promiscuous in their binding. We also used different ratios of WT:$EGFR^+$ cells, and different doses of EPN to test how

specific the EPN targeting can be at different EPN concentrations and target cell concentrations. Notably, even in the “pure” $EGFR^+/iRFP^+$ cells, there was some loss of the iRFP signal and likely EGFR expression as the cassette was not under selective pressure, which means that there was always some population of $EGFR^-/iRFP^-$ cells creating a small “negative control” population in each experiment.

DARPin-EGFR targeting results

We were able to successfully show specific targeting of the $EGFR^+/iRFP^+$ K562 cells by the EGFR-targeted EPN (Fig. 5F). We also showed a lack of EPN targeting to WT K562 cells, and a lack of targeting by the Her2-targeted EPN, although the Her2 DARPin sequence is a bit promiscuous. Furthermore, we showed efficient targeting of the $EGFR^+/iRFP^+$ cells, at high concentrations of target cells, at low concentrations of target cells, at high concentrations of EPN, and at lower concentrations of EPN. More work needs to be done in organizing the analysis of the data for future publication. An interesting avenue of further work could be to quantify the number of EPN particles and calculate how many EPN were directed to each cell, but that remains outside the scope of the work at this time, especially given the challenges in quantifying EPN particles.

5. EPN cage assembly state

Background-Membrane binding strength affects EPN release efficiency

As scientists we try to form reasonable and testable hypotheses, but when working in new fields our assumptions about the way systems work can bite us in the ass. CryoEM imaging of EPN-1 was relatively straightforward (at least by the difficulties and labor usually involved in CryoEM). From the results of hundreds of Western blots, we know that protected release of EPN cage proteins is higher for versions of EPN which have a stronger membrane binding potential. This work was well-evidenced in the 2016 Votteler publication, comparing different EPN constructs in their release efficiency. We rationally chose to use better-releasing EPN constructs in order to further advance our work on the EPN platform; we swapped from EPN-1 (single myristoylation group binding motif) to EPN-7 (myristoylation and palmitoylation), EPN-11 (myristoylation and palmitoylation), and EPN-24 (pleckstrin homology domain) based constructs to maximize the release of our constructs. The first five years of my work on this project was done using EPN-7/11/24 constructs.

A series of experiments was also carried out largely by another scientist (and not included in this work), Cassie Ogohara, showing that increasing membrane binding affinity results in more efficient EPN release. Changing the charge state of the membrane binding motifs to include differently-charged residues resulted in changes for release efficiency. We assume this to be due to EPN cages being more or less likely to locate to the inner side of the cell's plasma membrane prior to ESCRT-mediated budding and exocytosis.

Monocage EPN

Using EPN-7 as a base, the SpyCatcher motif was added, which allowed for the EPN-7-SpyCatcher construct to conjugate with the TMP-SpyTag (see section 3). This conjugation efficiency was relatively high (Fig. 3B). When imagining an EPN with the majority of the monomers being covalently linked to a TMP, we thought that the membrane might be pulled tightly around a single cage. Given the relative efficiency of the conjugation apparent by western blot, we looked to CryoEM imaging to search for single cage EPN (Fig. 3C).

Upon imaging, few intact vesicles were found. There were however a small number of vesicles that contained a single cage. This finding was notable because single-cage vesicles were never found in all of the cryoEM imaging. While these “monocage” EPN were still a minor species (Fig. 3D, E), it showed that wrapping a membrane around a single cage was possible. We had long thought that the small 25nm diameter of the I3-01 nanocage would be too difficult to wrap in a lipid bilayer, due largely to a belief that membrane bending energies would be too unfavorable, and that EPN could only exist as the larger multicage morphology. This finding of monocage EPN was exciting on a structural level. Previous EPN had relied on the membrane associative forces of hydrophobic residues (myristoylation or palmitoylation motifs) or a membrane-lipid-binder (the pleckstrin homology domain), whereas the covalent conjugation of the cage to a TMP protein was strong enough to fully bend the lipid bilayer around the cage.

Monocage EPN could be desirable, both for their inherent aesthetic beauty and symmetry, but also practically for future applications and FDA approval as a therapeutic

agent/class. Being able to consistently evaluate or dose a therapeutic with a heterogeneous size distribution, and containing different numbers of nanocage inside may be challenging. Production of single-cage vesicles may allow for more accurate and more precise purification and evaluation of EPN, and may help convince regulators that this platform is in fact modular and controllable.

A challenging facet of the work to image these monocage EPN has been that many of the monocages have required tomography to sort the EPN from other small vesicles. Tomography involves choosing a single point/object and taking images at multiple angles, which is significantly slower than imaging a whole field. It is also difficult to know if a small vesicle is empty or has a nanocage prior to doing tomography, with many monocage EPN being identified from what was thought to be an empty vesicle. Additionally, purifying EPN away from other cellular-derived vesicles has been difficult and inefficient. All of these factors have resulted in a slow, labor-intensive process to identify these monocage EPN.

EPN-1 vs EPN-7

We faced many struggles in trying to image EPN-7 based constructs, and sought to re-image EPN-7 itself to confirm we were still able to make EPN. Despite a directed, focused effort to simply image a “basic” EPN-7 prep, we were unable to show an interior cage reconstruction for the EPN-7 construct. Upon realization that we were not able to successfully reconstruct EPN-7, and that we had only ever imaged EPN-1, we once again did a small comparison of EPN-1 imaging (comparing to the cryoEM in the 2016 paper) and EPN-7 constructs (Fig. 8A). We were in fact still able to image and reconstruct the nanocages in EPN-1, and we also clearly showed a difference in interior

cage morphology when comparing the two EPN constructs. At a genetic and protein level, the only difference between these two different EPN cages is the addition of the palmitoylation group for EPN-7; EPN-1 has only a myristoylation group, with EPN-7 having both the myristoylation and palmitoylation motifs. We hypothesized that the (presumably small) additional membrane binding potential of the palmitoylation group overcame the cage formation strength, and essentially forced the trimers to the inner surface of the EPN, rather than maintaining cage competency. Closer inspection of the EPN-7 images showed what appears to be trimers forming a “mantle” or layer directly on the inside of the EPN membrane (Fig. 8B). This sets up a model where the single myristoylation motif present in EPN-1 results in whole cages with many contacts next to the EPN membrane, while the myristoylation plus palmitoylation motifs of EPN-7 result in only trimers next to the inner membrane surface and a complete lack of cages within the EPN vesicles.

Influence of SpyCatcher and GFP on EPN release efficiency

As has hopefully been conveyed thus far, production of a membrane-enveloped nanocage (i.e. EPN) is a delicate balance between nanocage formation strength (that is to say the propensity of the nanocage subunits to form and maintain the larger structure), the membrane binding strength, the ESCRT recruitment efficiency (a topic not touched upon in this dissertation), the interplay (and interference) between all of these units, and their impact on protein expression. It should come to no surprise then that addition of a bulky SpyCatcher motif could negatively impact the efficiency of expression and protected/enveloped release of these nanocages. When added to an EPN-1 construct (meaning EPN-1-SpyC), we see a drastic decrease in the overall

expression and protected release of the construct relative to EPN-1 (Fig. 8C). While we do see a moderate decrease in expression and protected release levels for the stronger membrane-binding EPN constructs (i.e. EPN-7, -11, -24) when adding the SpyCatcher motif, there is still protected release which contrasts to the case of EPN-1 which has significantly abrogated release, and none of which is protected. It is interesting to note that protected release of EPN-1-SpyC can be “rescued” if conjugation with a different TMP can be achieved, suggesting that the bulky SpyC motif inhibits efficient membrane binding, indeed EPN-1-SpyC looks very similar to the membrane binding deletion mutant EPNΔM-SpyC, but that conjugation to a TMP can help to make up for this effect (Fig. 8D).

Similarly, while investigating EPN-GFP constructs for use with the DARPin flow experiment, it was noticed that addition of GFP to the EPN-1-SpyC construct to make EPN-1-GFP-SpyC completely abolished the release of the EPN construct (not just protected release, but all release into the SN). It was also seen within the same sample set that conjugation of the EPN-1-GFP-SpyC to a TMP-SpyT resulted in the conjugated species being found in the SN fraction (Fig. 8E). These results show that the EPN-1-GFP-SpyC protein was in fact present in the cell, and we assume that it was unable to efficiently localize to the inner side of the plasma membrane for the ESCRT machinery to initiate budding and scission. Conjugation to the TMP likely rescued localization to the plasma membrane, and subsequent release. This line of investigation, while specific to individual constructs, still points towards the delicate balance that must be achieved when weighing the inclusion of functional motifs, and that the accessibility of each motif is critical for the correct function of this complicated

biological process.

I32 EPN

We also sought to make EPN from different cage symmetries, in an attempt to generalize the methods and principles of wrapping nanocages in a membrane. Being able to have more than a single component in the cage may also allow us to include more functions by increasing the places to add motifs, or we may find that changing the expression of two different components could be beneficial for EPN. We tried two different I32 (Icosahedral cage made of trimers and dimers) versions, and then largely focused on the I32-06 cage. The I32-06 nanocage requires both the trimer and dimer components for full assembly. We were successful in getting protected release of the cages, indicating true EPN morphology. We were also successful in getting release of either component individually if both membrane binding and the ESCRT motifs were present on the individual component. This means that we were able to get protected release of just trimer or just dimer components, and that the higher-order nanocage structure was not necessary for release. This work was done before our realization that it was trimers (rather than full nanocages) inducing release of EPN-7, and therefore the fact that trimers and dimers were able to induce release on their own was a surprise.

Complications for EPN production and assembly

The EPN platform has been developed in order to help display useful motifs on the outside of a vesicle. These vesicles may be able to display proteins which otherwise would require large amounts of stabilizing mutations in order to maintain conformation, or be able to bear fusogenic peptides to allow for delivery of biologics into recipient cells. While there are many methods to induce release of vesicles from mammalian

cells, we have found that getting nanocages to recruit the budding ESCRT machinery at the inner side of the plasma membrane to be relatively efficient in producing fully-enveloped and protected EPN. However, the production of EPN have been challenging and it is important to note some of the complications that come with producing the hybrid biological nanomaterials. It's known that overexpression of VSV-G can result in the budding of small VSV-G-decorated vesicles from cells, called "gesicles"(Mangeot et al. 2011; Breakefield, Frederickson, and Simpson 2011; Campbell et al. 2019). VSV-G is also relatively expressible, and it was challenging ensuring that our EPN were in fact cage-bearing vesicles and not simply empty, VSV-G presenting gesicles. When working with the PDGFRβ-based TMP's (i.e. p#65), we were able to get release of vesicles with only high expression of the TMP in the absence of any cage. Similarly, we were able to get release of a single component of the two component I32-06 nanocages, meaning that in certain circumstances we were able to induce vesicles containing only trimers or dimers, something which was also demonstrated in the EM images of the EPN-7 trimers. It is clear that vesicles can be induced to release from HEK-based cell lines, and that all of these factors are important:

1) Membrane binding strength of the cage component
2) Expression level of any TMP
3) Interactions between TMP and cage
4) Cell line specific factors

It's clear that more work can be done to help elucidate some of the mechanics which influence EPN biogenesis, and that many of these same mechanics may pertain to the biogenesis of other vesicles, or even budding viruses.

6. Technical challenges of EPN production

The production of EPN for this project has been highly challenging and time consuming. While it is not uncommon for the bulk of a project's time to be spent setting up and optimizing experiments, timely EPN preparations have been hindered for almost a decade by substantial problems in production, harvest, and evaluation.

Cell line specific effects on EPN production

Initial experiments for EPN production were carried out in 10cm TC-treated culture (Tissue Culture) dishes, with the initial CryoEM average in the 2016 paper requiring a prep of 40 dishes to produce enough concentrated sample for proper imaging. In an effort to reduce the time required and ease of cell passaging and bulking, the speed of EPN production and purification, and the scalability of prep sizes, we looked at different HEK 293-based cell lines.

We wanted to transition to a suspension cell type, which we thought would reduce the labor required for maintaining and bulking up the cells for transfections and be more straightforward in scaling up production volume. As an example, the 40 10cm dishes equates to approximately 400mL of EPN-containing cell supernatant. For this 293T-based preparation, the 293T cells were bulked up to approximately 30 plates, then harvested individually with washes, trypsinization, homogenization, and then counting of the cell-media mixture prior to plating; meanwhile each of the individual 40 plates requires coating with a cell adhesion liquid (in our case 0.1% gelatin). On the day of transfection, each of the 40 plates would need to be carefully given the transfection media, and the media aspirated off each individual plate and carefully replaced without disrupting the cell monolayer later that day or following morning. The harvest 24-72h

post-transfection, requires that the EPN-containing supernatant would be individually transferred into a bulk bottle, then filtered to remove cell debris. We did slightly improve this workflow by transitioning from 10cm to 15cm dishes (Figure 9A).

In contrast to the labor-intensive and sensitive process of adherent cell transfections and harvest, it is significantly easier to take an appropriate volume of suspension cells, dilute into fresh media, and then transfect (Fig. 9B). If necessary, it would be possible to centrifuge the cells down and replace the media (as is the case with adherent cells), but it does not seem necessary. Harvest is exceedingly easy, where you can simply pellet the cells, pour off the EPN-containing supernatant, and sterile filter.

For our general purposes, I estimate that a transition to suspension cells would save approximately 75% of the total time of maintenance, transfection, and harvest compared to adherent cells. Towards this end, we tested suspension Freestyle 293F as a replacement for adherent HEK 293T cells, and later compared the Freestyle 293F to expi293F (Fig. 9D). Due to their ease of maintenance and high protein yields, amplified by their ability to sustain high cell densities during transfections (compare high-cell lanes between expi293F and Freestyle in Figure 9D), we settled on the expi293F system for both our EPN work and general mammalian-based protein production at the IPD.

We thought that expi293F-derived EPN preps may have a small increase in cell debris and “junk” when compared to 293T-derived EPN preps, but that the increased overall expression and ease of throughput greatly made up for the shortcomings of the expi29F system for our EPN production. We also assumed that the difficult, technically

challenging, and time-consuming EPN harvest, purification, and concentration protocol (Fig. 9C) would be sufficient to overcome any increase in “junk” that was introduced by the expi293F system. Regardless of the cell type, after cell SN has been 0.45uM filtered, 25mL of SN is put into an ultracentrifuge, and 5mL of a solution of 20% sucrose in PBS is added slowly as a cushion to the bottom of the tube, making sure to try to maintain the integrity of the cushion. After a 70min spin at 100,000g, the EPN will be pelleted through the sucrose cushion, therefore all of the liquids are decanted. If increased concentration is desired, additional supernatant+sucrose cushion can be placed on top of the existing pellet and spun again. If an EPN prep is to be imaged via CryoEM, then a PBS wash with a 30min 100,000g spin is used to eliminate any residual sucrose as it may cause issues during the freeze. The remaining EPN pellet is usually not visible, but it is resuspended in an appropriate amount of PBS usually such that the ending amount is 200x concentrated (i.e. 300mL of cell SN down to 1.5mL of concentrated EPN) although this can vary. From here the EPN can be used for assays, typically starting with a western blot to ensure that the correct proteins are present (i.e. anti-myc bands at expected sizes).

After many years of production and struggles to recreate CryoEM images of similar quality to the 2016 paper, we went back and compared EPN-1 preps from both 293T and expi293F cells. What we found was that despite the intensive purification protocol, the expi293F-derived preps were significantly “dirtier” when compared to the 293T-derived EPN (Fig. 10A). Interestingly, the overall number of vesicles does not seem to be dramatically different, but the large increase in cellular debris, un-enveloped “naked” cages, and partial vesicles confirmed that expi293F-derived EPN preps are not

suitable for EM imaging. Since this finding in June 2022, we have fully transitioned back to 293T-based production, which has dramatically slowed progress, both in terms of greatly reduced throughput per time, and in time spent rebooting a “new” cell line and workflow which had been abandoned for 5 years.

Challenges in producing membrane-bound nanocages

In addition to problems deriving from cell line specifics, and all production work requiring time in a BSC, we thought that EPN might also have lower yield resulting from their requirement to exocytosis, as opposed to just being secreted. In the King Lab in particular, secreted proteins have been routinely made for many different projects, and high secreted protein yields have been achieved particularly with produced antibodies. In an effort to try to quantify this difference, I prepared 3 different EPN preps and compared them to 3 different secreted I3-01-based constructs (JW-20, -21, -22). The results (Fig. 10B) showed that EPN are significantly lower in overall non-cell-bound production (sum of the R and SN-P bands) than strictly-secreted nanocages. This blot also shows that a significant portion of the overall EPN protein production remains cell-associated and is not released (C lane), and that a large portion is also not spun down into the EPN pellet (R lane). Furthermore, even the pelleted EPN have a large portion of trypsin-sensitive fraction (T lane, smaller bands), although the caveat is that these preps were made in expi293F cells, so this result is not unexpected and may change in the context of 293T cells.

Successful plasmid production and QC is not something that is only an issue with the EPN system, but the aforementioned production challenges and low overall yields mean that any issues with plasmids become exacerbated. Early in the project many

times there would be an unexpected and unexplained failure in transfection. While this problem still exists to some extent, a large portion of the problem was eliminated when plasmids were run post-purification to ensure that they not only had good concentration by Nanodrop, but also looked as expected in a DNA gel (Fig. 10C). With one particular plasmid purification kit in general, there was a large incidence of sheared DNA (whether genomic or plasmid, it's unknown), across several different lab members. This large increase in sheared components results in transfections which consist mostly of transfecting in junk, and result in very low protein yields. Traditional plasmid quantification and QC methods such as A260/A230 generally capture contamination and not pDNA size. This is a finding applicable to transient protein production in general, however the effect was disproportionately severe during the many different co-transfections (i.e. in a 2x2 transfection scheme if only 1 of the 4 plasmids is of poor quality then two of the four transfections will be affected) and low overall protein production in EPN preps.

In addition to unexpected plasmid quality issues, the quality of our transfection reagent varied across the last 7 years of the project. The effects here are similar to that of the plasmid quality issue, in that generally it was a rare occurrence, but it did happen multiple times. Even if not of catastrophic significance (Fig. 10D), the overall impact on EPN production throughput was large while it remained an undetected issue.

Challenges in EPN detection and quantification

In a similar vein to some of the challenges listed above, the western blot process which was routinely used was occasionally prone to failure. In addition to any transfection or purification issues which could cause a real problem in the EPN

samples, oftentimes the western blot process or reagents would lead to a failure in detection or inaccurate assessment of results (such as a failed construct when in fact it was simply a failure to image correctly). There have been different imaging reagents and kits, different instruments for imaging, and different transfer machines used throughout the process, all of which have had unexpected errors or complications. The most frequent cause of failure for western blotting in our lab has been due to degradation of the primary antibodies, typically when starting up western blotting again after a month or two of not needing to use the technique. In an effort to reduce the number of antibodies that were required, I transitioned to a ladder which has horseradish peroxidase (HRP) directly conjugated, thereby eliminating the required anti-ladder antibody which we had been using. In data now shown here, the first three years of the project involved a different ladder whose antibody also detected some substrate in the cell and SN fractions around 50kDa, which often complicated interpretation of the blots (and would have to be explained each time a new collaborator saw it). All of our EPN and TMP constructs include a myc tag for easy detection in blots. In further efforts to cut down on the different antibodies necessary, direct anti-myc-HRP antibodies were used, which would allow us to eliminate the anti-mouse-HRP secondary antibody we had commonly used in conjunction with a primary mouse anti-myc antibody. While many of these antibodies worked, we of course first tried one which did not, the CST direct anti-myc-HRP antibody (Fig. 10E right-most set). We did eventually find a suitable replacement in the Invitrogen anti-myc-HRP antibody, but even in lots sourced just a few months apart and both kept at the recommended conditions, we still see very large differences in detection in the same exact blot imaged simultaneously

(Fig. 10F). Additionally, we have struggled to come up with a suitable and rapid quantification method for EPN, and have settled for comparing the relative band intensities of a 1wa3 trimer which was previously purified and quantified, and comparing a particular EPN construct against dilutions (Figs. 5B, 6A, or 7E for example). Many other groups have figured out methods for better quantifying exosomes, and it may be beneficial to try to adopt or adapt some of their techniques.

Almost all of these production and quantification issues are surmountable and have been effectively dealt with via rigorous troubleshooting. One of the issues with production of enveloped particles, such as vesicles or retroviral vectors has been the difficulty in scaling up and purifying sufficient amounts of materials. Part of the challenge of the EPN project within the King Lab and IPD in general has been the requirement to do work in a BSC, with a large input of effort and time, and relatively production, purification, and quantification. Methods have evolved over time (such as plasmid QC post-purification or comparing EPN preps to a quantification standard), but working in isolation on a very different system with a very large amount of failure points between production and evaluation has been quite difficult and taxing. While each of these roadblocks represent a small, perhaps less than 10%, failure rate, they work multiplicatively such that on average an EPN prep usually will fail for non-”scientific” reasons rather than because the construct inherently will not work. Additionally, the challenges of doing the workflow alone means that design, production, verification, and analysis cannot realistically be maintained alongside other duties. It is significantly more error-prone to stop and start processes (such as cell culture lines, or batches of western blots) than to keep a steady pace which helps to identify error points quickly. As an

example, if a set of western blots are failing and you don't have a good positive control from recent production, it can take a day to find out if it's due to an antibody losing detection capability, the rest of the week if it was due to improper protease treatment or some other assay problem, the whole next week if it was a problem of the transfection or harvest, and then the whole month if it's deemed to be a problem stemming from a cell line. In contrast, if changing to a new aliquot of an antibody suddenly results in failed images, it can be pinpointed with confidence much more quickly. While these troubleshooting methods are typically done sequentially, it still amounts to a large amount of lost time, materials, and effort.

7. Future Directions

As with any successful project, in particular one focused on technology platform development, there exists a large array of possibilities for follow-up study. The following avenues of research and development are things which I believe to be a combination of fundamental technology development (for example increased production and characterization), and more interesting and therapeutically-relevant applications (such as drug delivery). Allocation of research resources, specifically within the context of an academic environment is of course essential in assigning the priority to which avenues should be investigated and developed.

Addition of detargeted VSV-G

There is currently a large interest in targeted delivery to different cellular subset. The EPN platform allows for packaging of different protein cargoes, and the use of designed TMP can allow for specific targeting of certain cellular subsets. In the near future, we plan on taking the logical next step in the DARPin-mediated EPN targeting project by going from the current cellular association assay and showing delivery of functional protein into target cells. Using the same assay, we will also leverage a de-targeted VSV-G construct (Yu et al. 2021) combined with our current DARPin-conjugated-EPN's. VSV-G has both broad cellular targeting and endosomal escape/intracellular delivery capabilities which has made it an invaluable tool for gene therapies; however the broad tropism of VSV-G also precludes its use for targeted delivery. By knocking out the cell targeting capability, the EPN will instead be targeted to cells solely through the DARPin constructs. This will allow for the targeting capability of the DARPin to dictate which cells the EPN will be targeted by, while still retaining the

endosomal escape and intracellular delivery properties of VSV-G. We can then load the EPN with beta lactamase (BLaM) and the recipient cell population with the CCF4 dye used in the 2016 BLaM delivery assay, but crucially we should see BLaM activity solely in the cells targeted by the DARPin (in this case $EGFR^+$ cells). This will be a complicated assay which will almost certainly be difficult, but combines two assays we have successfully used before: the BLaM assay and the DARPin-targetting assay, both of which are flow cytometry assays and can be run in parallel with the use of selected dyes. We anticipate colocalization of GFP with iRFP (showing EPN going towards only $EGFR^+$ cells), also colocalizing with the cleaved CCF4 dye (indicating successful delivery of the EPN contents). Negative controls include the off-target Her2 DARPin, and WT ($EGFR^-$) cells, while the WT VSV-G will offer a positive control which should successfully deliver the BLaM to both on-target ($EGFR^+$) and off-target ($EGFR^-$) cells. Constructs are already in-hand, with experiments planned shortly.

Eventually, different fusogenic peptides should be used, with a focus on proteins which are not inactivated by human serum. We have considered other viral fusogens including measles, influenza, and Nipah viral fusogens, but establishing an assay and successful workflow will help to rapidly evaluate other fusogenic proteins. Testing in various animal models, or switching the payload to a therapeutic molecule would be a logical step after successful delivery to cell lines. The eventual goal is for efficient and specifically targeted *in vivo* delivery via EPN.

TMP-EPN

The use of the “degreased” I3-01 sequences has allowed for single-polypeptide cage-TMP constructs. These constructs may allow for more efficient production of

TMP-EPN, which could be of big consequence when it comes time to manufacture EPN at any scale, including the ongoing animal studies for the EPN-based CoV-2 mRNA vaccine. Reducing the number of constructs helps to decrease complexity, and within just our own EPN work, we have seen that increasing complexity (by introducing TMP into EPN production) drastically increases the failure rates. We would like to continue developing TMP-EPN, and try new cage architecture and different TMP, with the goal of recapitulating the functions of the TMP-conjugated-EPN such as immunogenicity and cell-specific targeting. While the conjugation technique has proven to be relatively reliable and efficient, a TMP-EPN ensures that there is exactly one TMP for each cage monomer, which could be advantageous in circumstances where maximal display of a TMP is desired. One downside of the TMP-EPN strategy is that you cannot decouple or re-tune expression levels, however it seems that combining constructs and reducing the total possible points of failure in the production and expression of EPN warrants exploration and further development.

Re-test CoV-2 mRNA EPN

While there has already been success in using “naked” protein nanocages to display antigens for vaccines, development of EPN-based vaccines may present several advantages. We would like to continue to investigate EPN as a vaccine platform, especially given the rise of mRNA-based delivery for vaccines. More-carefully controlled experiments, as well as testing with DNA-based plasmid vaccines is currently planned. We are very interested in determining how EPNs, designed as free-floating carriers of antigen in the native context of the membrane, perform against secreted antigen, membrane-bound antigen, and even antigen similarly-presented in an array on

“naked” protein nanocages. Additionally, while we have reason to believe that the conjugating EPN are able to more-efficiently display CoV-2 antigen, comparing a “passive” pseudotyping approach with the “specific” conjugation approach to CoV-2 spike decoration of the EPN could help show whether differences that we see on a Western blot translate to a functional difference in an animal model. It will be interesting to retest the CoV-2 mRNA EPN vaccine with a more comprehensive experimental design

Production of HIV-EPN

Given the ability for EPN to successfully incorporate and display TMP of interest, and the ability of the mRNA CoV-2 vaccine to elicit a response in mice, we are very interested in attempting to display HIV Env on the surface of the EPN, as a potential vaccine candidate. HIV has long been a difficult target for vaccines, partially due to the difficulty in expressing the Env trimers, and further complicated by the relatively low-abundance of the Env on the natural HIV particles (Klein and Bjorkman 2010). but the field has continued to make progress, and is now focusing on advances in designed HIV Env for vaccine purposes, with new interest membrane-bound VLP as a method to display the Env antigens in a more immunologically-similar way to a natural HIV particle (Gonelli et al. 2021).

This is a very ambitious project, and the first step we’d like to take is to look for expression of stabilized Env constructs on our exocytosed EPN. Following that, we would like to use flow cytometry analysis of these EPN and producer cells with antibodies which recognize the specific conformations of the Env trimer to see if the trimers we’re producing are in fact biologically relevant. If we’re successful in

recapitulating the correct orientation and conformations of the Env trimers in our EPN system, we can begin to envision ways to test these as vaccine candidates, particularly with an mRNA delivery system similar to what was used in our CoV-2 experiment.

Evaluation of EPN production in the context of other EV's

While not as interesting scientifically as trying to introduce new fundamental capabilities or proofs of function, EPN still must meet or overcome certain producibility problems which currently plague other membrane-bound particles such as EV's and retroviral vectors. It is clear that the EV field as a whole is still sorting out best practices for production, evaluation, and functionalization of these particles. What would be illuminating would be to compare EPN against other classes of membrane-bound particles, such as LV's, cell-derived EV's, or LNP's for ease of production, amount produced, and relative capabilities. This seems like a broad, undefined goal however it seems that the best way to try different production, purification, and quantification practices would be to compare technologies directly and evaluate whether or not EPN offer distinct advantages. As an example, production of LV's which are much larger and may be more destructive to the host cell could be balanced against the less-evolutionarily-refined EPN platform with smaller particles and likely more robustly-produced by cells. In addition to rough quantification, using VSV-G to pseudotype both particles would allow quick evaluation of relative delivery capabilities. While the EPN technology has promise in this new and rapidly-developing field, it's still unknown where exactly it stands relative to other technologies of varying maturity. Many groups are beginning to incorporate Size Exchange Chromatography (SEC) in addition to ultracentrifuged-based purification strategies, and it will be useful to keep abreast of

the field and see if there are applicable methods which may allow higher concentrations, more pure EPN fractions, or even a move back to the “messy” expi293F or other suspension cell lines which would greatly reduce the labor involved in EPN preps. While it is important to try to optimize EPN production, it is also important to realize that an academic lab may not be the most suitable space to optimize difficult production problems, and that introducing new fundamental capabilities, even if it takes a large amount of labor to produce, may be more appropriate; it may be that an interested commercial partner would be the most efficient way to optimize production.

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9. Figures

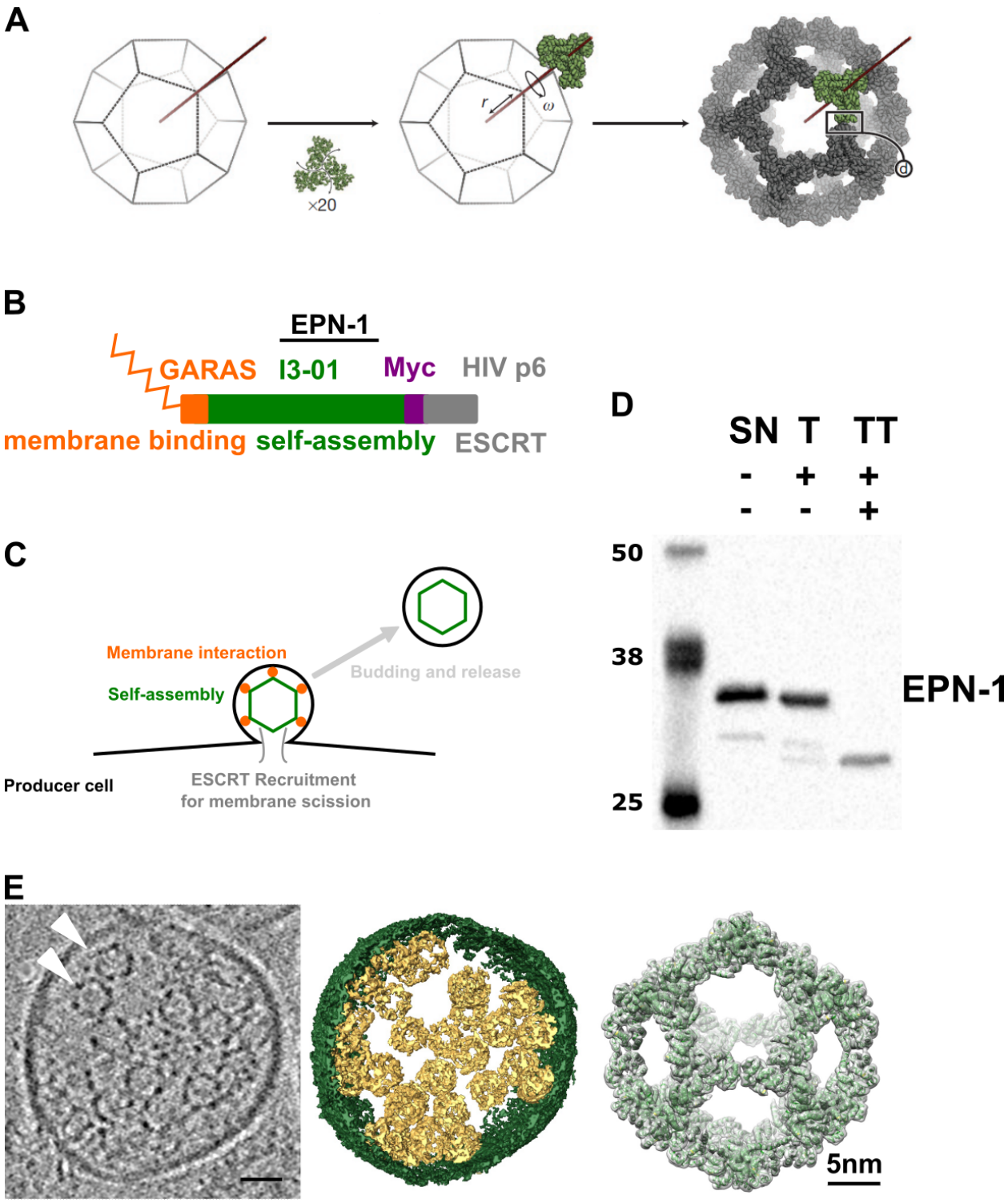


Figure 1. Basis of EPN design, release, and morphology.

A) Adapted from Hsia et al. *Science* (2016): A self-assembling trimer with designed trimer-trimer interfaces forms a 25nm icosahedral nanocage **B)** The EPN-1 construct is comprised of the I3-01 self-assembling nanocage (green), a myristoylation motif which directs the nanocage towards membranes (orange), a myc tag for identification on a western blot (purple), and the ESCRT recruitment motif for budding of the nanocage from the producer cell (gray). **C)** visual representation of a single cage interacting and budding from the producer cell into the supernatant. **D)** Western blot of released EPN harvested from cell supernatant. Lanes showing supernatant (SN), trypsin-treated supernatant (T), and trypsin and triton-treated supernatant (TT). **E)** Adapted from Votteler et al. *Nature* (2016): Central slice from a cryo-EM tomographic reconstruction of a released EPN; two internal protein nanocages are marked with arrowheads. Isosurface model of the 3D cryo-EM reconstruction from the first image, with the EPN membrane in green and individual protein nanocages are gold. Single-particle cryo-EM reconstruction of the I3-01-based nanocages released from EPNs following detergent treatment. Charge density from the 5.7 Å resolution electron microscopy reconstruction is shown in grey (contoured at 4.5σ). The I3-01 computational design model9 (green ribbon) was fitted into the density as a rigid body.

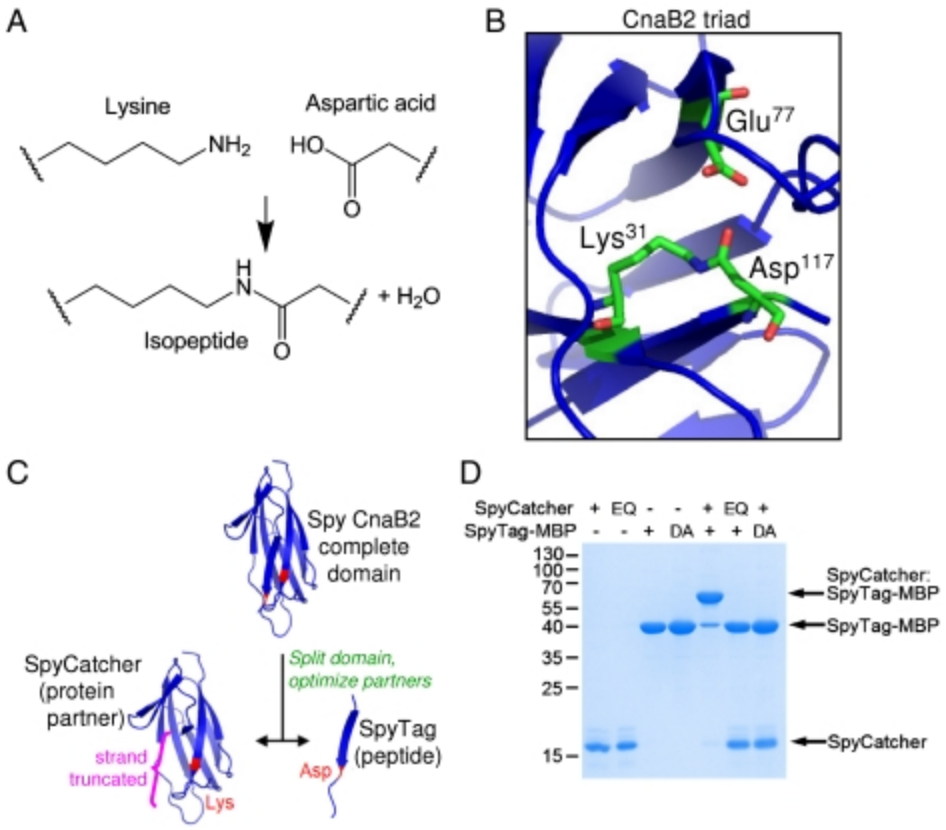


Figure 2. From Zakeri et al. *PNAS* (2012): Spontaneous intermolecular amide bond formation by SpyTag.

(A) Amide bond formation between Lys and Asp side chains. (B) Key residues for amide bond formation in CnaB2 shown in stick format, based on PDB 2X5P. (C) Cartoon of SpyTag construction. Streptococcus pyogenes (Spy) CnaB2 was dissected into a large N-terminal fragment (SpyCatcher, left) and a small C-terminal fragment (SpyTag, right). Reactive residues are highlighted in red. (D) SpyTag and SpyCatcher associated covalently. SpyTag-MBP and SpyCatcher were mixed each at 10 μM for 3 h and analyzed after boiling by SDS-PAGE with Coomassie staining, alongside unreactive controls, SpyCatcher E77Q (EQ) and D117A (DA) SpyTag-MBP.

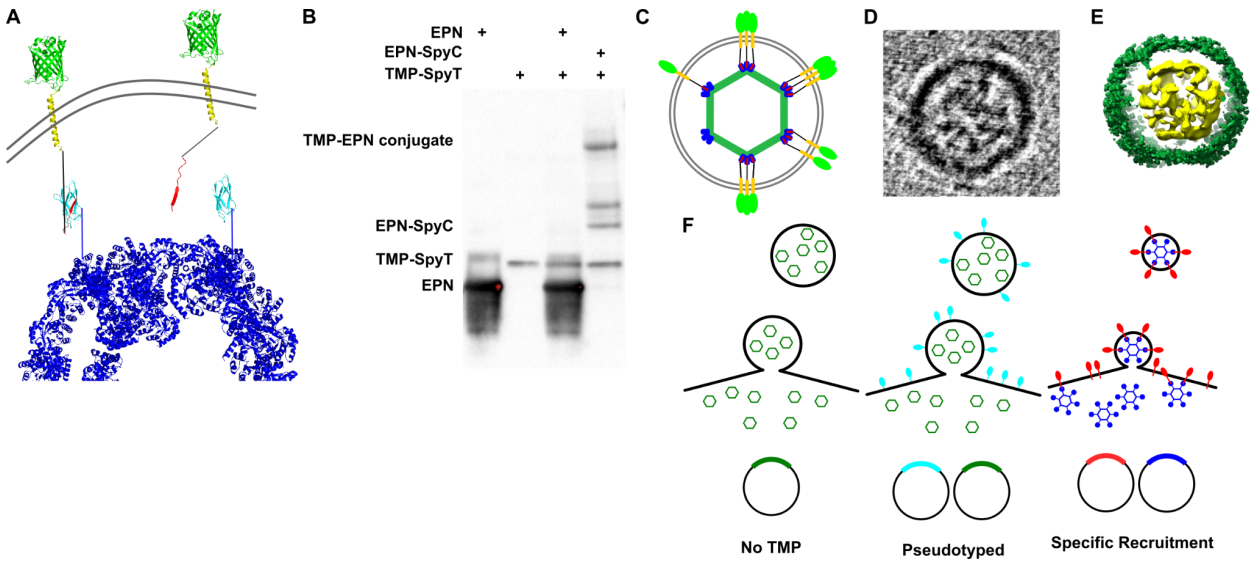
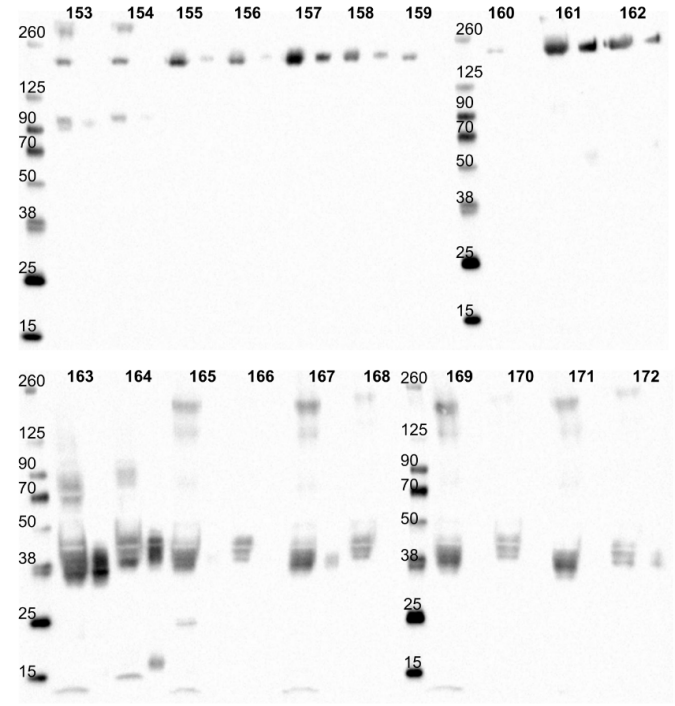


Figure 3. SpyCatcher-Tag conjugation allows for decoration of EPN with designed TMP, and can alter EPN morphology.

A) 2-D schematic of a Transmembrane Protein Conjugated EPN (TMP-C-EPN) with the I3-01 nanocage (blue) fused to a SpyCatcher (cyan) covalently conjugated to the intracellular SpyTag (red) of the designed TMP also consisting of a minimal PDGFRβ TMD (yellow) and extracellular GFP (bright green). B) Western blot of harvested SN fractions of EPN preps showing EPN, TMP-SpyT, EPN pseudotyped with TMP-SpyT, and resulting conjugated species upon transfection with both EPN-SpyCatcher and TMP-SpyTag. C) Cartoon depicting a monocage EPN. Nanocage protein (green) is genetically fused to a spyCatcher (blue), and is covalently conjugated to a SpyTag (red) fused to the intracellular tail of a PDGFRβ TMD (yellow) which has an extracellular moxGFP (green). D) CryoEM tomography image of a monocage TMP-C-EPN and E) 3-D reconstruction of image in D with the cage (yellow) and membrane (green) densities. F) Schematic depicting the different released and EPN morphologies for an EPN transfection, a pseudotyped EPN co-transfection, and co-transfection with specific

recruitment between the cage and TMP.

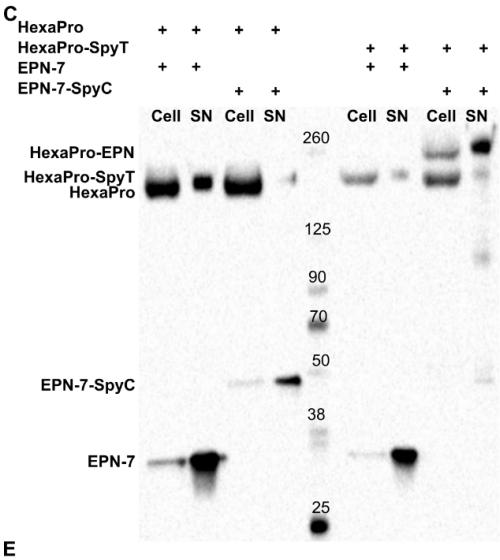
A



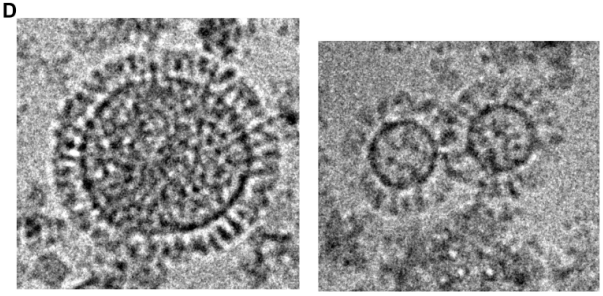
B

Sample ID	Construct Design Elements	Design kDa
153	[CoV2-S]-myc	143
154	[CoV2-S]-myc-12GS-SpyTag	146
155	[CoV2-S_ZXC21S_furinmutant]-myc	143
156	[CoV2-S_ZXC21S_furinmutant]-myc-12GS-SpyTag	145
157	[CoV2-Start]-[CoV2-Stab_Tri]-[S_TMD_CT]-myc	143
158	[CoV2-Start]-[CoV2-Stab_Tri]-[S_TMD_CT]-myc-12GS-SpyTag	146
159	[CoV2-Start]-[CoV2-Stab_Tri]-[PDGFRβ]-myc	142
160	[CoV2-Start]-[CoV2-Stab_Tri]-[PDGFRβ]-myc-12GS-SpyTag	144
161	[CoV2-Start]-[CoV-2-HexaPro]-[S_TMD_CT]-myc	143
162	[CoV2-Start]-[CoV-2-HexaPro]-[S_TMD_CT]-myc-12GS-SpyTag	146
163	[CoV2-Start]-[CoV2_RBD]-[S_TMD_CT]-myc	33
164	[CoV2-Start]-[CoV2_RBD]-[S_TMD_CT]-myc-12GS-SpyTag	36
165	[CoV2-Start]-[CoV2_RBD]-[PDGFRβ]-myc	32
166	[CoV2-Start]-[CoV2_RBD]-[PDGFRβ]-myc-12GS-SpyTag	36
167	[CoV2-Start]-[CoV2_RBD-Rpk4]-[PDGFRβ]-myc	32
168	[CoV2-Start]-[CoV2_RBD-Rpk4]-[PDGFRβ]-myc-12GS-SpyTag	35
169	[CoV2-Start]-[CoV2_RBD-Rpk5]-[PDGFRβ]-myc	32
170	[CoV2-Start]-[CoV2_RBD-Rpk5]-[PDGFRβ]-myc-12GS-SpyTag	35
171	[CoV2-Start]-[CoV2_RBD-Rpk9]-[PDGFRβ]-myc	32
172	[CoV2-Start]-[CoV2_RBD-Rpk9]-[PDGFRβ]-myc-12GS-SpyTag	35

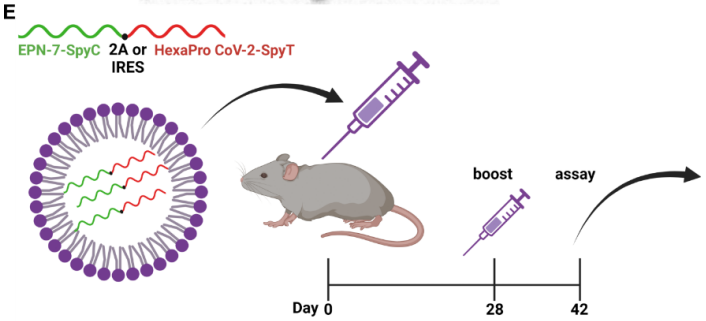
C



D



E



F

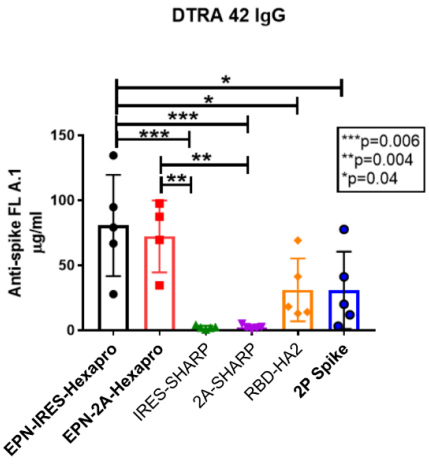


Figure 4. SARS-CoV-2 conjugated EPN show some level of protection in early mouse trials.

A) Western blot showing the expression levels of different tested SARS-CoV-2 TMP. For each sample the first lane shows the protein in the cellular fraction and the second shows the protein levels in the supernatant fraction. B) Table detailing the construct ID, design features, and calculated molecular weights for each of the samples in the blots in A). C) The lead vaccine construct candidate HexaPro was selected from the set tested in A, and was further tested with an EPN component to look for efficient decoration of the EPN surface. Conjugation of the EPN-SpyCatcher with the HexaPro-SpyTag improved the amount of TMP found in the released EPN fraction in the supernatant. D) EM images of particles of the conjugated EPN-7-SpyC and HexaPro-SpyT EPN particles. E) Schematic of the mRNA transcripts enveloped in a LNP. Mice were injected with 1ug of mRNA, and boosted with another 1ug dose 4 weeks later. Six weeks post start mice sera were analyzed. The EPN-7-SpyC and the HexaPro-SpyT components were separated by either an IRES or 2A motif. F) ELISA results from the vaccine study, including the first two EPN samples using either an IRES or 2A motif.

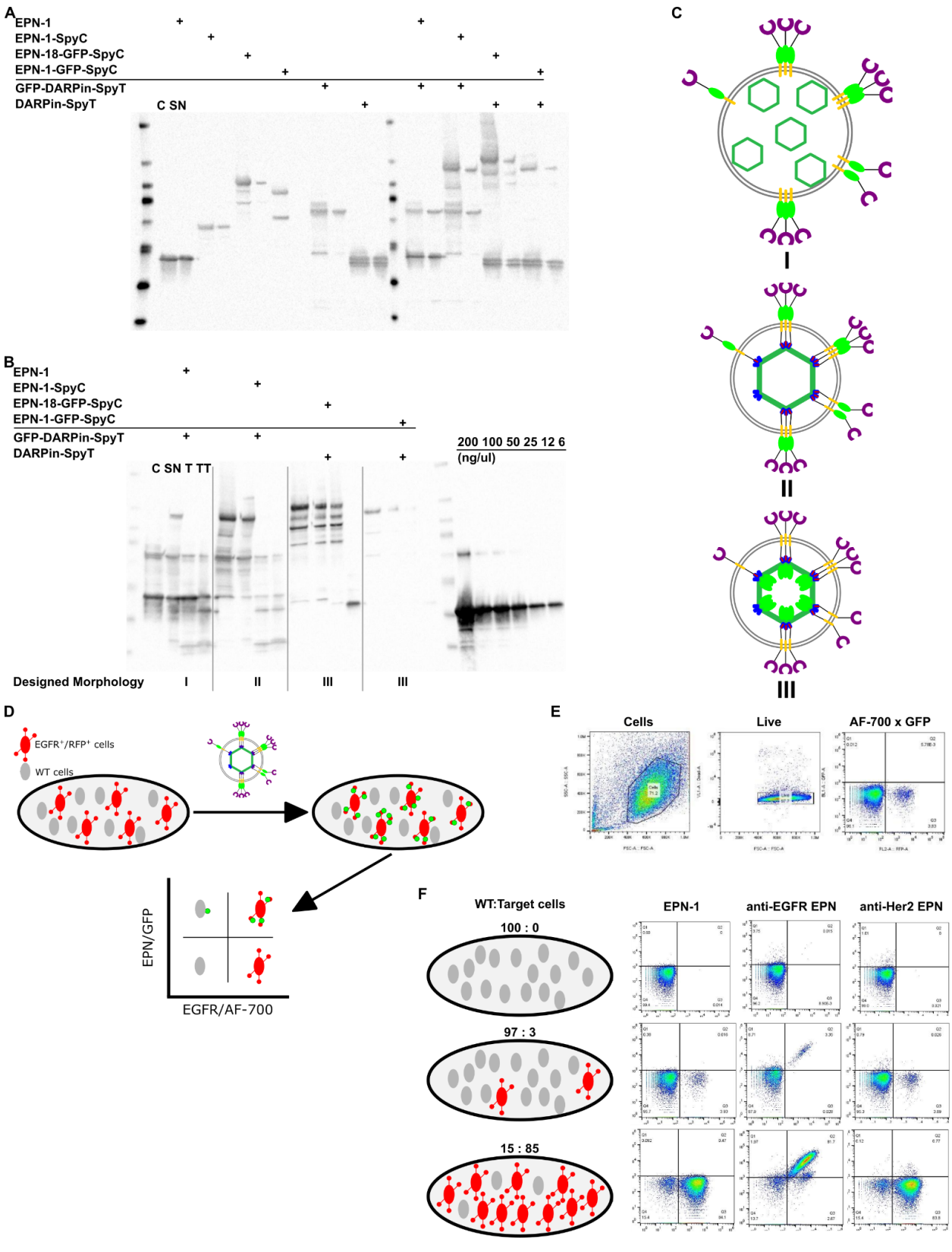
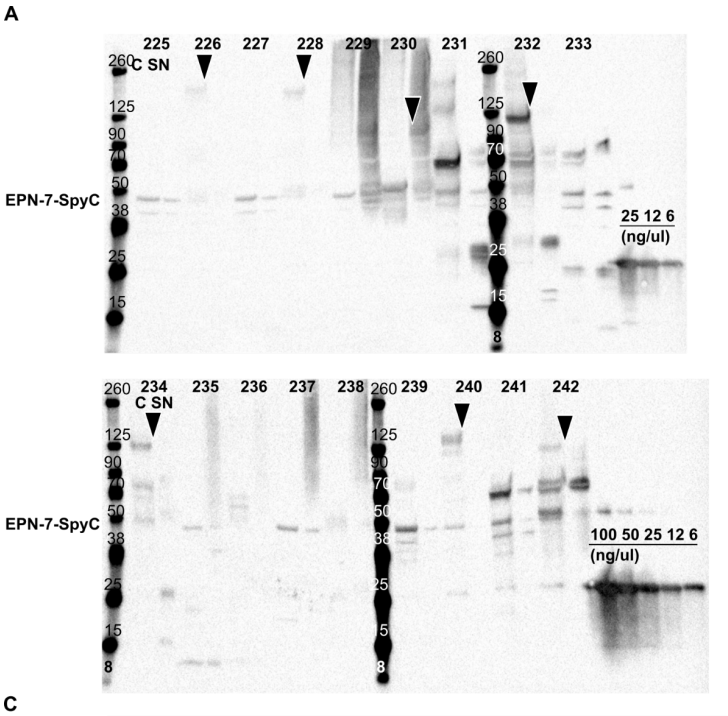


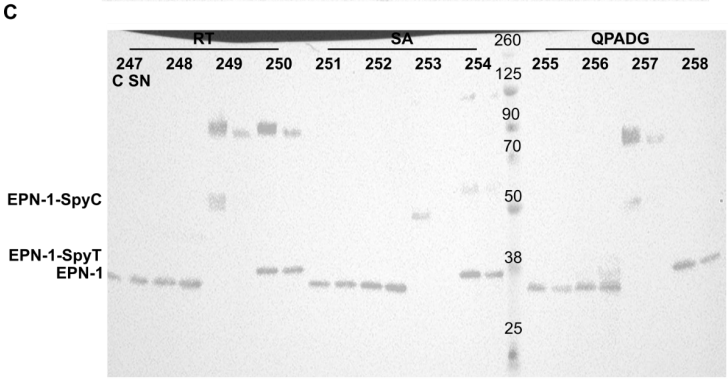
Figure 5. Inclusion of an EGFR-binding DARPin targets EPN to EGFR$^+$ cells.

A) Western blot showing the different varieties of cage and TMP in both the Cell (C) or Supernatant (SN) fractions for the individual components and then some combinations of cage and TMP. B) Combinations of selected components from A probing the cell, supernatant, trypsin-treated supernatant, and triton and trypsin-treated supernatant (C, SN, T, TT respectively). Designed morphology row refers to C. C) The schematic mockup of an idealized DARPin-conjugated-EPN with I consisting of a multi-caged pseudotyped EPN, II consisting of a conjugated EPN with extravesicular GFP on the TMP component, and III a conjugated EPN with intravesicular GFP on the cage component. The anti-EGFR DARPin is in purple, the mox-GFP is in bright green, the minimal PDGFRβ TMD in yellow, SpyTag in red, SpyCatcher in blue, and I3-01 cage in dark green. D) Schematic of a flow experiment with a mixture of WT K562 cells and $EGFR^{+}/iRFP^{+}$ K562 cells. The GFP+ DARPin-conjugated-EPN should preferentially bind the EGFR+ K562 cells and result in a double positive $GFP^{+}/iRFP^{+}$ overlap if there is successful targeting. E) Example flow gating for live cells showing a partial mixture of WT and $iRFP^{+}$ cells. F) Flow cytometry results for the control EPN-1, EFGR-targeted EPN, and off-negative control Her2-targeted EPN. The experiment was done for fully WT, a low target cell pool (~3%), and a high target cell pool (~85%).



B

Sample ID	TMP Design Elements	TMP kDa	TMP-Cage kDa
225	B8 (BG505 v8.1 SC TM-HIV)-myc	80	-
226	B8 (BG505 v8.1 SC TM-HIV)-myc-8GS-SpyT	82	125
227	ConM v7 SC TM-HIV-myc	78	-
228	ConM v7 SC TM-HIV-myc-8GS-SpyT	80	123
229	CD47-myc	36	-
230	CD47-myc-8GS-SpyT	38	81
231	SYCY1-myc	61	-
232	SYCY1-myc-8GS-SpyT	63	106
233	SYCY2-myc	61	-
234	SYCY2-myc-8GS-SpyT	63	106
235	CD195 (CCR5)-myc	42	-
236	CD195-myc-8GS-SpyT	44	87
237	CD184 (CXCR4)-myc	41	-
238	CD184-myc-8GS-SpyT	43	86
239	MI_15_HA-myc	65	-
240	MI_15_HA-myc-8GS-SpyT	67	110
241	MI_15_NA-myc	53	-
242	MI_15_NA-myc-8GS-SpyT	55	98



D

Sample ID	CSP Design	Cage Architecture	CSP + Cage kDa	CSP-Cage kDa
247	RT-PfGPI	EPN-1	27 + 30	-
248	RT-hsGPI	EPN-1	28 + 30	-
249	RT-SpyT	EPN-1-SpyC	26 + 44	70
250	RT-SpyC	EPN-1-SpyT	37 + 33	70
251	SA-mut-CSP-PfGPI	EPN-1	45 + 30	-
252	SA-mut-CSP-hsGPI	EPN-1	47 + 30	-
253	SA-mut-CSP-SpyT	EPN-1-SpyC	44 + 44	88
254	SA-mut-CSP-SpyC	EPN-1-SpyT	55 + 33	88
255	QPADG-sRT-PfGPI	EPN-1	25 + 30	-
256	QPADG-sRT-hsGPI	EPN-1	26 + 30	-
257	QPADG-sRT-SpyT	EPN-1-SpyC	24 + 44	68
258	QPADG-sRT-SpyC	EPN-1-SpyT	34 + 33	67

Figure 6. The EPN platform can allow for rapid pseudotyping of extracellular vesicles with a variety of natural and designed

TMP. A) Western blots showing the Cell (C) and Supernatant (SN) fractions for different TMP without and with an additional C-terminal SpyTag motif coexpressed with EPN-7-SpyCatcher. Sizes for different TMP vary, and not all TMP were successfully expressed but TMP-EPN conjugation generally increased recovery of the TMP from the SN fraction, as compared to the TMP pseudotyped fraction. B) Table correlating the sample ID with the different TMP design elements and expected molecular weights,

including conjugated products. C) Blot showing different Malaria CSP antigens RT, SA, and QPADG designed to include either a plasmodium GPI-anchor sequence (-PfGPI), a human GPI-anchor sequence (-hsGPI), a SpyTag (-SpyT), or SpyCatcher (-SpyC) in the Cell (C) and Supernatant (SN) fractions. D) Table correlating sample ID with CSP design, cage architecture, and expected molecular weights for the blot shown in C.

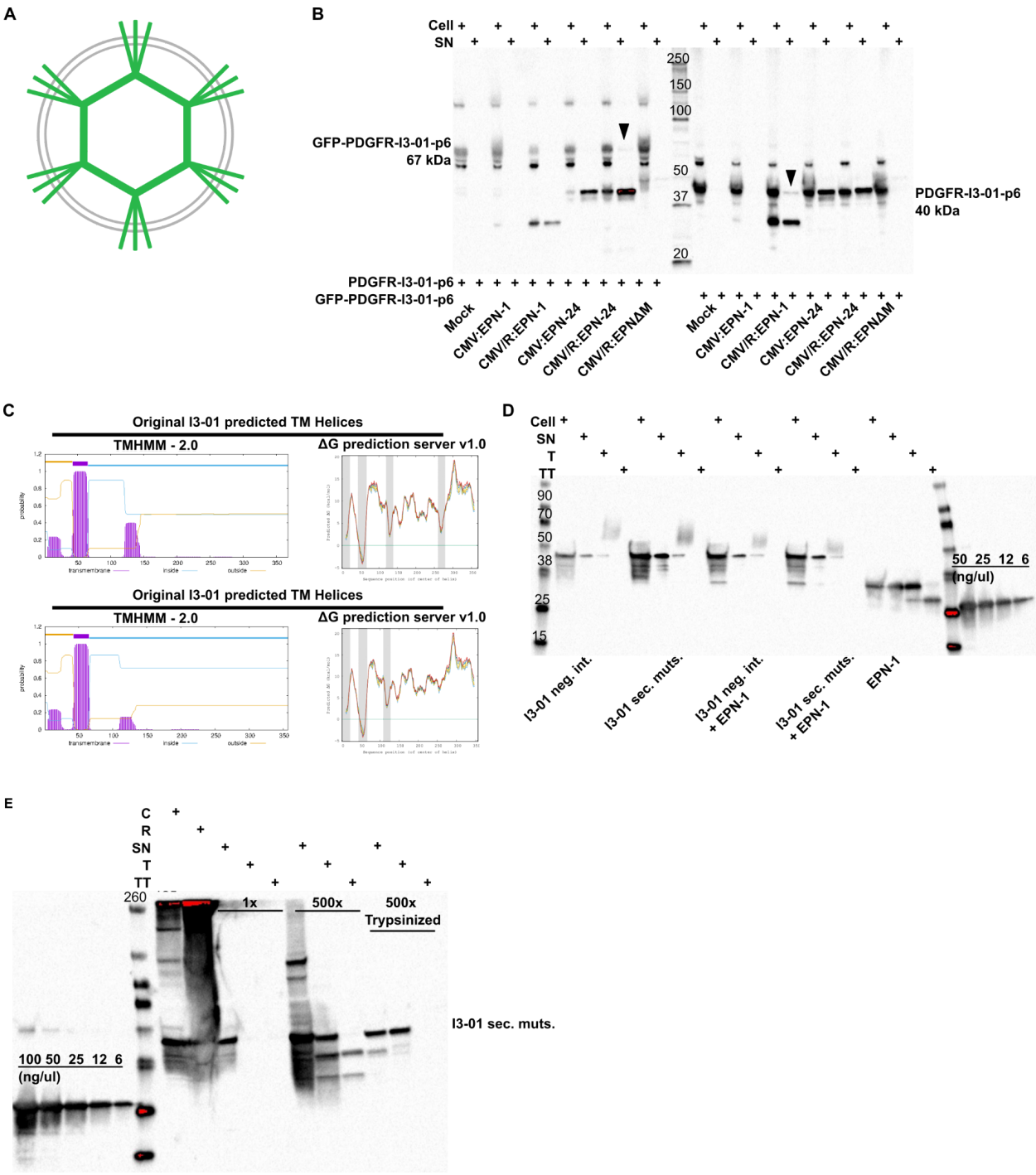


Figure 7. One-component TMP-EPN

A) Simplified schematic of a 1-component TMP-EPN. Green is the I3-01-based cage component, as well as the genetically fused transmembrane domain, with lipid bilayer in gray. B) Western blot showing that initial designs of PDGFR TMD fusions to I3-01

showed very little or no expression, even when co-transfected with “helper” EPN constructs. Black arrows show two combinations which resulted in very low levels of released 1-component TMP-EPN. Cell (C) and Supernatant (SN) fractions. C) Two different analysis programs showing an earlier TMP-EPN design from the experiment in B (top), compared to the newer “degreased” designs used in D (bottom). Note the first secretion signal and the designed TMD, compared to the third (and fourth in upper right panel) unintended TMD’s. D) Western blot with a “degreased” (I3-01 neg. int.) and a “degreased” with secretion optimized sequences (I3-01 sec. muts.) construct with robust escape into the SN fraction. E) Western blot showing various fractions for a highly-concentrated TMP-EPN prep. Cell fraction (C), Retained in supernatant after spinning (R), Supernatant (SN), Trypsin-treated SN (T), and Triton and Trypsin-treated SN (TT).

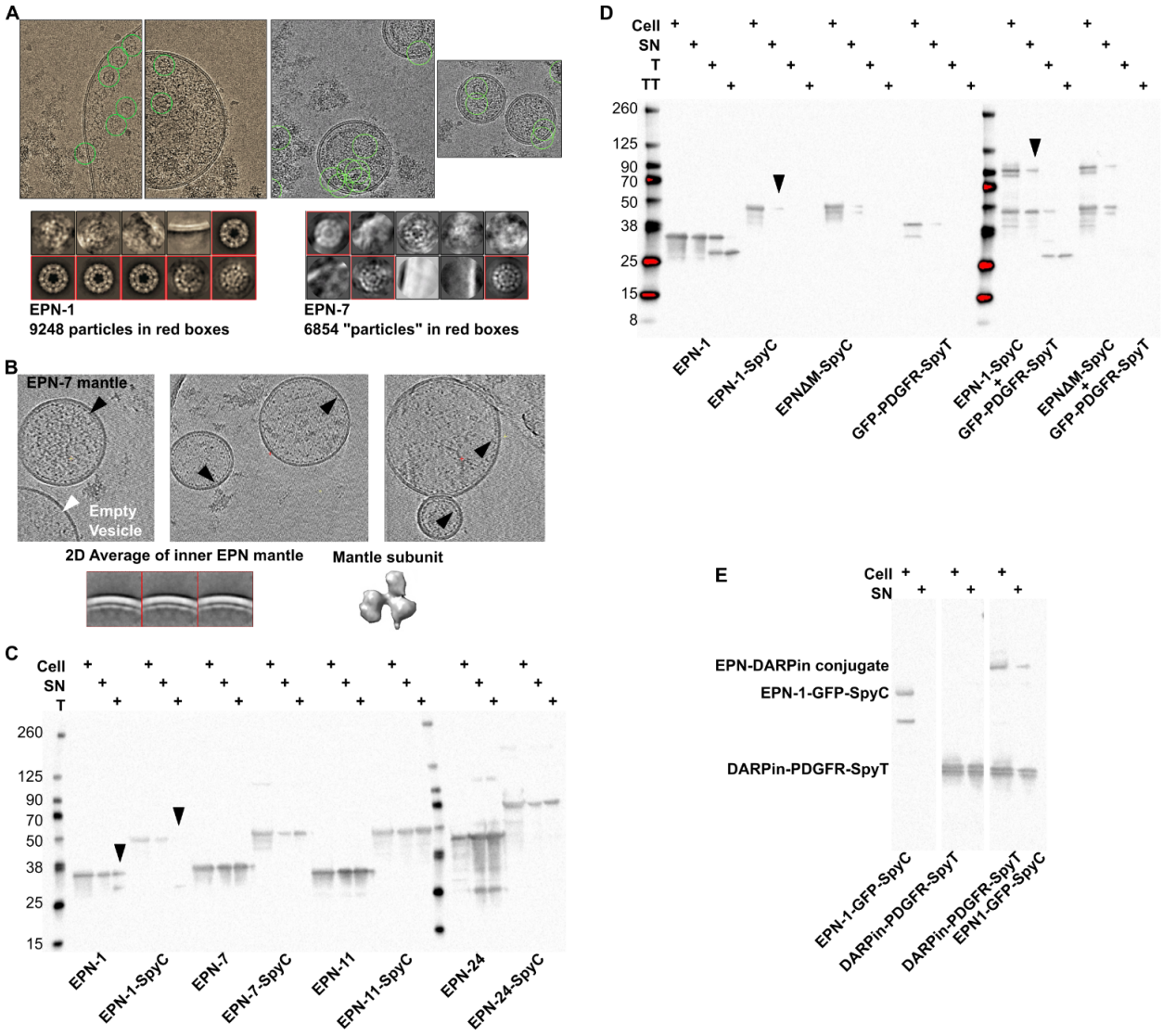


Figure 8. EPN motifs influence cage (dis)assembly states and release efficiency.

A) CryoEM of EPN-1 (left) and EPN-7 (right). Note the selected cages in green and the resulting particle averages. The averages for EPN-1 resulted in symmetrical particles, whereas the auto-picked particles for EPN-7 resulted in nonsensical averages. B) CryoEM of EPN-7 focusing on the “mantle” of proteins apparent on the inner leaflet of the lipid bilayer. Compare mantle apparent in EPN-7 particles with the empty vesicles. Resulting 2D average showed organized particles, and the reconstructed subunit shows a remarkable similarity to the 1wa3 trimer which EPN-7 is based on. C) Addition of

-SpyCatcher motifs to different EPN constructs results in a decline in overall expression across each EPN, and notably eliminates protected release of the EPN-1-SpyC construct (arrows for emphasis). D) Conjugation of EPN and TMP can result in increased release of both the EPN and the TMP protein. EPN-1-SpyC is very low in the SN fraction and completely unprotected, however when conjugated with GFP-PDGFR-SpyT the released fraction shows higher amounts of both released and protected EPN. E) Selected lanes of a western blot showing lack of EPN-1-GFP-SpyC release into the SN, however conjugation with a DARPin-PDGFR-SpyT construct allows for release of the conjugated EPN-TMP into the SN fraction.

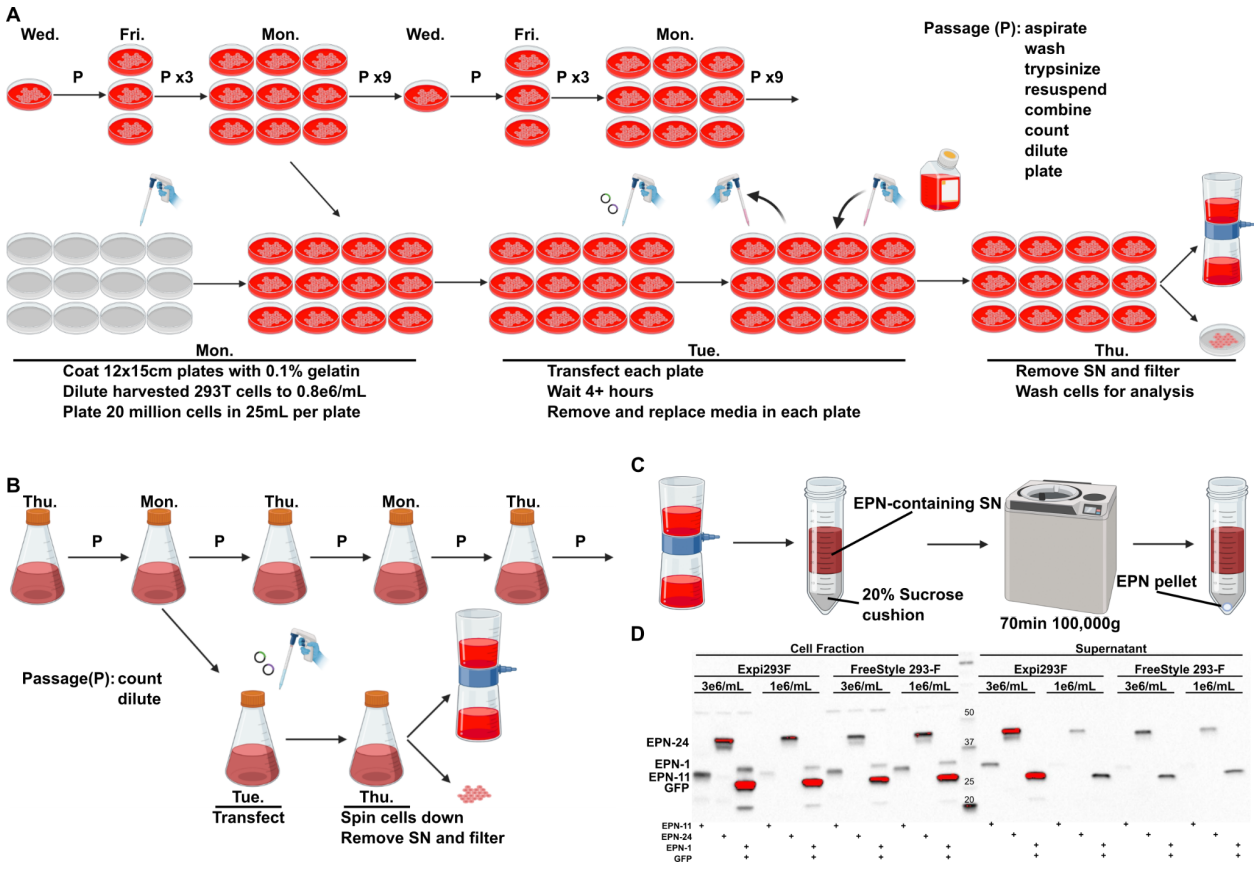


Figure 9. EPN production workflow

A) Schematic of HEK 293T adherent cell production of EPN. Cells passaging (P) occurs

Mondays, Wednesdays, and Fridays and consists of aspiration of the use growth medium, careful addition of PBS to wash cell monolayer, aspiration of PBS and addition of trypsin with 10min incubation at 37C. Growth media containing FBS is added and cells are resuspended and counted. Cells are then pooled, counted, diluted, and plated either for continued passaging, and for plating for EPN production onto gelatin-coated plates Mondays. For production plates, transfection occurs Tuesday morning and media change Tuesday afternoon. Harvest occurs Thursday morning. Each plate must be individually handled and care must be taken not to disrupt the cell monolayer. B) Suspension cell production of EPN involves passaging of cells Mondays and Thursdays only, with splits for EPN production occurring Monday. Transfection occurs Tuesday, and harvests Thursday (without the need to change media). C) EPN harvest and purification schematic, where cell SN is separated, put into ultracentrifuge tubes, a sucrose cushion is added below the SN, and then the preparation is spun for 70min at 100,000g. The SN and sucrose are decanted from the tube, and EPN pellet is resuspended. An additional wash step can be introduced after pelleting by adding PBS and spinning for 30min at 100,000g. D) Comparison of two different suspension cell types, expi293F and Freestyle293F, each at both 1 million and 3 million cells per ml of culture at time of transfection, for both the cell fraction and the released SN fraction. Protein bands are significantly higher at the higher cell density in the SN fraction for the expi293F cells. Red bands show overexposure.

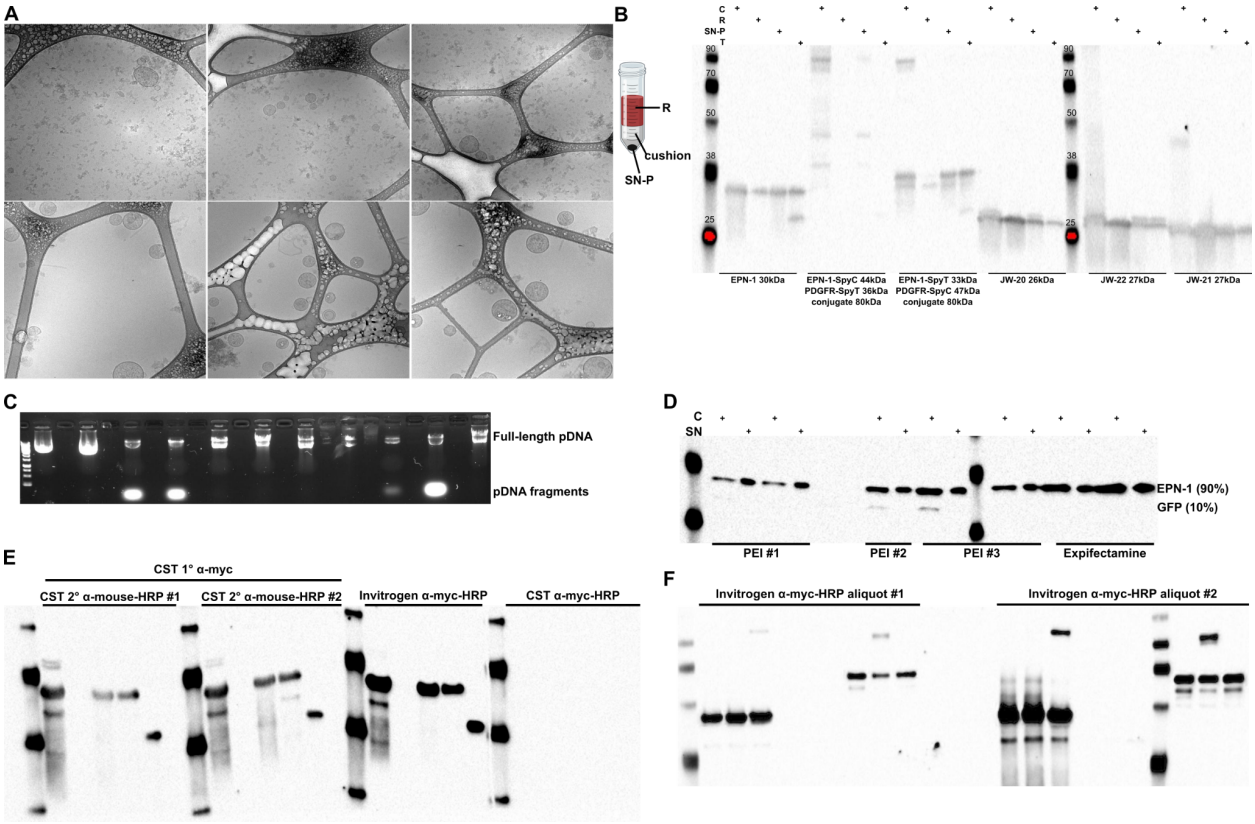


Figure 10. Challenges in EPN production

A) CryoEM micrographs showing expi293F-derived EPN vesicles (top) and 293T-derived EPN vesicles (bottom). B) Western blot comparing relative expression levels of EPN constructs (left three sample sets) and secreted non-enveloped cage constructs (right three sample sets JW-20, -22, -21). Cell (C), retained supernatant (R), supernatant pellet (SN-P), and trypsin-treated supernatant pellet (T) fractions. C) Agarose gel stained with SYBR safe dye visualizes different plasmid prep qualities. Full-length plasmids are large and at the top in various concentrations, while contaminating fragmented DNA under 200bp is present in 4 of the 11 samples. D) Western blot showing the effect of different transfection reagents (PEI compared to Expifectamine) and lots (PEI #1, #2, and #3) when used with a single plasmid mastermix of 90% EPN-1 and 10% GFP. Duplicate and triplicate samples were separate

transfection flasks each using the same plasmid mastermix, with both cell (C) and supernatant (SN) fractions. E) Western blot showing the differences in antibodies for detection of EPN samples. The ladder included HRP and was not antibody-dependent, and served as an interior positive control. Samples were from the same tube, run on the same gel, and were separated only for incubation with their respective antibodies. Membranes were recombined for exposure to imaging reagents and the imaging process. F) Western blot comparing samples exposed to two different tubes of the same anti-myc-HRP antibody. Sample volume loaded was identical for both blots, and the halves of the blot were separated only during incubation with their respective antibody.