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Current Opinion in Biotechnology

Virus-like particles against infectious disease and cancer: guidance for the nano-architect

Rory A Hills and Mark Howarth

Virus-like particles (VLPs) can play important roles in prevention and therapy for infectious diseases and cancer. Here we describe recent advances in rational construction of VLP assemblies, as well as new approaches to enhance long-lasting antibody and $CD8^+$ T cell responses. DNA origami and computational protein design identified optimal spacing of antigens. Chemical biology advances enabled simple and irreversible VLP decoration with protein or polysaccharide antigens. Mosaic VLPs co-displayed antigens to generate cross-reactive antibodies against different influenza strains and coronaviruses. The mode of action of adjuvants inside VLPs was established through knock-outs and repackaging of innate immune stimuli. VLPs themselves showed their power as adjuvants in cancer models. Finally, landmark clinical results were obtained against malaria and the SARS-CoV-2 pandemic.

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Introduction

The study of virus-like particles (VLPs) represents an exciting nexus of nanotechnology, immunology and synthetic biology. VLPs have potential to protect against some of the most important medical challenges, but are also powerful tools for basic understanding of the immune system. Initial work on VLPs hijacked proteins from viral capsids, but it was soon found that similar behavior was achieved with non-viral self-assembling nanocages [1]. Here we focus principally on non-enveloped VLPs and their recent application against infectious disease and cancer. We highlight insights on building different VLP architectures through directed evolution and computational design. The effect of different architectures for induction of the immune response is considered, including the impact of surface glycosylation and interior cargo. There have been striking advances in the use of VLPs to protect against highly variable pathogens, through the use of mosaic VLPs. We also consider how VLPs have been employed against SARS-CoV-2 and momentous advances against malaria.

How different protein architectures affect the immune response

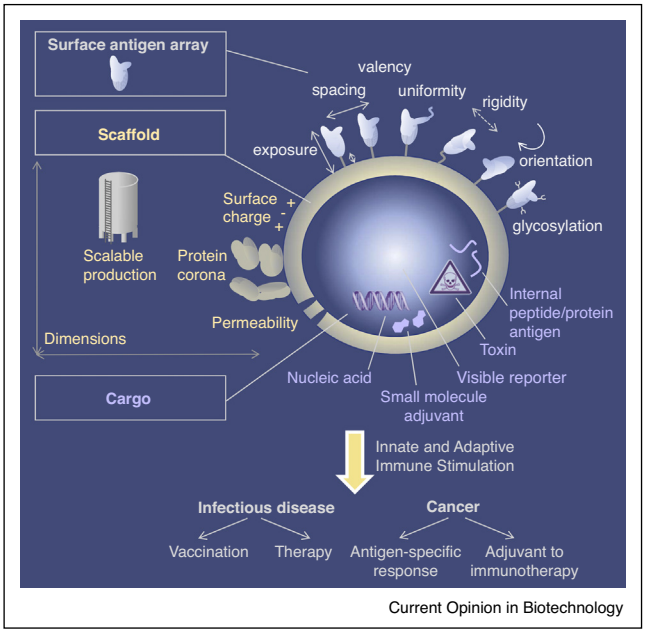
It has long been known that clustering an antigen greatly enhances induction of an immune response [1]. For deeper understanding of the effect of antigen multivalency, an HIV immunogen was injected as a monomer, 4-mer, 8-mer or 60-mer [2]. High multimerization facilitated strong induction of transcription factors interferon regulatory factor 4 and Bcl6 in B cells. High multimerization also led to activation and division of B cells with a broader range of antigen binding affinities. Activating a diverse B cell pool may induce broadly protective immune responses to combat the viral diversity in HIV [2].

An important question for VLP architecture is what is the optimal spacing between antigens (Figure 1) [1]. DNA origami allows excellent spatial control: icosahedral or 6 helical bundle DNA nanoparticles were used for precision display of an HIV antigen. Antigen pairs held rigidly on DNA bundles with 28 nm spacing gave the strongest activation of calcium flux in a B cell line (Figure 2a). Antigen pairs on flexible single-stranded DNA or polyethylene glycol (PEG) chains were less stimulatory [3••]. The caveat at this stage is that these studies used a B-cell receptor with subnanomolar antigen affinity, not typical during immune priming. Also, the study did not look at immune induction in an animal, where DNases and innate immune activation by DNA may cause further complexity.

Enhanced coupling to VLPs

Modular VLP assembly describes when the antigen and scaffold are expressed separately and coupled post-purification. This approach avoids many of the challenges posed by genetic fusion (e.g. misfolding of complex antigens, Figure 1) [4]. Modular coupling can also facilitate coupling of personalized neoantigens for cancer therapy. We previously developed SpyTag/SpyCatcher for plug-and-display VLP coupling. More recently we generated the SpyTag003/SpyCatcher003 system, giving 400-fold faster reaction than the original pair [5]. It was

Figure 1



Architectural elements in VLP design. Tuning the surface, underlying scaffold and cargo can all have major effects on VLP biological efficacy.

unclear whether antigens of different symmetries could couple efficiently to the VLPs or would provoke catastrophic aggregation (Figure 2b). We found that a panel of antigens of different sizes and symmetries could effectively couple to the SpyCatcher003-mi3 platform, including trimeric influenza hemagglutinin or tetrameric neuraminidase (Figure 2b) [5]. Another emerging conjugation approach is the incorporation of cobalt porphyrin–phospholipid (CoPoP) into a liposome, which facilitates stable interaction with His-tagged proteins (Figure 2c) [6]. The malaria antigen Pfs230 has been coupled to liposomes in this manner and elicited antibodies that inhibit parasite transmission [7].

SnoopLigase allows specific irreversible coupling between the peptides DogTag and SnoopTagJr (Figure 2d). The malarial antigens CyRPA and PfEMP1 or the cancer-associated Telomerase peptide were generated as fusions to SnoopTagJr. SnoopLigase catalyzed the formation of an isopeptide bond to couple the antigen to a DogTag-linked scaffold [8]. The related DogTag/DogCatcher system can proceed to 98% coupling with faster reaction than SnoopLigase. The β-hairpin formed by DogTag is conducive to loop insertions [9]. Modular VLP display from loops rather than termini may help to focus antibody responses to desired regions of the antigen (Figure 1).

Polysaccharides are important antibody targets for protection against a range of bacterial diseases. Polysaccharides on their own do not efficiently induce helper T cells and so are often chemically conjugated to protein antigens, to generate conjugate vaccines. Efficient display of polysaccharides on VLPs could further enhance vaccine potency. The 'Glycoli platform' expresses glycosyltransferases in the *Escherichia coli* cytoplasm, for generation of protein-linked oligosaccharide chains. Glycans could be enzymatically coupled to protein cages that were fused with an acceptor sequence and co-expressed in the cytoplasm [10•]. Synthesis of some bacterial oligosaccharides may be more tractable in the periplasm. Li Zhu's group expressed PglL in the inner membrane of *Shigella flexneri*. PglL allowed *Shigella* oligosaccharides to be coupled onto transfected SpyCatcher bearing a glycosylation sequence in the periplasm. The purified SpyCatcher-oligosaccharide could then react *in vitro* with SpyTag-fused AP205 or Qβ VLPs. Multimerization of the protein-oligosaccharide conjugate greatly increased the antibody response and protection to *Shigella* infection [11••].

Building new architectures

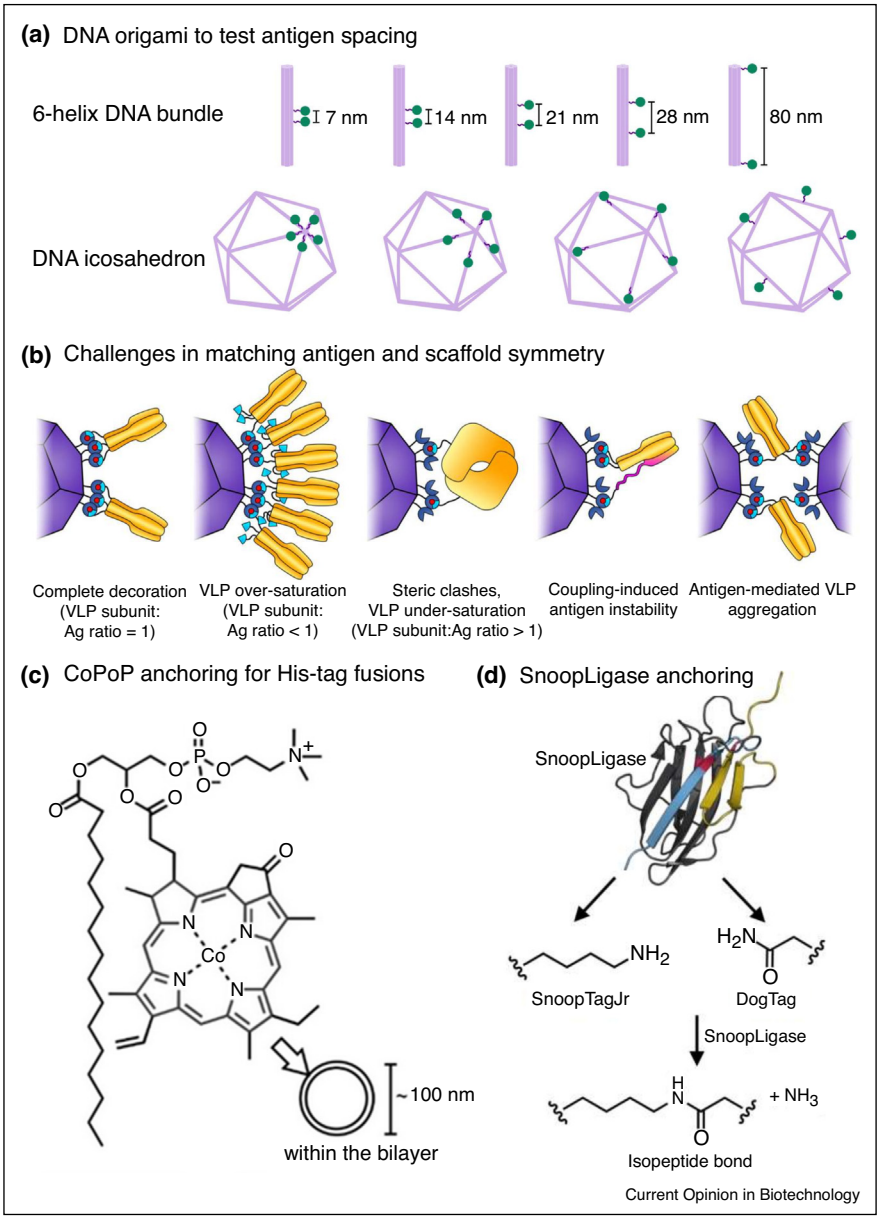
In the era of synthetic biology, we should not just rely on nature to furnish us with VLP architectures. The Hilvert group used directed evolution, starting from the lumazine synthase 60-mer that does not bind nucleic acids. Rounds of selection for RNA encapsulation led to an icosahedral 240-mer packaging its encoding mRNA, with spontaneous appearance of stem-loop packaging motifs in the mRNA [12•].

Computational design optimized the assembly of tetrahedral, octahedral, and icosahedral nanoparticles for efficient display of trimeric antigens from HIV, influenza and Respiratory Syncitial Virus (RSV). These scaffolds matched the natural spacing of the antigen termini to maximize antigen stability. In addition, designed scaffolds concealed regions of the antigen, to favor induction of antibodies against the tips of the antigen (Figure 3a) [13•].

Assembly of cages within a cage was shown by packing ferritin nanoparticles within the 56 nm diameter bacteriophage P22 capsid [14]. Simultaneous control of the interior and exterior decorations of a VLP was shown by Sebyung Kang's group. Split intein reaction coupled reporter proteins on the inside of encapsulin and SpyTag/SpyCatcher allowed outside functionalization with a cancer-directed affibody [15].

Metagenomic sequences were mined for new single-stranded RNA bacteriophage coat proteins. From *E. coli* expression of 110 different sequences, including 11 new classes, 80 led to VLP assembly [16].

Figure 2

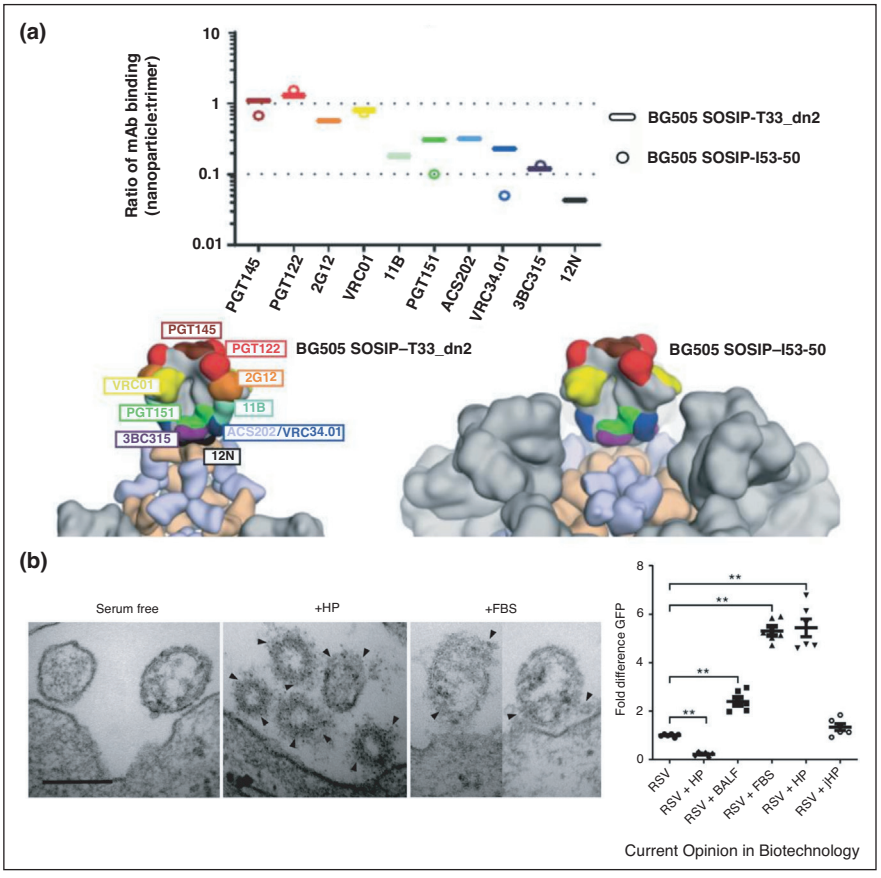


Advances in antigen display technologies. **(a)** DNA origami testing of antigen spacing. 6-helix DNA bundles were prepared with two copies of antigen anchored at different spacing, before testing of B cell activation. Also, DNA icosahedra were constructed with 5 copies of antigen at different spacing. Based on Ref. [3••]. **(b)** Challenges in matching of symmetry between the nanoparticle and antigen. For modular coupling, display of multimeric antigen (in orange) on the VLP (purple) may lead to different densities of display. There is potential for coupling to destabilize the antigen (misfolding shown in magenta) and for bridging of different VLPs by the antigen. Taken from Ref. [5]. **(c)** Cobalt porphyrin phospholipid (CoPoP) for anchoring His-tagged antigen to the bilayer of liposomes, taken from Ref. [6]. **(d)** SnoopLigase (dark gray) directs formation of an isopeptide bond (red) from a lysine of SnoopTagJr peptide (blue) to an asparagine of DogTag peptide (yellow), for linking antigen to nanoparticles. Adapted from Ref. [8].

How VLPs provide adjuvant effects

Injecting antigen alone does not usually induce a strong immune response — it is important to activate the innate immune system. Vaccines are nearly always given with an adjuvant to increase innate stimulation [17]. Beyond multimeric presentation on VLPs, other internal and external VLP features affect the size and quality of the immune response. VLPs from phage capsids would naturally package nucleic acid from the expressing organism, which stimulates the innate immune system. The coat protein of Qβ phage was expressed in *E. coli* and disassembled with reducing agent, before different RNAs

Figure 3



Changing the environment around the antigen at the VLP surface. **(a)** Masking of antigen by surface VLP architecture. Epitopes at the base of HIV antigen (BG505 SOSIP) are less exposed when displayed on I53-50 nanoparticles compared to T33_dn2 nanoparticles, based on binding by different monoclonal antibodies (target sites on antigen color-coded), taken from Ref. [13•]. **(b)** Protein corona interaction with the viral surface. Transmission electron microscopy of RSV close to the cell surface, incubated in serum-free medium, human plasma (HP) or fetal bovine serum (FBS). Arrows point to protein corona bound at the virus surface. On the right is quantification of RSV infectivity (leading to GFP expression) in serum-free conditions compared to different media (BALF, bronchoalveolar lavage fluid; jHP, juvenile human plasma). Taken from Ref. [27••].

(from *E. coli*, HEK 293T, or yeast tRNA) were packaged inside. Packaging of bacterial RNA in VLPs induced the greatest protection in an influenza model, altering antibody isotypes. This difference depended on innate immune activation by Toll-like receptor (TLR) 7 and not mitochondrial antiviral signaling protein (MAVS) or retinoic acid-inducible gene-I-like receptor (RIG-I) [18••].

TLR agonists can also facilitate anti-cancer immune responses. A small molecule TLR7/8a agonist linked to neoantigen peptides enhanced $CD8^+$ T cell-mediated responses in different mouse tumor models [19]. TLR5 and the Naip5 inflammasome can be stimulated by flagellin, the building block of the bacterial flagellum. Flagellin attachment to a ferritin-based nano-cage enhanced Th1 immune responses [20].

VLPs can be a potent adjuvant even without antigen loading. Human Papillomavirus-16 VLPs, conjugated with a photoactivatable drug, were injected intravenously. Then the tumor was illuminated with near infra-red light to induce necrotic cell death and stimulate long-lasting $CD8^+$ T cell-mediated anti-tumor responses [21]. Different VLP types were compared for their adjuvant effects following intratumoral injection. Cowpea mosaic virus nanoparticles outperformed other plant, phage or mammalian-derived nanoparticles [22]. TLR reporter assays and knock-out mice indicated that Cowpea mosaic virus nanoparticles activate TLR2, 4 and 7 and promote secretion of type I interferons [23]. The value of recruiting other arms of innate immunity was shown by packaging 2′,3′-cyclic GMP-AMP (cGAMP) in VLPs, which increased $CD4^+$ and $CD8^+$ T cell responses and enhanced influenza protection [24].

To supercharge anti-tumor efficacy, VLPs have even been equipped with micro-motors. Qβ VLPs were coupled onto 25 μm particles containing magnesium metal, which reacts with water to generate hydrogen bubbles and drive propulsion. These motorized assemblies for treatment of ovarian cancer were injected into the mouse peritoneum, where micro-motors increased the Qβ in the tumor [25•].

VLPs certainly can induce good $CD8^+$ T cell responses, but have rarely reached the potency of viral vectors. Recent priming and boosting with different arenavirus vectors induced anti-tumor responses up to 50% of the circulating $CD8^+$ T cell pool [26].

We note that animal studies often focus on inducing the strongest immune response without worrying much about inflammatory side-effects. Clinical translation, at least for infectious disease vaccines, requires a balance of mild side-effects with strong induction of a specific immune response [17].

Fundamental understanding of the immune system and its interaction with VLPs

There has been extensive work on how the localization and biological activity of non-biological nanoparticles (e.g. liposomes, quantum dots) depends on the protein corona — the set of proteins that spontaneously associate with a nanoparticle surface exposed to biological fluids. A protein corona also forms on viruses and influences viral infectivity (Figure 3b) [27••], so this surface complexity is an important concept for VLP engineers.

VLP glycosylation can also be an important signature for the immune system (Figure 1). Increasing the glycosylation on Hepatitis B surface antigen (HBsAg) enhanced immunogenicity, when particles were expressed in a human cell-line and ~40% of glycans were oligomannose [28]. Mannose-binding lectin recognition of oligomannose-rich surfaces on VLPs activated deposition of complement C3 and enhanced transport to follicular dendritic cells, promoting a strong immune response [29••]. Fluorescent imaging of ferritin-based nanoparticles indicated the involvement of a dendritic cell population expressing SIGN-R1 (specific intercellular adhesion molecule-3-grabbing nonintegrin-related 1) in VLP delivery to follicular dendritic cells. These particles generated activity in a model for therapy of chronic Hepatitis B Virus [30].

The route of immunization is also important. To understand the correlates for induction of a strong anti-cancer $CD8^+$ T cell response, intravenous VLP vaccination led to a superior response to subcutaneous delivery. Here neoantigen peptides were induced to self-assemble by conjugation to a hydrophobic polymer linked to a TLR7/8 agonist. T-cell specific transcription factor 1 (TCF1) was identified as an important feature of the arising $CD8^+$ T cells, maintaining a stem cell-like state where cells keep self-renewing [31].

Improved analysis of VLP assembly and composition

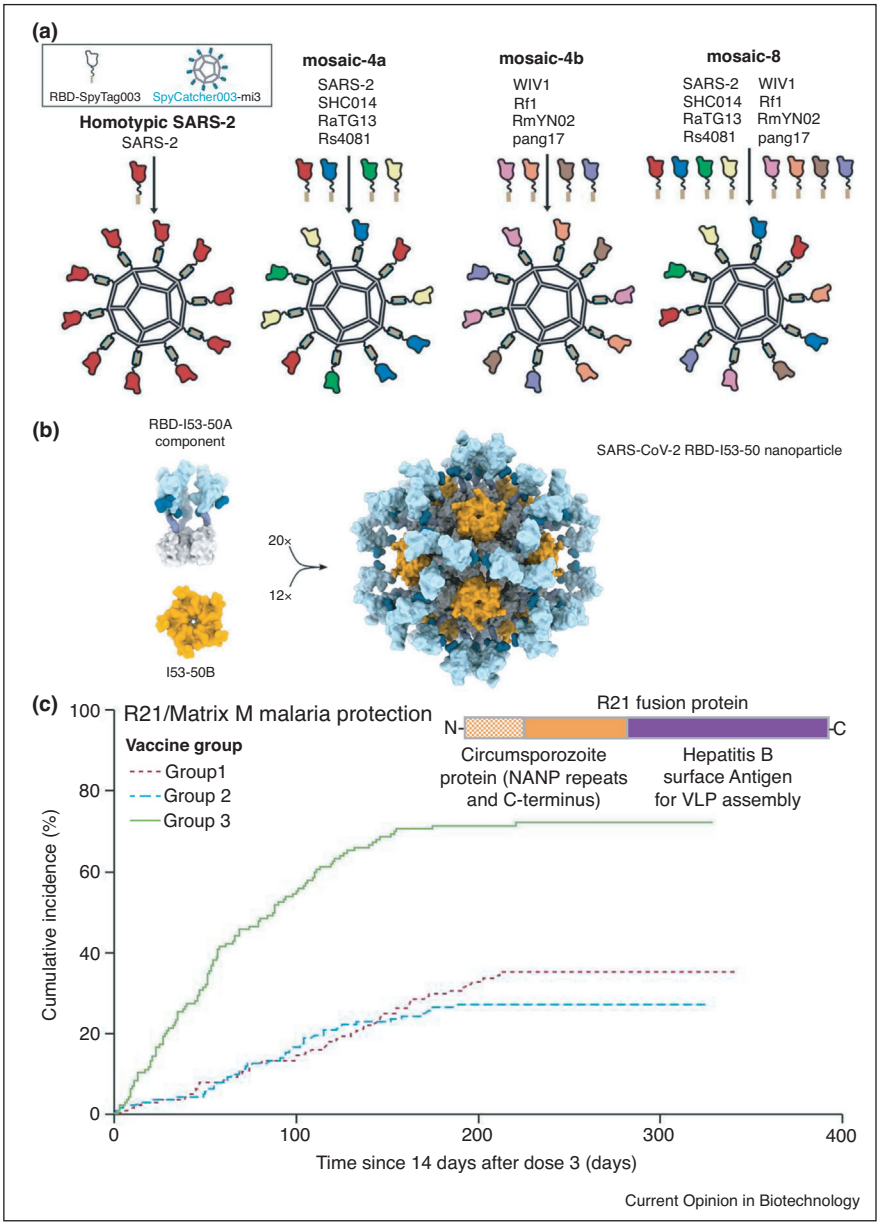
In nanotechnology, understanding what has been built is often as much of a challenge as building new structures. Precise antigen conformation is crucial to eliciting protective antibodies. It is now relatively standard to obtain high resolution structures of protein antigens in isolation. However, when antigens are attached to a nanoparticle, it is much harder to use structural analysis. Advanced solid-state nuclear magnetic resonance (NMR) established that the structure of an influenza hemagglutinin variant was retained, following coupling to Qβ particles via thiol-based cross-linking [32]. AFM now enables real-time visualization at the single molecule level of nucleation and reversibility in capsid formation [33]. Many expect perfection in the assembly of a protein nanoparticle. However, cryo-electron microscopy, along with statistical mechanics modeling, showed that mistakes in VLP assembly are normal and hard to avoid, with >15% particles mis-shapen from Hepatitis B Virus capsid [34]. Nonetheless, assembly of computationally designed two component nanoparticles could be uniform and complete, as assessed by dynamic light scattering and native mass spectrometry [35•].

How to improve the antigens displayed on VLPs

Recent innovations provide strategies to tackle a central challenge — how to generate a vaccine against divergent and evolving pathogens. Mosaic nanoparticles have been produced with several different but related antigens displayed on a single particle. B-cell receptors that bind regions conserved between the different antigens on the VLP should receive the strongest stimulus. Thus mosaic particles encourage the production of antibodies binding a wider variety of pathogen strains [36••]. Mosaic nanoparticles displayed a panel of up to eight different sarbecovirus receptor-binding domains (RBDs) (Figure 4a). These VLPs elicited a good response against evolutionarily related viruses not represented on the particle, suggesting potential against new SARS-CoV-2 variants and emergent zoonotic coronaviruses [36••]. Mosaic nanoparticles have also been developed for a more broadly neutralizing influenza response, with different hemagglutinin trimers displayed through genetic fusion [37••] or isopeptide coupling [38].

Antigen has been modified to focus the antibody response, using an innovative strategy called nanopatterning. Zika is an especially difficult vaccine target because non-neutralizing antibodies cause antibody-dependent enhancement (ADE), increasing viral entry into cells. The unnatural amino acid p-azido-L-phenylalanine was introduced at specific sites of Domain III of Zika

Figure 4



VLP assemblies against coronavirus and malaria. **(a)** Mosaic vaccine for broad coronavirus protection. RBD variants from SARS-CoV-2 alone or other sarbecoviruses were expressed bearing SpyTag, allowing covalent coupling onto the SpyCatcher003-mi3 nanoparticle. Display of different RBDs on the same VLP elicited antibodies with broad reactivity to sarbecoviruses. Taken from Ref. [36••]. **(b)** Two component SARS-CoV-2 VLP vaccine. Model of the SARS-CoV-2 RBD (light blue) fused to the trimeric I53-50A component (gray) and the pentameric I53-50B component (orange). These components are expressed separately and then mixed, to yield a nanoparticle bearing 60 copies of RBD for immunization, taken from Ref. [47]. **(c)** Malaria VLP vaccine trials results. Incidence of malaria is plotted over time following the third vaccination. Group 1: 5 μg R21/25 μg Matrix M. Group 2: 5 μg R21/50 μg Matrix M. Group 3, rabies control vaccination. The organization of the R21 fusion protein is indicated. Adapted from Ref. [50••].

envelope glycoprotein, allowing click coupling to PEG. When displayed on VLPs, the antibody response was targeted away from the PEGylated region, to favor induction of effective Zika-neutralizing antibodies [39].

Important clinical results from VLPs

The first vaccines approved against SARS-CoV-2 were adenoviral vectors, mRNA vaccines, or inactivated SARS-CoV-2 [40]. A huge range of nanoparticle

strategies have been tested towards a COVID-19 vaccine, since VLPs are effective at inducing neutralizing antibodies and may have advantages in stability and scalability [41••,42,43]. NVX-CoV2373 from Novavax is a synthetic lipid nanoparticle displaying SARS-CoV-2 spike, stabilized in pre-fusion conformation, and has successfully completed Phase III trials [41••]. Another lipid nanoparticle candidate employs CoPoP coupling [44•], undergoing Phase I/II [45] (Trial ID: NCT04783311).

Genetic fusions to VLPs have produced promising results against COVID-19. Stabilized spike on ferritin is undergoing Phase I [46] (Trial ID: NCT04784767). RBD was fused to I53-50A and mixed with I53-50B, to assemble into a two-component nanoparticle (Figure 4b) [47] which has entered Phase I/II (Trial ID: NCT04742738). COVID-19 vaccine candidates have been developed using Tag/Catcher ligation, coupling to the protein nanoparticles mi3 [42], AP205 [48] (Trial ID: NCT04839146), or HBsAg [43] (Trial ID: ACTRN12620000817943). Also, VLPs displaying spike trimer, budded from intracellular membranes of *Nicotiana benthamiana*, entered Phase II/III (Trial ID: NCT04636697) [49].

Against malaria, rational design of VLPs has led to a major advance. R21 is a genetic fusion between a portion of *Plasmodium falciparum* Circumsporozoite protein and HBsAg, adjuvanted with Matrix-M (Figure 4c). Phase IIb trials demonstrated good safety and a landmark 77% efficacy after 1 year (Figure 4c) [50••]. A Phase III trial is now testing a larger population over a longer time-period (Trial ID: NCT04704830). The antibody response to the NANP repeats correlated to vaccine efficacy [50••]. R21/Matrix-M was previously shown to induce substantial $CD4^+$ T cells but very low $CD8^+$ T cell responses against Circumsporozoite protein [51]. Antibody responses to the HBsAg scaffold (which would provide some protection to Hepatitis B Virus) were very low in response to R21/Matrix-M [51]. Unlike for viral vectors which need to infect cells, the immune response to antigens displayed on VLPs is not blocked by pre-existing antibodies against the VLP scaffold [4,52].

Conclusions

Advances now make it routine to array complex antigens on VLPs, which are scalable for clinical development and induce protective antibody responses for infectious disease vaccination. Data are now accumulating to compare the effect of specific VLP features on the immune response in pre-clinical models. Multiple trials against SARS-CoV-2 will help to uncover how VLP features impact human immunity. Persistent infectious diseases and cancer provide the most challenging targets and it is here that testing of new VLP designs may achieve the greatest benefit.

Conflict of interest statement

M.H. is an author on patents or patent applications for Tag/Catcher/Ligase technologies (UK Intellectual Property Office 1002362.0, 1509782.7, 1705750.6, 1706430.4, 1903479.2, 2104999.4.) and a SpyBiotech co-founder, shareholder and consultant. R.A.H. has no conflicts of interest.

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