



New Routes and Opportunities for Modular Construction of Particulate Vaccines: Stick, Click, and Glue

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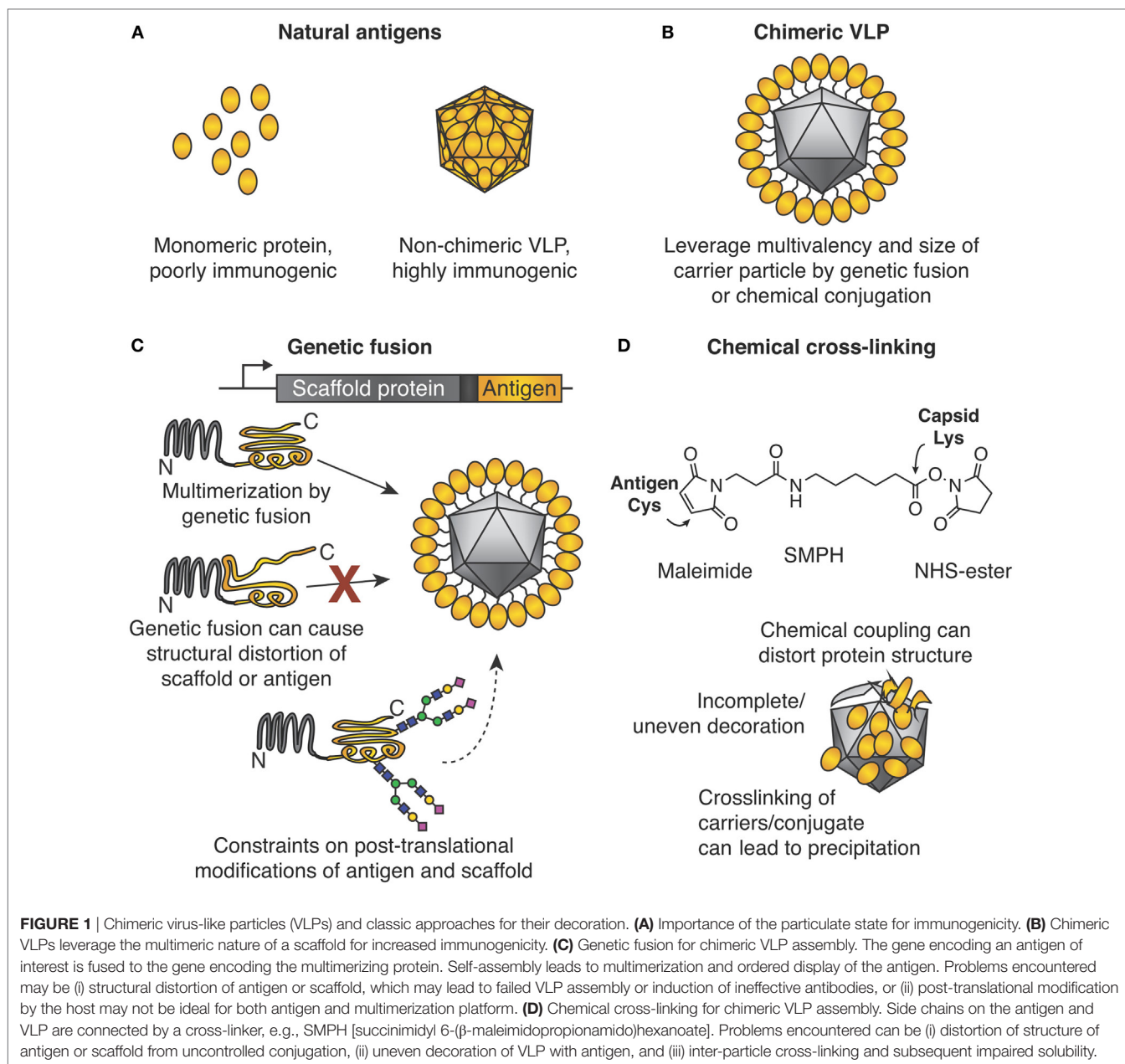
Vaccines based on virus-like particles (VLPs) can induce potent B cell responses. Some non-chimeric VLP-based vaccines are highly successful licensed products (e.g., hepatitis B surface antigen VLPs as a hepatitis B virus vaccine). Chimeric VLPs are designed to take advantage of the VLP framework by decorating the VLP with a different antigen. Despite decades of effort, there have been few licensed chimeric VLP vaccines. Classic approaches to create chimeric VLPs are either genetic fusion or chemical conjugation, using cross-linkers from lysine on the VLP to cysteine on the antigen. We describe the principles that make these classic approaches challenging, in particular for complex, full-length antigens bearing multiple post-translational modifications. We then review recent advances in conjugation approaches for protein-based non-enveloped VLPs or nanoparticles, to overcome such challenges. This includes the use of strong non-covalent assembly methods (stick), unnatural amino acids for bio-orthogonal chemistry (click), and spontaneous isopeptide bond formation by SpyTag/SpyCatcher (glue). Existing applications of these methods are outlined and we critically consider the key practical issues, with particular insight on Tag/Catcher plug-and-display decoration. Finally, we highlight the potential for modular particle decoration to accelerate vaccine generation and prepare for pandemic threats in human and veterinary realms.

Keywords: vaccinology, virus-like particle, bioconjugation, malaria, synthetic biology, click chemistry, SpyCatcher

INTRODUCTION: THE IMPORTANCE AND CHALLENGE OF NANOPARTICLE VACCINES

The medical and commercial success of virus-like particle (VLP) vaccines against hepatitis B virus (HBV, vaccine ~22 nm in size) and human papillomavirus (HPV, vaccine ~55 nm in size) is attributed to their particulate nature (1, 2). The medical significance of VLPs is highlighted by the Nobel Prize in 1976 to Baruch Blumberg for work on HBV and identifying VLPs. Also, the 2017 Lasker Award in Clinical Medical Research went to Douglas Lowy and John Schiller for HPV vaccine development. These vaccines are non-chimeric, i.e., HBV VLPs protect against HBV infection (**Figure 1A**). The advent of genetic engineering allowed genetic fusion of a coat protein from one VLP to a protein antigen derived from a different pathogen (**Figures 1B,C**). Such a chimeric VLP acts as a scaffold to gain the benefits of multimerization. Lacking pathogenic nucleic acids, such vaccines show an excellent safety profile, although the immunogenicity may be less than live-attenuated vaccines (3).

Reasons for the potency of particulate vaccines have been reviewed (1, 4). Briefly, particulate vaccines mimic the size (>20 nm) and structural organization of viruses. The particulate vaccine efficiently enters lymphatic vessels and drains to lymph nodes (1). Here, the high-level B cell receptor



cross-linking, caused by the repetitive high-antigen density on the particulate, can directly activate B cells without T cell help (4). In addition, IgM and complement interactions with the particulate vaccine may result in increased follicular dendritic cell (FDC) uptake of the particle (4). Increased FDC uptake then increases presentation of antigens in the lymph node, causing further B cell induction (2, 4). VLPs can also induce cytotoxic T cell responses *via* major histocompatibility complex (MHC) cross-presentation (4). Antigen-processing and cross-presentation have been reviewed elsewhere in detail (5). Certain VLPs have encapsulated nucleic acids, which can serve as toll-like receptor (TLR) ligands for cytokine signaling and further enhance the immune response (1, 4). Hereafter, we will refer to VLPs and other nanoparticles collectively as virus-like nanoparticles (VLPNs).

THE DIFFICULTY OF INSTALLING COMPLEX ANTIGENS ON VLPNS

To capitalize on the above advantages of particulate display, much effort has been directed to impart these advantages on monomeric or oligomeric antigens.

Genetic Fusion and Its Challenges

In many areas of molecular biology, genetic fusion of two proteins is robust, and the first construct may be expected to retain the desired functions. For example, GFP fusions have been performed genome wide (6). For reasons discussed below, fusion of pathogen-derived antigens to VLP monomers for multimerization has not been as successful (Figure 1C).

The Termini of Coat Proteins Are Often Important for VLP Assembly

For many VLPs, one or both termini of the coat protein subunit are involved in inter-subunit interactions, e.g., Q β or hepatitis B core antigen (7, 8). Termini at interfaces may impart stability, since the termini are the part of the protein most likely to flex. Fusion of a new protein at such a terminus is likely to destabilize the VLP. Alternatively, a terminus may face the inside of the VLP, so fused antigens are less likely to induce antibodies. Insertion in a loop of the capsid subunit may be tolerated for some short peptides, but insertion of a protein antigen in a loop of a capsid subunit will often disrupt the fold of the capsid and/or protein antigen (8, 9).

VLP Assembly Is Often Metastable

In natural infection, the virus capsid should not assemble as soon as the protein is made: assembly should be synchronized to packaging, e.g., of viral nucleic acid (10). Therefore, even though many VLPs are highly stable once assembled, small changes to the VLP (such as fusion) can cause major interference in the assembly pathway (11).

Cooperative VLP Assembly Means That Errors Propagate

If 5% of a monomeric protein is misfolded, that fraction can typically be purified away without consequence. If 5% of a VLP subunit is misfolded, those defective subunits may dock on to partly formed VLPs and prevent completion of the VLP assembly. Therefore, much greater than 5% malformed VLPs may result, giving dominant negative cooperativity. Folding of chimeric VLPs is highly sensitive to errors, e.g., from misfolding, mistranslation, incomplete post-translational modification, or unexpected interaction with other cellular proteins (11). Unmodified viral coat proteins have faced evolutionary pressure over many generations to fold and assemble optimally, so that such challenges have been addressed.

The Complexity of the Antigen

Multimeric State of the Antigen

Some antigens of interest are monomeric, but many are oligomeric. Viral glycoproteins are frequently trimeric (e.g., HIV gp120, influenza hemagglutinin). Given that many VLPs are icosahedral, the symmetry at the fusion site should match the symmetry of the intended fusion. Other important antigens are heteromultimeric, such as in herpesviruses.

Post-Translational Modification of the Antigen

A wide variety of VLNP scaffolds have been developed based on viruses and multimeric protein complexes from bacteria, insects, plants, and mammals. The merits of each VLNP expression system have been reviewed (12–14). However, the optimal expression host for a specific VLNP may not be optimal for expressing the specific target antigen. Phage-derived VLPs can be expressed at gram per liter scale in *Escherichia coli* (13). However, expression in *E. coli* does not typically allow for correct formation of disulfide bonds or complex glycosylation (15).

Plant expression provides high yield at low production cost and improved disulfide formation, but may differ in glycosylation of antigens (14). Mammalian expression is generally considered the best platform for folding and post-translational modification of complex proteins. However, mammalian expression is the most expensive approach (16) and also susceptible to passenger viruses (e.g., simian vacuolating virus 40 or porcine circovirus-1), requiring stringent quality-control monitoring (16).

Antigens May Have Multiple Conformations

Often target antigens need to convert between conformations in their functional role, e.g., pre-fusion, fusion-active, and post-fusion conformations of viral glycoproteins (17). In some cases, display of the wrong conformation on the VLNP is not just neutral but harmful, as for respiratory syncytial virus vaccination (18). Sometimes, instability of antigen structure may have evolved to reduce the stability of antibody complexes (19).

Antigen Sequence Variability

Annual strains of influenza are a regular reminder that vaccine development must keep pace with a changing genetic landscape of pathogens (17, 20). HIV is one of the most variable human pathogens; major advances have been made in designing an HIV gp120 variant with a stabilized disease-relevant conformation to generate broadly neutralizing antibodies (17, 21).

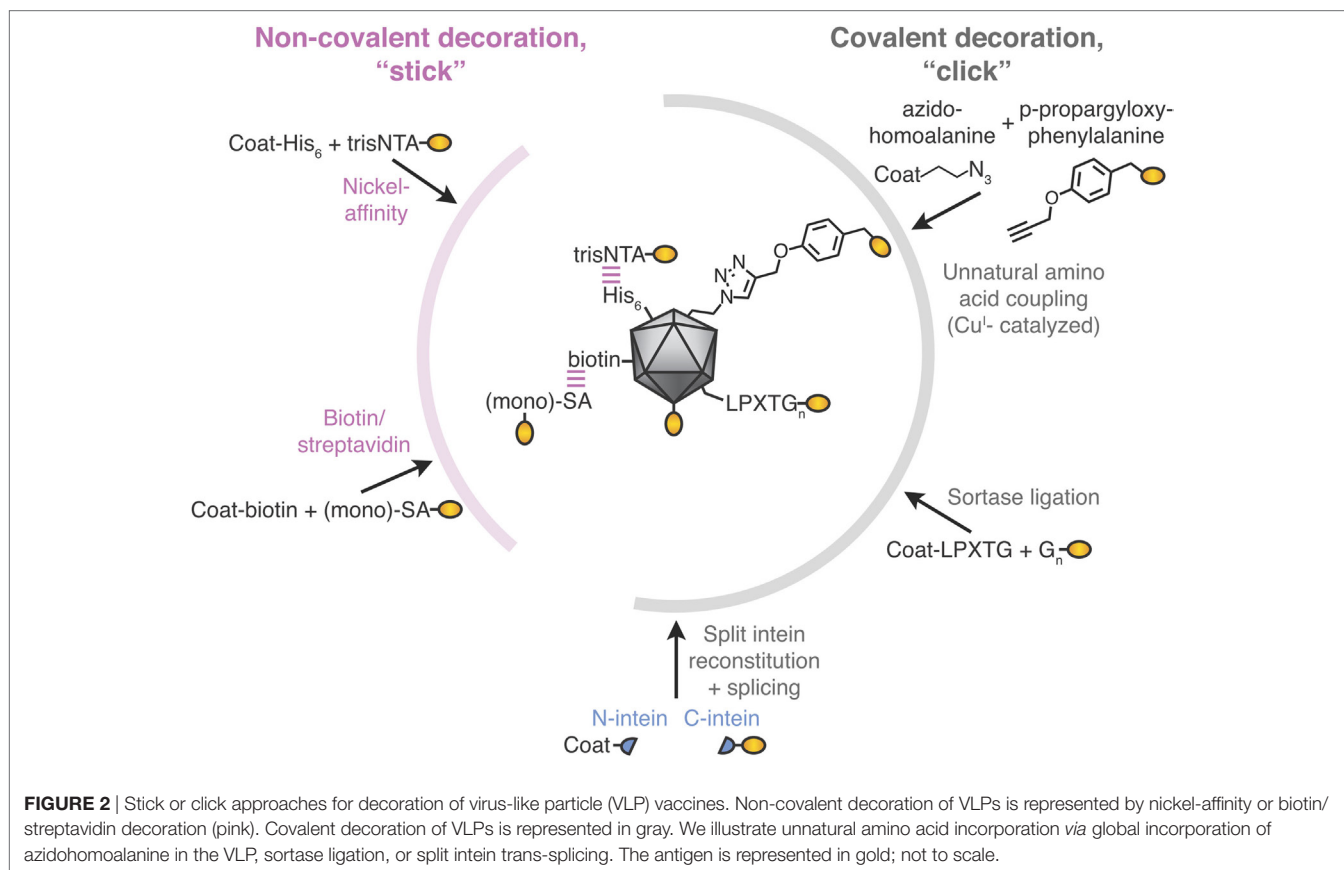
Enhancing Genetic Fusion to VLPs

Various innovative approaches have been taken to overcome the above challenges for genetic fusion to VLPs. Fusion to ferritin at its threefold axis has enabled display of trimeric viral glycoproteins (22). Genetic dissection of the hepatitis B core antigen generated an exposed terminus, which was better able to display fusions on the VLP (SplitCore HBcAg) (8). However, sometimes compromises in genetically fused VLPs have decreased the chances of long-term success: if full domains cannot be genetically fused, fusion of VLPs to short peptides has often been tried or antigens were genetically dissected and distributed across the carrier (23, 24). Such peptide-decorated VLPs can generate antibodies that cross-react with the parent protein. However, compared with full domains, peptides on VLPs often decrease the proteolytic stability and solubility of the assembled VLP, as well as reducing the titer of neutralizing antibodies (25, 26). Also, genetic fusion of peptides to VLPs can sometimes cause toxicity to the expressing cells (27).

Modular Vaccine Assembly

Modular VLNP assembly refers to generating a VLNP-based vaccine in more than one stage. In particular, the VLNPs and antigen are generated separately and then coupled (**Figures 1D** and **2**). Compared with genetic fusion, modular VLNP assembly adds an extra step, so in theory is more complex. In practice, modular assembly can side-step many of the challenges of genetic fusion. The VLNP and antigen can be generated in different systems, choosing whatever gives the optimal yield, conformation, and post-translational modification for each.

The difficulty of manufacturing a vaccine is a major factor in the overall vaccine cost (16). The Bill and Melinda Gates Foundation



have set a target of US \$0.15 per human vaccine dose, while even lower costs may be needed for veterinary and food-safety vaccines (28, 29). Mass production of an abundant, stable, and easy-to-conjugate VLNP scaffold could accelerate vaccine generation. Such a nanoparticle scaffold could also allow production costs to be shared across multiple antigens and diseases (30). Quickly and reliably multimerizing libraries of pathogen-derived antigens should speed up development of vaccines against the most challenging diseases, such as tuberculosis or malaria (31, 32).

Modular assembly is also attractive because personalized vaccines and pandemic threats will require an ever shorter time from development to manufacturing (33, 34). Pre-production of modular scaffold particles, that only need to be mixed with conjugation-enabled antigen or good manufacturing practice (GMP)-grade synthetic peptide, may be helpful for pandemic preparedness (35). As an indication of how governments prepare for pandemics, 40 million doses of a drug, oseltamivir, against influenza were purchased by the UK government (UK population ~66 million people) (36). UNICEF maintains 2 million doses of the oral cholera vaccine (37), while the World Health Organization has 35 million doses of smallpox vaccine stockpiled (38).

Classic Approaches to Modular VLNP Decoration

What we term the classic approach to modular VLNP decoration is the use of cross-linkers to react with nucleophilic amino acid side chains on the VLNP and the antigen (Figure 1D). The most

common reaction is acylation of an amino group from lysine or the N-terminus on the VLNP by the *N*-hydroxy succinimide arm of the cross-linker. This is followed by alkylation of a cysteine on the antigen by the maleimide arm of the cross-linker. Cysteine is rare on the surface of proteins, so often a single exposed cysteine can be introduced by site-directed mutagenesis (39, 40). VLP platforms for classic chemical coupling include the bacteriophage Q β and tobacco mosaic virus and have entered a number of clinical trials, including for diabetes and addiction (41, 42). VLNP decoration with chemical cross-linkers has virtues of scalability and simplicity. Challenges arise when introducing a reactive cysteine interferes with formation of pre-existing disulfide bonds in the antigen (39). Since there are multiple lysines on the surface of the VLNP, coupling is usually heterogeneous and difficult to predict or analyze (Figure 1D). Less commonly, carboxylic acid residues are activated and then reacted with amines (43). It has been suggested that non-uniform coupling of antigen to VLNP may result in sub-optimal immunogenicity (see Catcher/Tag Technology for VLNP B Cell Vaccines).

LATEST APPROACHES IN POST-ASSEMBLY DECORATION OF PARTICLES

Chemical conjugation approaches to proteins in general have been reviewed elsewhere (44). Here, we specifically consider approaches used to decorate VLNPs and discuss their strengths and limitations, in contrast to the classic approaches above.

Non-Covalent VLNP Decoration His-Tag/Ni-NTA Affinity

The His-tag is the most commonly employed peptide motif for recombinant protein purification. Exploiting the interaction between a His-tag and nickel-loaded tris-nitrilotriacetic acid (trisNTA), the Hytönen lab decorated norovirus VLPs (**Figure 2**) (45). trisNTA was chemically linked to dye, vesicular stomatitis virus peptide or biotin. The His-tag binds to Ni-NTA with moderate affinity, which is reduced at lower pH because of its increased His protonation (46). Hence, re-shuffling of VLP assemblies may be an issue during storage or in the lower pH endosomes after vaccine injection.

Biotin–Avidin Affinity

To leverage one of the strongest non-covalent interactions, VLNPs may be biotinylated chemically or using biotin ligase. Biotinylated VLNPs are subsequently recognized by a member of the avidin family (**Figure 2**). Avidin and streptavidin are both tetrameric, which may cause challenges with VLNP cross-linking. However, one can use monovalent streptavidin (four subunits but only one subunit binds biotin) or monomeric streptavidin (one subunit but lower biotin affinity) (47–50). Early studies only coupled short peptides to VLNPs (51, 52), but the approach has since been adopted for full-length proteins up to 85 kDa (49). Even though biotin's interaction to avidin/streptavidin has a high-affinity, half-times for biotin-conjugate dissociation are on the scale of hours (53), so re-shuffling is expected upon storage.

Anthrax Toxin Receptor Decoration

Flock house virus coat protein was genetically fused to part of the human anthrax toxin receptor (ANTXR2). This fusion allows decoration with anthrax protective antigen, which binds with 170 pM dissociation constant (54). High titer neutralizing antibodies to the affinity-displayed protective antigen were observed after administration. No significant antibody response was observed against endogenous ANTXR2, indicating that this platform did not break self-tolerance (54).

Novel Covalent VLNP Decoration Methods

Covalent reaction between the target antigen and particle brings the obvious advantage of stability. This includes not only stability

during storage but also stability after injection, where the lower concentration and altered conditions (e.g., endosomal pH, shear force in the circulation) favor dissociation of non-covalently linked antigen from the particle. Covalent conjugation also facilitates analysis of decoration. A simple SDS-PAGE can quantify what proportion of particle subunits are coupled to antigen and how much non-covalently associated antigen remain (55). Covalent coupling methods are described below, compared in **Table 1**, and illustrated in **Figure 2**.

Incorporation of Unnatural Amino Acids to Enable Click Chemistry

Several groups have decorated VLNPs by translationally introducing unnatural amino acids as uniquely reactive chemical handles. For example, the unnatural amino acid azidohomoalanine was incorporated into the coat proteins of MS2 or Q β bacteriophage *via* cell-free protein synthesis (56). Using this global replacement strategy, >85% of methionines was replaced with azidohomoalanine in the coat protein of the VLP (**Figure 2**) (56). Alternatively, unnatural amino acids can be site-specifically incorporated when an amber stop codon in the mRNA is recognized by a tRNA incorporating the unnatural amino acid (56, 57). The most widely adopted unnatural amino acids utilize click chemistry, which refers to reactions of functional groups that occur rapidly, selectively and in high yield. The most commonly used click-chemistry reactions are alkynes with azide in the presence of Cu^I catalysis (44). Faster and catalyst-independent click reactions have also been developed (44). VLNP decoration using unnatural amino acids has the advantage of requiring minimal change (1 amino acid) to the antigen. Challenges for scaling up come from the cost and side reactions of the unnatural amino acid (58, 59). Also, assembly of a VLNP is cooperative and so a few percent of mistranslated or misfolded subunits may impair a majority of the resultant capsids (see Genetic Fusion and Its Challenges).

HaloTag

HaloTag, a modified haloalkane dehalogenase, binds irreversibly to haloalkane ligands. HaloTag has been used to decorate the 60-mer E2p from *Geobacillus stearothermophilus* with DNA strands and to decorate other nanoparticles with short peptides

TABLE 1 | Features of covalent protein–protein coupling methods.

Method	Terminus restriction	Redox dependence	Orthogonal pairs	Scar after coupling	Other features or challenges
Split intein	N-intein at C-terminus, C-intein at N-terminus	Yes, for Cys-dependent split inteins	Yes	0–3 aa	–
Sortase	C-terminal -LPXTGX and N-terminal oligoglycine/alanine	No	<i>S. aureus</i> and <i>S. pyogenes</i> enzymes	Small (7 aa)	Need high conc. of oligoglycine reactant; challenging to reach complete conjugation
Tag/Catcher	Tag or Catcher at N- or C-terminus or internal	No	SpyCatcher, SnoopCatcher	Catcher (≥ 84 aa) and Tag (12–13 aa) retained	High reaction yield
Electrostatic interaction lock	Should not be restricted	Reducing before reaction; oxidizing after reaction	In principle: several coiled-coil pairings known	Moderate (typically 18 aa)	Disulfides may reverse
Unnatural amino acid coupling	None	No	Yes	1 aa in Ag and 1 aa in virus-like particle	Cost/complexity of expression

(60, 61). Strengths of this approach are that HaloTag does not require cofactors or post-translational modifications for its coupling reaction and HaloTag reacts specifically in cellular systems. A possible drawback is the 33-kDa size of HaloTag and the requirement to install a synthetic haloalkane ligand on the antigen or VLNP substrate protein (62).

SNAP-Tag

SNAP-Tag is a 20-kDa protein which binds irreversibly to alkyl-guanine ligands. SNAP-Tag has been used to conjugate single-chain variable fragments of antibodies (scFvs) to nanoparticles (63). SNAP-Tag has also been used to derivatize HIV-1 virions (64). These virions were capable of assembly and infection but displayed reduced replication (64).

Sortase

Sortase is an enzyme from Gram-positive bacteria that covalently joins proteins with a C-terminal LPXTGX motif to other proteins with an N-terminal oligoglycine motif (Figure 2) (65). Sortase A-mediated ligation was used to conjugate to a genetically installed LPETGG motif on the surface of HBV core antigen VLPs and has since been adopted to other carriers (8, 66). Decoration efficiencies can be high (90%), but may require 6- to 12-fold molar excess of antigen (up to 480 μ M) (66). Sortase was used to decorate E2p nanoparticles with proteins up to 150 kDa, but yielded only sparsely decorated 60-mers (60, 67).

Split Inteins

Split inteins are a naturally occurring protein chemistry, where N- and C-terminal intein regions associate non-covalently and then splice each other out, leading to the attached fragments (exteins) being joined by a peptide bond (Figure 2) (68). Split inteins have subsequently been enhanced by rational design and evolution. Note that in the literature SpyTag/SpyCatcher (see Spontaneous Isopeptide Bond Formation) has sometimes been mistakenly referred to as a split intein. Split inteins have been used to generate a C-terminal thioester on a protein, with subsequent conjugation to flock house virus *via* native chemical ligation (54). Split inteins have also been used to functionalize adeno-associated virus particles with scFvs or DARPins, showing a coupling yield up to 10% (69). Some of the fastest split inteins operate *via* a reactive cysteine, which may be problematic with disulfide-containing antigens (see Classic Approaches to Modular VLNP Decoration). However, in other split inteins, the key reactive residue is serine or threonine (68).

Electrostatic Interaction Locks (EILs)

Electrostatic interactions have been utilized to align cysteine residues to form preferred disulfide bonds, yielding EILs (70). Attempts at locking larger proteins, such as antibody Fv fragments to viral coat protein suffered from <20% decoration efficiencies (71). More recent EIL-mediated peptide display on bovine papilloma virus was successful but reduced particle uniformity (9).

Spontaneous Isopeptide Bond Formation

Catcher/Tag technology was engineered from certain cell-surface proteins of Gram-positive bacteria, which form autocatalytic

intramolecular isopeptide bonds (72). Such isopeptide bonds can confer resilience to chemical (pH 2), thermal (>100°C), and mechanical (>1,000 pN) challenges (73). The SpyTag peptide (13 amino acids) forms a spontaneous isopeptide bond upon mixing with the SpyCatcher protein (116 amino acids) (Figure 3) (73). SpyTag/SpyCatcher reaction can proceed to >99% efficiency on VLNPs at modest excess (1.5-fold) (55, 73). SpyTag/SpyCatcher reacts under diverse conditions, such as pH 5–10 and 4–37°C, in the presence or absence of reducing agent (55, 73). Similarly, the SnoopTag peptide forms a spontaneous covalent bond to the SnoopCatcher protein and has no cross-reaction with SpyTag/SpyCatcher (74). Catchers and Tags react *via* side chains, so each partner can react independently of the location within a protein, i.e., either at the N- or C-terminus, or internally within a protein sequence (72). For many pathogen-derived antigens, there is good understanding where antibodies should bind for optimal neutralization (17, 19). Therefore, the ability to position SpyTag at various locations provides control in how the antigen will extend from SpyCatcher-linked VLNPs.

Given our greater expertise on Catcher/Tag VLNP decoration, we discuss the progress, opportunities, and limitations of this approach in more detail below.

ADOPTION OF CATCHER/TAG TECHNOLOGY FOR VACCINE ASSEMBLY

Catcher/Tag Technology for T Cell Vaccines and Cytotoxic Cancer Immunotherapy

The first application of Catcher/Tag technology toward immune modulation was the development of SpyAvidins—variants of streptavidin bearing either SpyTag or SpyCatcher on specific subunits of the tetramer. SpyAvidin-MHC eicosamers (bearing 20 streptavidin subunits) surpassed conventional MHC tetramers in stimulating T cell activation (76). In another study, SpyTag was fused at the C-terminus of anti-DEC205 scFv, to allow modular targeting of antigens to dendritic cells (DCs) (77). SpyCatcher-OVA8-TBEV-ED3 is a fusion protein that includes a CD8⁺ T cell epitope (OVA8, residues 257–264 from ovalbumin) and a B cell epitope (Tick-borne Encephalitis Virus ED3). Linking antigen to scFv *via* SpyTag/SpyCatcher allowed targeting of antigen to DEC205-positive DCs for cytotoxic T cell stimulation (77). The same DC-targeting platform subsequently induced potent cytotoxic T cell responses against the HPV oncogene E7, translating to therapeutic antitumor responses. Use of checkpoint inhibitors further enhanced the therapeutic effect (78).

Catcher/Tag Technology for VLNP B Cell Vaccines

Brune et al. and Thrane et al. independently developed Spy-VLPs (Figure 3A) (55, 75). N-terminally truncated SpyCatcher (79) was fused to the coat protein of the bacteriophage AP205 (55). SpyCatcher-VLP reaction could proceed to quantitative yield (Figure 3C) (55). SpyCatcher-VLPs (~20–43 nm in size, undecorated) were efficiently decorated with SpyTagged complex antigens, such as two *Plasmodium falciparum* blood-stage CIDR

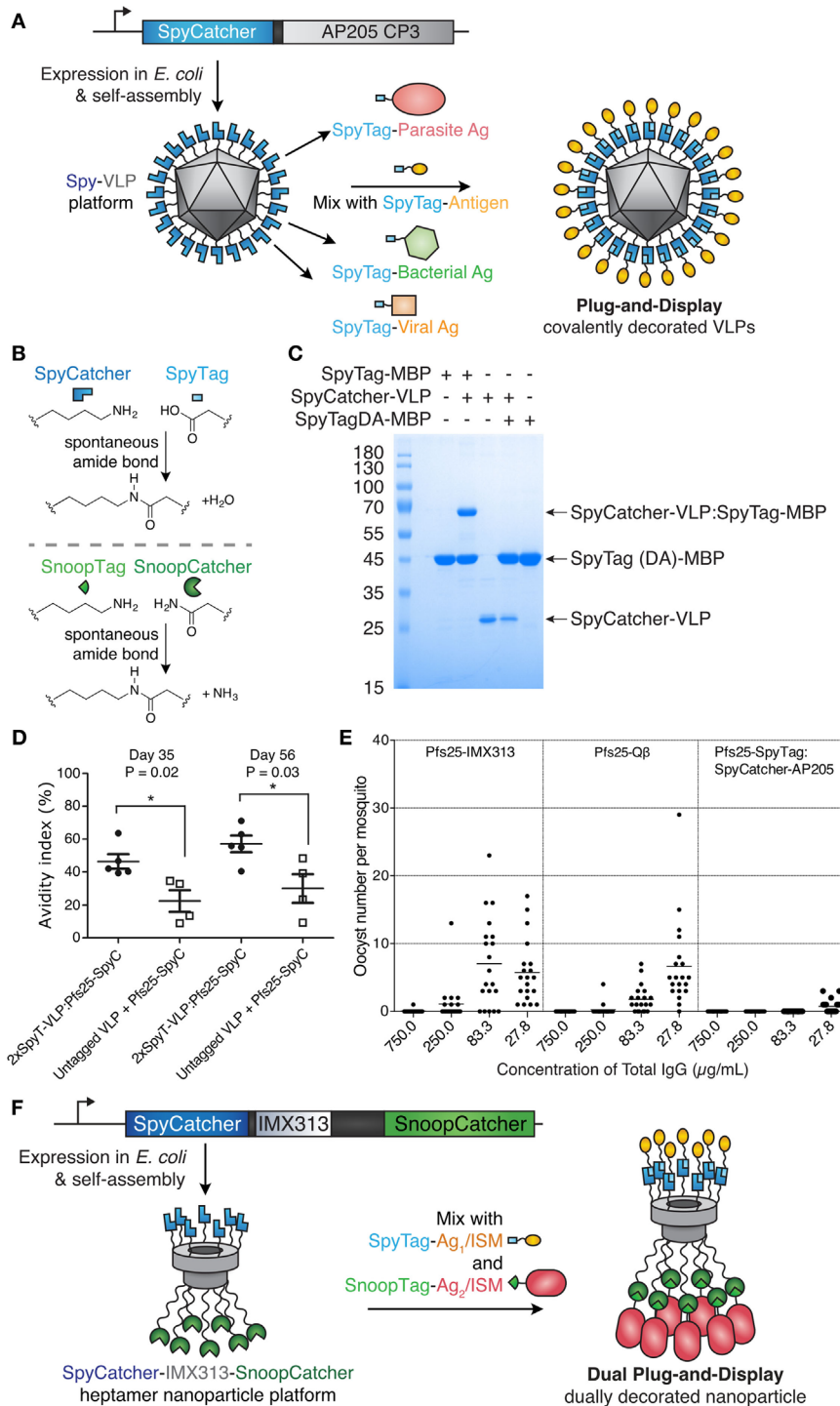


FIGURE 3 | Plug-and-display virus-like particle (VLP) decoration. **(A)** Plug-and-display principle. SpyCatcher was genetically fused to the AP205 coat protein and expressed in *Escherichia coli*. Upon mixing, different SpyTag antigens form spontaneous isopeptide bonds, yielding decorated particles for immunization. **(B)** Catcher/Tag chemistry. SpyCatcher/SpyTag side chains form a covalent bond with release of water. Reaction of SnoopCatcher with SnoopTag yields a covalent bond and release of ammonia. **(C)** Quantitative coupling of Spy-VLPs with antigen. SpyCatcher-VLPs were incubated with SpyTag-maltose-binding protein (MBP) or the negative control SpyTagDA-MBP, before SDS-PAGE with Coomassie staining. Data from Ref. (55) with permission. **(D)** Improved antibody quality by Spy-VLP display. Antibody avidity (based on resistance to 8M urea) was assessed on days 35 and 56 in serum from mice immunized with Pfs25-SpyCatcher and untagged or dual SpyTag-VLPs. Data from Ref. (75) with permission. **(E)** Malaria transmission blocking with Spy-VLPs. Pfs25-IMX313 is a genetically fused nanoparticle. Q β -Pfs25 is a chemically decorated VLP. Pfs25-SpyTag:SpyCatcher-AP205 is a plug-and-display conjugate. Total IgG, purified from pooled serum after immunization, was analyzed by standard membrane feeding assay. Data points represent oocysts in a mosquito, and the lines show the arithmetic mean. Data from Ref. (39) with permission. **(F)** Dual plug-and-display. Expression in *E. coli* and spontaneous multimerization yields SpyCatcher-IMX313-SnoopCatcher nanoparticles. SpyTag linked to antigen 1 (Ag₁) or an immunostimulatory molecule (ISM) react with SpyCatcher, while the other nanoparticle face is decorated by SnoopTag/SnoopCatcher reaction.

antigens or the transmission-blocking ookinete antigen Pfs25 (55). Spy-VLPs induced high titers against Pfs25 after a single immunization (55). Spy-VLP-induced antibodies showed high avidity (**Figure 3D**) (75).

There is a shortage of side-by-side comparisons of vaccine platforms in the literature. As an initial test of different immunization platforms, mice were immunized with Pfs25 displayed on (i) SpyCatcher-AP205 VLPs using plug-and-display conjugation, (ii) Pfs25-VLPs from chemical decoration of Q β , or (iii) genetic fusion of Pfs25 to the nanoparticle IMX313. Q β display generated the highest anti-Pfs25 titer. However, SpyCatcher-VLP display gave the highest antibody quality, assessed by the ability of a given concentration of antibody to block malaria transmission to mosquitoes, using a standard membrane feeding assay (**Figure 3E**) (39, 55). Given the difficulty of varying one feature of a nanoassembly at a time, the molecular features determining the size and specificity of antibody responses need further investigation.

Thrane and co-workers demonstrated fusion of SpyTag to the N- and C-termini of the AP205 coat protein. Spy-VLPs (36–42 nm in diameter, undecorated) were conjugated to a range of antigens from diverse viral and bacterial pathogens (up to 118 kDa) (75). Spy-VLP display enabled breaking of tolerance against self-antigens (including PD-L1, CTLA-4, IL-5, and HER2) (75, 80). Immunization using SpyTag-AP205-SpyTag VLPs decorated with SpyCatcher-HER2 (extracellular domain) induced autoantibodies against human HER2. Four out of five mice were protected against a HER2-positive cancer challenge after prophylactic vaccination (80). Furthermore, the HER2-decorated Spy-VLPs (~66 nm in diameter, decorated) were also 100% effective in preventing the onset of mammary carcinomas in a mouse model expressing the Delta16 isoform of HER2, a splice variant more oncogenic than full-length HER2 (80). It will be interesting to explore the Spy-VLP approach against other cancer immunotherapy targets.

The range of antigens and platforms linked using Tag/Catcher at the time of submission is summarized in **Table 2**.

Decoration of VLNP With More Than One Different Component

Decoration of VLNPs with multiple ligands is technically difficult, but may be important for protecting against different strains of a pathogen or even against distinct diseases (86). For classic approaches to VLNP decoration (see The Difficulty of Installing Complex Antigens on VLNPs), the challenge of dual display increases substantially, because of increased misfolding of larger genetic constructs and the difficulty in finding compatible chemistry without cross-linking particles. In one example, a cytokine (GM-CSF) and a scFv (each bearing alkynes) were simultaneously displayed on an azido-derivatized MS2 VLP, by carefully controlling ratios of both alkyne ligands (56). The resulting VLP may represent a range of slightly differently decorated particles with different immunological properties, since the precise location and spacing of multiple ligands cannot be controlled when a single reaction handle is used for attaching multiple proteins.

To avoid heterogeneity, it is paramount to identify conjugation strategies that are orthogonal to each other. Click chemistry and His-tag protein display, for example, have been combined on

gold nanoparticles, enabling simultaneous display of influenza hemagglutinin and immunostimulatory flagellin (87). We set out to expand the Catcher/Tag VNLP approach to dually addressable Catcher/Tag protein nanoparticles (**Figure 3B**) (74). The multimerization domain IMX313 was recombinantly expressed with SpyCatcher at its N-terminus and SnoopCatcher at its C-terminus, yielding dually addressable protein NPs (~16 nm in diameter, undecorated) (**Figure 3F**). This VLNP allowed independent decoration of SpyCatcher with Pfs25-SpyTag and decoration of SnoopCatcher with Pfs28-SnoopTag. Immunization with this dually decorated VLNP yielded antibodies to both these *P. falciparum* transmission-blocking antigens, enhancing the response compared with monomeric antigen (82).

Practical Aspects of Catcher/Tag Technology

Tag/Catcher-Fusion Effects on Antigen Expression

An important consideration for any vaccination platform is how easily the method can be adapted to different antigens. Ideally, it should be possible to express the modified protein with a yield and quality that is comparable to the untagged antigen. There are only a few fusion partners, such as glutathione-S-transferase and GFP, where solubility and expression data are available on thousands of fusions (6, 88). For SpyCatcher/Tag, these data are still limited but growing quickly (**Table 2**). There are promising signs that fusion of SpyTag (13 aa) to a protein often has little effect on its expression. SpyCatcher fusion, on the other hand, might be expected to cause more issues because of its bigger size (84–116 aa). Surprisingly, SpyCatcher fusion enhanced soluble expression in *E. coli* of the disulfide-rich Pfs25 from *P. falciparum* (75). Fusions to Catcher and Tag sequences have been expressed in various systems, including Gram-positive and Gram-negative bacteria (73, 89, 90), mammalian cells (73), insect cells (75), plant cells (91), and yeast (92).

Improving Vaccine Stability

The need for thermostable vaccines is widely recognized. Failure of the cold-chain has often led to wasting of vaccines or administering despite loss of activity (93). Important advances have been made by optimizing the engineering and chemistry of vaccine formation. However, the intrinsic stability of the protein components may also have profound effects on the magnitude and quality of the immune response (94, 95).

The dually addressable SpyCatcher-IMX-SnoopCatcher particles (**Figure 3F**) remained soluble after incubation at 99°C, while efficient Tag-antigen reaction was retained following incubation up to 60°C (82). Immobilization of enzymes onto potato virus X VLPs *via* SpyCatcher/SpyTag technology also suggests improved heat tolerance up to 50°C (91). Intramolecular cyclization *via* “sandwiching” enzymes using Tag/Catcher can impart high resilience to heat inactivation and even allow purification of an enzyme *via* boiling (89, 96). One could contemplate harnessing this resilience for purification and sterilization for cheap production of monomeric antigens (16, 29).

Similarly, cyclization of coxsackievirus B3 (CVB3) coat protein VP1 *via* split inteins averted irreversible heat aggregation, increased the proteolytic resilience, and reduced degradation

TABLE 2 | Disease-related proteins assembled using Catcher/Tag technology.

Category	Antigen	Antigen expression	Platform	Platform expression	Reference
Self	Peptide-major histocompatibility complex class I multimers	<i>Escherichia coli</i>	Streptavidin-SpyTag, Streptavidin-SpyCatcher	<i>E. coli</i>	(76)
Cancer (Neo-self)	EGFRvIII-SpyTag	Chemically synthesized	Δ N1SpyCatcher-AP205	<i>E. coli</i>	(55)
Cancer (Neo-self)	SpyTag-Telomerase	Chemically synthesized	Δ N1SpyCatcher-AP205	<i>E. coli</i>	(55)
Cancer (HPV)	SpyCatcher-E7	<i>E. coli</i>	Anti-DEC205-SpyTag	Mammalian	(78)
Cancer (Breast)	SpyCatcher-HER2	Insect	SpyTag-AP205-SpyTag	<i>E. coli</i>	(80)
Malaria	SpyTag-CIDR	<i>E. coli</i>	Δ N1SpyCatcher-AP205	<i>E. coli</i>	(55)
Malaria	Pfs25-SpyTag	Mammalian	Δ N1SpyCatcher-AP205	<i>E. coli</i>	(39, 55)
Malaria	SpyCatcher-CIDR	Insect	SpyTag-AP205	<i>E. coli</i>	(75)
Malaria	CSP-SpyCatcher	Insect	SpyTag-AP205	<i>E. coli</i>	(75)
Malaria	Pfs25-SpyCatcher	<i>E. coli</i>	SpyTag-AP205-SpyTag	<i>E. coli</i>	(75)
Malaria	SpyTag-VAR2CSA	<i>E. coli</i>	SpyCatcher-AP205	<i>E. coli</i>	(75)
Malaria	CSP-SpyCatcher (Full-length 3d7 CSP)	Insect	SpyTag-AP205	<i>E. coli</i>	(81)
Malaria	Simultaneous display of: Pfs25-SpyTag, Pfs28-SnoopTag	Mammalian	Δ N1SpyCatcher-IMX-SnoopCatcher	<i>E. coli</i>	(82)
Malaria	SpyCatcher.R0.6C (GLURP fusion of partial Pfs45/48)	<i>Lactococcus lactis</i>	SpyTag-AP205-SpyTag	<i>E. coli</i>	(83)
Malaria	SpyCatcher-6C (fragment of Pfs45/48)	<i>L. lactis</i>	SpyTag-AP205-SpyTag	<i>E. coli</i>	(83)
Malaria	CSP-SpyCatcher conjugated to SpyTag-DBL1x-DBL2x-ID2a	Insect	N/A	N/A	(84)
Pneumococcal	SpyCatcher-(PspA α '-domain)-SnoopTag	<i>E. coli</i>	HbpD(Δ d1)-SpyTag	<i>E. coli/Salmonella Typhimurium</i>	(24)
Pneumococcal	SpyCatcher-SP1690-SnoopTag	<i>E. coli</i>	HbpD(Δ d1)-SpyTag	<i>E. coli/S. Typhimurium</i>	(24)
Pneumococcal	SpyCatcher-PsP α -SP1960-SnoopTag	<i>E. coli</i>	HbpD(Δ d1)-SpyTag	<i>E. coli/S. Typhimurium</i>	(24)
Self	SpyCatcher-IL-5	Insect	SpyTag-AP205	<i>E. coli</i>	(75)
Self	SpyCatcher-HER2 extracellular domain	Insect	SpyTag-AP205	<i>E. coli</i>	(75)
Self	SpyCatcher-Survivin	Insect	SpyTag-AP205-SpyTag	<i>E. coli</i>	(75)
Self	SpyCatcher-IL-5	Insect	SpyTag-AP205-SpyTag	<i>E. coli</i>	(75)
Self	JoTag-transmembrane helices of GPCRs (C5aR)-InCatcher	<i>E. coli</i>	N/A	N/A	(85)
Self (heart disease)	PCSK9-SpyCatcher	Insect	SpyTag-AP205	<i>E. coli</i>	(75)
Self (T cell immunity)	CTLA-4-SpyTag	Insect	SpyCatcher-AP205	<i>E. coli</i>	(75)
Self (T cell immunity)	PD-L1-SpyTag	Insect	SpyCatcher-AP205	<i>E. coli</i>	(75)
Tick-borne encephalitis virus	SpyCatcher-OVA _s -ED3	<i>E. coli</i>	Anti-DEC205-SpyTag	Mammalian	(77)
Tuberculosis	SpyCatcher-Ag85A	<i>E. coli</i>	SpyTag-AP205	<i>E. coli</i>	(75)

N/A, not applicable.

in vivo (94). Upon immunization, circularized VP1 gave increased CVB3-specific antibody and cellular immune responses, improving protection against viral myocarditis (94).

LIMITATIONS AND WHAT IS NOT YET KNOWN FOR CATCHER/TAG TECHNOLOGY

Here, we describe some key issues for modular decoration, moving from proof of principle toward real world application. We illustrate these challenges for SpyTag/SpyCatcher-based assembly, but many issues may be generalized to other decoration technologies.

Structural Compatibility of Catcher/Tag-Antigen Fusions

Catcher/Tag decoration appears feasible for a range of non-enveloped VLPs (55, 75, 91, 97). Also, a wide range of antigens have now been displayed for immunization using SpyTag/SpyCatcher (Table 2). However, non-functional fusion proteins are much less likely to be published, so the literature is tilted toward functional fusion. In the future, it will be important to perform systematic studies on how the intrinsic multimeric state of an antigen affects VLNP display efficiency and the characteristics of the decorated particles. One may expect that antigens forming higher-order homomultimers would not be suitable for plug-and-display, because of the danger of cross-linking between particulates.

However, IL-5 and survivin, typically dimeric (98, 99) have been successfully displayed on Spy-VLPs (75). It is possible that strategic placement of SpyCatcher/SpyTag close to multimerization interfaces may minimize particle cross-linking.

Purification and Characterization of VLNPs

Yields for multimeric scaffolds of Catcher/Tag-VLPs (AP205 or IMX313) are still at the research scale of 1–20 mg/L using shaking-flask culture (55, 82, 83). Even though fermentation will increase biomass, it will be important to optimize these yields or identify other more scalable VLPs.

Molecules co-purifying with VLNPs are also an issue, when moving to clinical development. VLP platforms can package nucleic acids during assembly (100). Specific viral RNAs also act as scaffolds, guiding assembly of the viral capsid (10, 101). Intracellular nucleic acid detection is mediated particularly by the TLR family, e.g., TLR3 (dsRNA), TLR7 (ssRNA), and TLR9 (unmethylated CpGs in DNA) (100). AP205 VLPs contain 25–30 μ g of host cell RNA per 100 μ g of coat protein (102). Efficient removal of endotoxin from *E. coli*-expressed Spy-VLPs has been demonstrated, important for well-controlled comparison between immunogens (55). Endotoxin may be easily removed from Spy-VLPs, either by washing with Triton X-114 when the VLPs are immobilized on purification-resin or by phase separation (55).

Virus-like nanoparticle purity also depends on the yield of any coupling reactions. SpyTag/SpyCatcher in isolation gives a high yield of reaction (>99%) (Figure 3C) (74); the mechanism allows little possible side-reactions. Complete coupling of antigens to SpyCatcher-VLPs after 3 h was achieved in some cases, as assessed by SDS-PAGE using 1.5-fold molar excess of SpyTag-antigen over SpyCatcher-coat protein (55). In other cases, where there was a shorter spacer between the SpyCatcher and coat protein, coupling was less complete (75, 81, 83). Coupling may become slower on the crowded VLP surface, since already-coupled antigens sterically inhibit access of unattached antigens. SpyCatcher coupling may be optimized by exploring reaction conditions, in particular decreasing to pH 6 (55, 73). Faster variants (named SpyTag002/SpyCatcher002) have been engineered, which may allow faster VLNP coupling (103). In other cases, adjustment of the spacer between SpyCatcher/coat protein and SpyTag/antigen may be needed for large antigens. Alternative Catcher/Tag pairs have also been engineered that can cater to different pH-dependent conjugation optima, which may be more suitable for a particular demanding antigen or expression system (74, 85, 104–106).

Does the Immune Response to the VLP Platform Help or Hinder Vaccination?

Chimeric VLPs such as RTS,S (the malaria vaccine based on HBsAg VLPs) will contain immunologically relevant sequences other than those of the pathogen of primary interest. It is preferable that such sequences are not of human origin, to minimize the risk of autoimmune disease. For RTS,S, one vaccine construct raises antibodies against HBV and malaria. Would pre-existing antibodies against the platform prevent a new vaccine providing protection? For RTS,S in a typical three dose treatment, booster

doses do improve the malaria protection even though anti-HBsAg antibodies are already present (107). Similar results were found in mouse models with Spy-VLPs (39, 55, 81, 82). A recent human trial found that pre-existing cross-reactive antibodies from the inactivated Japanese encephalitis virus vaccine enhanced the response to subsequent live-attenuated yellow fever vaccination (108). Nonetheless, further testing of these questions will be important.

There is limited information whether SpyCatcher or SpyTag contains MHC class II epitopes, which might contribute to B cell memory. Deletion of the N-terminal region of SpyCatcher to give Δ N1SpyCatcher (92 aa, the construct used on AP205 by Brune et al.) reduced antibody titers against SpyCatcher (77). We showed that antibodies were raised to SpyCatcher-VLPs after immunization, but antibody levels were substantially reduced after conjugating SpyTag antigen (55).

SUMMARY AND FUTURE OPPORTUNITIES

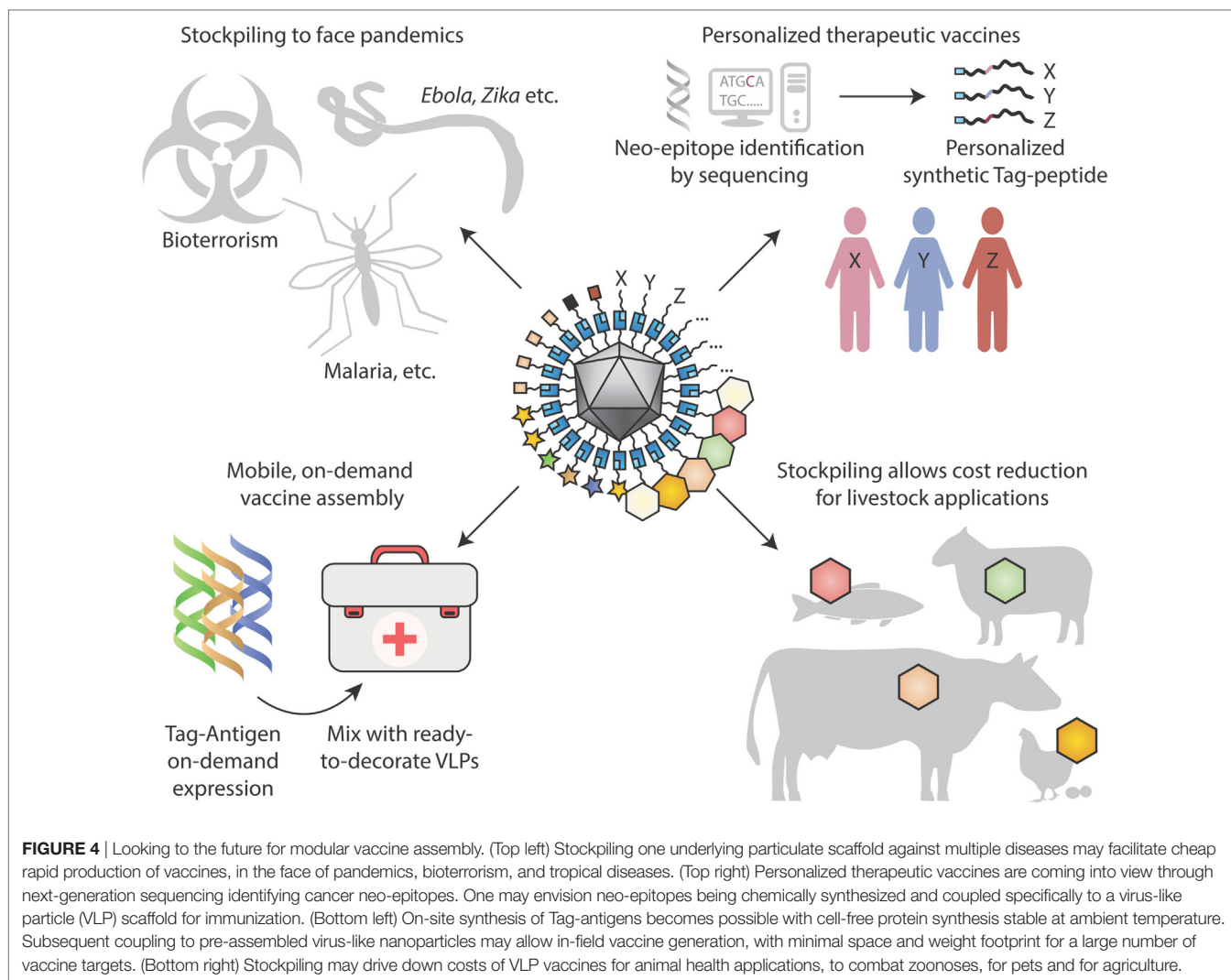
Decoration Opportunities on Other Platforms

Assembly scaffolds other than non-enveloped VLPs have valuable properties for immune stimulation and could benefit from modular decoration with other antigens. Outer membrane vesicles (OMVs) are spherical membranous nanoparticles released from the outer membrane of Gram-negative bacteria. OMVs can be produced at large scale, displaying proteins of interest on their surface or packaging proteins stably inside. SpyTag was first used for OMV display through expression of a SpyTag-OmpA fusion, allowing conjugation of an enzyme for detoxification of nerve agents (109). SpyTag was subsequently displayed on hemoglobin protease, yielding OMVs (30–200 nm in diameter) from *E. coli* and *Salmonella Typhimurium* displaying SpyCatcher-linked pneumococcal antigens (24). In addition, this platform was amenable to SnoopTag/SnoopCatcher antigen display (24). The same authors also introduced the concept of attenuated, Spy-displaying live bacteria as vaccine carriers (24).

In the future, Tag/Catcher display may facilitate comparison between other non-protein vaccination platforms. Such platforms are beyond our scope to cover in detail, but include emulsions, inorganic NPs, immune-stimulating complexes, and lipid-based NPs (e.g., liposomes and spheroplasts) (4, 110, 111).

Virus-like particles are pre-eminent vaccine platforms for inducing antibody responses; a central challenge for the field is to develop platforms giving potent T cell and B cell responses, important for the most intractable pathogens such as tuberculosis or HIV (21, 112, 113). Though existing Catcher/Tag-VLNP platforms have not yet been thoroughly assessed for T cell responses, recent work has enhanced T cell induction based on co-delivered adjuvants (114), additional helper T-cell epitopes (115), or incorporating cell-permeable peptides (116).

Where VLNPs may not be able to provide a sufficient T cell response, viral platforms are leading the way at inducing cytotoxic T cell responses. SpyTag fusion has recently enabled modular modification of certain virus models, although immune stimulation has not been tested. The envelope protein GP64 from baculovirus was fused with SpyTag and labeled with SpyCatcher-labeled



quantum dots for tracking and visualization in insect cells (117). SpyTag was also used in lentivirus targeting: target-cell tropism was redirected upon conjugating SpyTagged Sindbis virus E2 lentiviral envelope protein with SpyCatcher-linked anti-HER2-DARPin (118).

Modularity Could Enhance VLP Vaccines for Chronic Diseases and Personalized Medicine

Beyond infectious diseases, VLP platforms may be adopted for therapeutic use. Allergens on VLPs may suppress allergic immune responses, while avoiding side-effects from direct activation of mast cells (40). New therapeutic antibodies continue to achieve clinical success, including landmark results of checkpoint inhibitors against cancer (119). Rather than recombinantly expressing antibodies and then infusing, there may be advantages in inducing people to generate their own blocking antibodies (75, 80). Particulate vaccines presented here may be employed for non-infectious chronic diseases, such as Alzheimer's disease or hypertension (120). With plummeting costs of DNA sequencing, personalized mutation-specific cancer therapy will advance

rapidly (121), providing further drive for rapid assembly of personalized cancer vaccines (Figure 4).

In all fields of science, progress can come faster when one can move from patient crafting of success to a reproducible assembly line (122–126). Vaccines must satisfy many criteria, including efficacy, safety, manufacturability, and cost-effectiveness, so vaccinology will never be easy. But the gains in pathogen genomics (31) and structural vaccinology (17) can be complemented by the automation of antigen multimerization, as modular VLP decoration becomes routine.

AUTHOR CONTRIBUTIONS

KB and MH conceived and wrote the manuscript.

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Conflict of Interest Statement: KB is a consultant and co-founder of Genie Biotech Ltd. MH is an inventor on a patent concerning peptide targeting via spontaneous amide bond formation (EP2534484) and co-founded SpyBiotech Ltd.

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