ACS APPLIED BIO MATERIALS

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Surface Functionalization of Rod-Shaped Viral Particles for Biomedical Applications

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Cite This: https://doi.org/10.1021/acsabm.1c01204 Read Online

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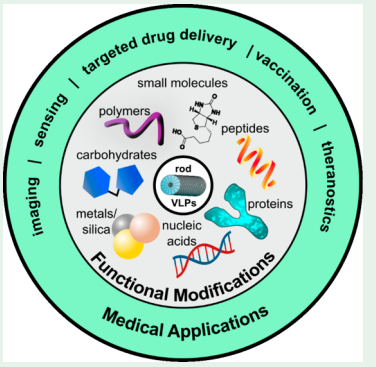
ABSTRACT: While synthetic nanoparticles play a very important role in modern medicine, concerns regarding toxicity, sustainability, stability, and dispersity are drawing increasing attention to naturally derived alternatives. Rod-shaped plant viruses and virus-like particles (VLPs) are biological nanoparticles with powerful advantages such as biocompatibility, tunable size and aspect ratio, monodispersity, and multivalency. These properties facilitate controlled biodistribution and tissue targeting for powerful applications in medicine. Ongoing research efforts focus on functionalizing or otherwise engineering these structures for a myriad of applications, including vaccines, imaging, and drug delivery. These include chemical and biological strategies for conjugation to small molecule chemical dyes, drugs, metals, polymers, peptides, proteins, carbohydrates, and nucleic acids. Many strategies are available and vary greatly in efficiency, modularity, selectivity, and simplicity. This review provides a comprehensive summary of VLP functionalization approaches while highlighting biomedically relevant examples. Limitations of current strategies and opportunities for further advancement will also be discussed.



KEYWORDS: *virus-like particle, surface functionalization, click chemistry, protein engineering, rod-shaped plant virus, bioconjugation*

INTRODUCTION

Nanotechnology is rapidly transforming modern medicine and advanced particle platforms are now central to most biomedical applications. Various particles of diverse compositions are regularly used for imaging, diagnostics, drug delivery, vaccination, and upcoming treatments such as gene therapy and photothermal ablation.[1,2] Particles are designed to carry small molecules, metals, polymers, proteins, nucleic acids, and other functional motifs to support these applications. However, particle structure and physicochemical properties play an increasingly appreciated role. For example, particle size and shape are known to dictate properties such as retention and biodistribution and can be tuned to target disease locations such as tumors.[3] Surface properties including charge, polarity, hydrophobicity, and architecture are also crucial parameters that control cellular and molecular interactions *in vitro* and *in vivo*.[4,5] While common, synthetic particles offer some control over size, shape, and surface functionality, their chemical production methods often involve toxic solvents and/or catalysts that can lead to undesirable side effects in patients.[6] Furthermore, synthetic particles are often polydisperse in size and surface composition. This limitation not only blurs the relationship between particle properties and function but also makes it difficult to tightly control these properties for optimal medical performance. Among the more mature materials for particle design are lipid nanoparticles that carry current mRNA-based vaccines. Nonetheless, these materials still face important drawbacks including cold-chain requirements due to low room-temperature stability and limited biodistribution.[7,8] There are thus many areas to improve on current particle platforms, including biocompatibility, stability, and dispersity. Biomacromolecules such as proteins and nucleic acids exhibit exceptional monodispersity and often self-assemble into nanostructures with inherent stability and compatibility in biological environments. Nanoparticles derived from biological macromolecules thus have numerous advantages over synthetic and other nonviral nanoparticles and are a natural choice for next-generation particle platforms.

Viruses are an assembly of biological macromolecules that are extremely well adapted for many biomedical applications. Their biological origin promises sustainable bioproduction, cold-chain independent stability, and biodegradability, thus enhancing translational potential. Viruses are also natural carriers for DNA and RNA and thus have tremendous potential for nucleic acid protection and delivery. Virus-like particles (VLPs) can be prepared without native genomes thereby removing infectivity to enhance safety and bio-

Special Issue: Early Career Forum

Received: November 28, 2021
Accepted: January 31, 2022

security.[9] Viruses are also known to self-assemble into monodisperse nanoparticles with precise, hierarchical structures with hundreds to thousands of protein subunits in a single particle. Each subunit in these particles can potentially be coupled to small molecules or other macromolecules to modify particle function. Such extreme multivalency is very useful for high-capacity drug loading, sensing, and antigen display for vaccination.[10,11]

Rod-shaped plant viruses offer unique advantages for medicine. First of all, they evolved to infect plant hosts and are typically noninfective and biocompatible in humans.[12] VLPs derived from these viruses encapsidate RNA templates of varying length with thousands of identical coat proteins that self-assemble helically into a tubular protein shell.[13] Produced particles have a diameter between ∼13 and 21 nm and control their length or aspect ratio by altering the number of coat proteins used. This tunable aspect ratio frees rod-shaped plant VLPs from the cargo capacity restrictions common to icosahedral viruses (e.g., adeno-associated viruses, AAVs) and offers a means to control particle size and shape, which affect biodistribution, pharmacokinetics, targeting, uptake, and intracellular trafficking.[3,14] Moreover, viral-surface exposed regions in the protein sequence, which typically include the C-terminus, provide important means of surface functionalization with peptides, proteins, and other ligands that improve targeting specificity and biological/therapeutic functionality.[15,16] While rod-shaped viruses are typically propagated in their respective plant hosts, heterologous expression in yeast and bacteria is also possible for scalable and sustainable production.[17,18] Moreover, heterologous expression improves engineering flexibility since production becomes decoupled from host plant infectivity. Flexible genetic engineering is particularly useful for the functionalization of viral particles with peptides and proteins. However, various chemical and biological functionalization approaches are established for these and other functional ligands including small molecules, metals, and polymers.

Current approaches vary in their ability to functionalize surfaces to high density in a modular manner with site-selectivity (Figure 1). A primary consideration is desired efficiency or degree of labeling. High density ligand display can enhance the avidity of particle interactions with cells or other structures and is generally useful for improved functional capacity. A modular approach enables modification with many diverse ligands with relatively high speed and ease. An example application is the modification of an established particle carrier with different antigens, which would be particularly useful for the rapid development of new vaccines against emerging pathogenic variants or other disease-associated epitopes. Modular functionalization approaches can be leveraged for high-throughput particle preparation and screening, and they are favorable when making more than one type of particle. Finally, selectivity is also a crucial feature for many applications. Site-selective approaches allow a specific region of the functional structure of interest to be linked at a specific residue of the viral coat protein. This not only preserves monodispersity in surface composition but also allows functional ligands to reach a proper orientation for function. Here, we compare current and emerging approaches for the functionalization of rod-shaped VLPs across critical parameters including efficiency, modularity, and selectivity to highlight opportunities and challenges for the development of next generation medical nanotherapies.

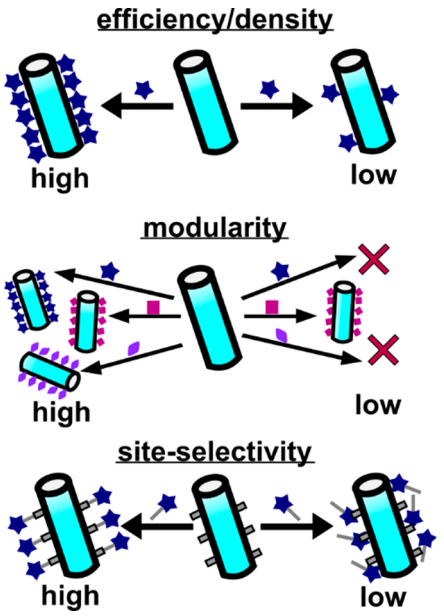


Figure 1. Features of viral particle surface functionalization that vary between different conjugation approaches.

DIRECT PROTEIN FUSION

Direct fusion through genetic engineering is commonly chosen for viral particle decoration with peptides and proteins due to its simplicity, efficiency, and selectivity (Figure 2). Fusions are

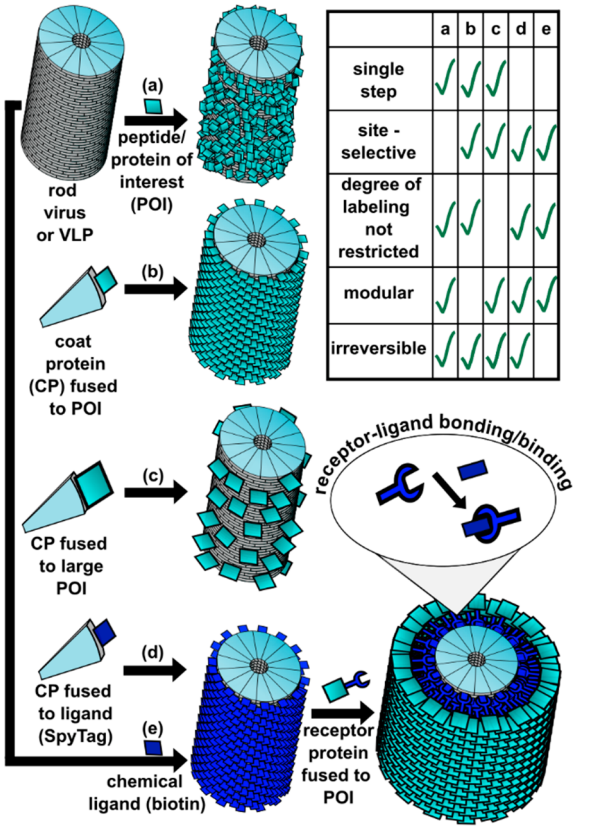


Figure 2. Decoration of rod virus particles with peptides and proteins via (a) chemical conjugation, (b) direct fusion, (c) coassembly of mutant fusion protein with wildtype coat protein, (d) SpyCatcher−SpyTag bonding, and (e) streptavidin−biotin binding.

https://doi.org/10.1021/acsabm.1c01204
ACS Appl. Bio Mater. XXXX, XXX, XXX−XXX

site-selectively inserted at surface-exposed regions, which are found at the coat protein's unstructured termini for several rod-shaped plant viruses via cryo-EM and X-ray diffraction.[15] Site-selectivity is especially important during functionalization with peptides and proteins, which often contain binding domains or active sites that must be exposed and accessible rather than buried near the particle surface. When every coat protein is fused to a peptide or protein of interest (POI), complete surface coverage is guaranteed (Figure 2b). Thousands of functional peptides can thus be displayed per nanoparticle, highlighting the utility of viral particles for high density decoration. Since functionalization occurs at the time of protein expression, standard protocols for particle purification alone will directly yield the final, functional product. However, the simplicity and efficiency of this approach is balanced by limited modularity. Modified coat proteins will not always form multivalent particles, since some fusions interfere with their self-assembly and stability. Nonetheless, many rod virus−POI fusion particles have been successfully constructed and tested in diverse applications.

Although a wide variety of peptidic structures have been directly fused to rod-shaped viruses and VLPs, these are generally restricted to ∼20 amino acids or less to reduce steric hindrance that may interfere with particle assembly and/or stability. For example, 23 amino acid peptide fusions compromised the stability of papaya mosaic virus particles. However, shorter peptide analogs up to 15 amino acids in length were successfully fused to form stable particles and ultimately develop an effective, single dose flu vaccine in mice.[19,20] Similarly, multiple peptide epitopes can be fused to a virus or VLP if their collective length does not exceed the ∼20 amino acid limit. For example, sperm-zona pellucida and spermatozoa-specific peptides totaling 20 amino acids in length were successfully cofused to the Johnsongrass mosaic virus for a contraceptive vaccine in mice.[21] As a very rare exception to the short fusion trend, a relatively large, 133-residue fragment of the immunoabsorbent protein A has been fused to TMV. The resultant proteins successfully assembled into rods and retained their infective and replicative capacity in plants, yielding inexpensively produced nanoparticles for antibody purification.[22] Fusion success relied on the presence of a 15 amino acid peptide linker between the coat protein and functional fragment. Both a flexible, neutral $(GGGS)_4$ and helical, charged $(EAAAK)_4$ linker peptide worked in this case, suggesting that linker flexibility and charge are not critical parameters. Clear design criteria remain elusive, however, as no other exceptions using these or other linkers were demonstrated with large POIs. While fusion success is difficult to predict *a priori* and experimental trial-and-error is thus necessary to validate all fusions, short peptides are generally more amenable to direct fusion approaches.

A few tricks have been developed to exceed the constraint on fusion size and accommodate relatively large peptides and proteins. For example, deleting four to six amino acids from TMV's C-terminus allowed an otherwise impossible 25 amino acid peptide fusion for immunization in swine and guinea pigs.[23] Reducing modification density can also mitigate steric hindrance to accommodate relatively large POIs (Figure 2c). One strategy to tune modification density leverages ribosomal skipping during expression of fusion proteins.[24] The 2A sequence of the foot and mouth disease virus acts similarly to a leaky stop codon, leading to expression of both separate and fused proteins whose ratio can be tuned by the 2A sequence. This system was used to construct VLPs with the 13 kDa, improved light, oxygen, or voltage sensing domain of *Arabidopsis thaliana* phototropin 2 (iLOV2) on their surface.[25] This photoreversible fluorescent protein was compatible with expression, VLP assembly, and replication in plant hosts.

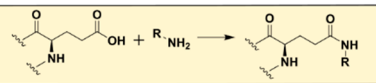
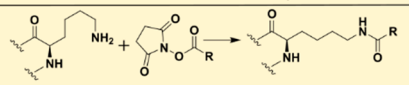
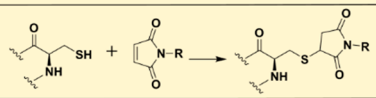
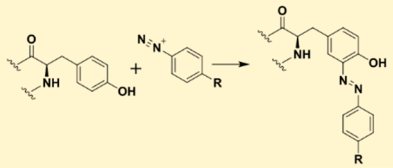
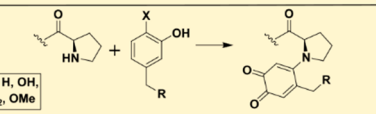
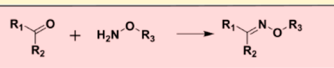
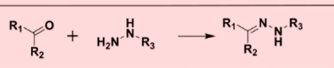
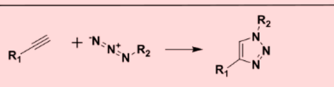
DIRECT CHEMICAL CONJUGATION

Chemical modification of rod viral particles is a very popular functionalization strategy with complementary advantages and disadvantages to direct protein fusion. This approach combines evolved biological functions with the vast structural diversity of synthetic chemistry, leading to enhanced or entirely new structures and functions. Chemical conjugation is therefore highly modular relative to genetic fusion, which is limited to amino acid chemistry. Chemical methods can still be used for functionalization with peptides and proteins (Figure 2a), but they accommodate much larger structures relative to genetic fusion.[26] In direct chemical conjugation, functional groups on the ligand of interest are reacted with natural, surface-exposed amino acid residues on the viral particle. By performing these reactions with previously assembled particles, the self-assembly process is decoupled from functionalization and ligands cannot interfere with particle assembly. Although chemical functionalization is thus highly modular relative to fusion, complete labeling is not guaranteed. Steric hindrance of functional ligands on neighboring subunits (∼3 nm apart) can slow down or even prevent reactions from taking place at every exposed reaction site.[27,28] Furthermore, not all chemical reactions are equally efficient. Most standard residues and their associated chemistry suffer from poor kinetics and can require a large excess of reagent to achieve high labeling densities.[29] This often makes downstream purification necessary, adding more production steps relative to direct genetic fusion. Click chemistries are very useful here due to their rapid kinetics and near quantitative conversion.[30] While many click chemistries, such as those involving thiols, are considered to be bioorthogonal, the common presence of these and other naturally occurring residues in biological structures can lead to undesired side reactions. Another major limitation of direct chemical conjugation is thus its lack of site-selectivity. Multiple reactive sites may be present on the viral particle surface and ligands of interest, which means not all ligands will be orientated in the same way. Suboptimal reaction sites are especially problematic when working with protein or peptide ligands, whose orientation may dictate functional performance. If chemically reactive impurities, which often derive from protein extractions, are present in the reaction mixture, they may also conjugate to the particles to occupy reactive sites and have a confounding effect on physicochemical properties. Despite these concerns, direct chemical conjugation is an important and widespread tool for rod-shaped VLP decoration. Many successful reactions have been demonstrated between diverse functional ligands and various residue chemistries (Table 1).

The acidic residues aspartate and glutamate are commonly found on the interior and/or exterior surfaces of rod viruses and VLPs and can be functionalized via carbodiimide-mediated amide coupling. This chemistry is frequently used for labeling VLPs via covalent modification with small molecule amines, including fluorescent reporters such as rhodamine B. Fluorescent labeling enables particle tracking in cells and animals, and has been recently used to probe the influence of particle aspect ratio on intracellular trafficking in mammalian

https://doi.org/10.1021/acsabm.1c01204
ACS Appl. Bio Mater. XXXX, XXX, XXX−XXX

Table 1. Established Chemical Coupling Strategies for Rod Virus Functionalization via Direct (Yellow) and Indirect, Bioorthogonal (Red) Chemistry[a]

Reaction / Residue	Reaction Scheme	Ref.
amide coupling / glutamic acid		31
amide coupling / lysine		5, 29
thiol-Michael addition / cysteine		29, 37
diazonium salt coupling / tyrosine		46, 44
oxidative coupling / proline		37, 40
oxime formation		44, 45
hydrazone formation		49
azide alkyne cycloaddition		34, 46

[a]These methods can couple functional groups to the indicated amino acid residues in any surface-exposed position for all but proline. Proline chemistries are specific to N-terminal residues.

cells.[31] Aspartate and glutamate can also react with hydroxyl groups on alcohols and other biomolecules including the amino acids serine and threonine, but these typically exhibit poor kinetics relative to stronger nucleophiles. Furthermore, the resulting ester is much less thermodynamically stable than amides, and products are susceptible to subsequent hydrolysis.[32] However, the amide coupling reaction displays poor kinetics without carbodiimide coupling reagents and benzotriazole catalysts, which are often explosive and dangerous to work with.[33] Acidic residues can also coordinate metals and have been used to encapsulate platinum-containing anticancer drugs such as cisplatin and phenanthriplatin in TMV.[34,35]

Amine and thiols, present in lysine and cysteine residues respectively, are very useful reactive groups that are sometimes naturally present on VLPs. These nucleophiles are so competent that VLP mutants presenting either residue on their outer surface are routinely used.[36] Both groups can react with reactive electrophiles including acids, acrylates, maleimides, and *N*-hydroxy succinimide (NHS) esters.[29] For example, a TMV-cysteine mutant was recently conjugated to the anticancer, topoisomerase II inhibitor doxorubicin with a maleimide group and an acid-cleavable linker, which facilitated drug release in cells.[37] Surface-exposed amines are most frequently used in amide coupling with acids, with the conjugation of viral surface-exposed acidic residues to four proteins: outer membrane protein A, the bacterial chaperone DnaK, dihydrolipoamide succinyl transferase, and the major membrane protein Tul4 as one example.[26] The resultant VLP-scaffolded tetra-antigen constructs induced long-term immunity against respiratory tularemia in mice.[38] Lysines are also very commonly reacted with NHS esters, and NHS-terminal PEG chains of varying length and degree of branching were recently conjugated to surface-exposed lysines on potato virus X (PVX) to investigate the influence of surface size and architecture on biodistribution and immune activation.[5] When both lysine and cysteine are present at the surface and selectivity is needed, amine reactivity can be suppressed via protonation at pH < 7.[29] Thiols can also selectively undergo radical-mediated click chemistry with alkenes, but this is yet to be tested for rod VLP decoration.[39]

Proline and tyrosine are less commonly used but very effective target sites for chemical reactions. N-terminal prolines containing secondary amines can undergo oxidative coupling with various phenols. While powerfully site-selective, this scheme is limited to N-terminal, surface-exposed proline residues which can be introduced via synthetic biology approaches if desired. Oxidative coupling of TMV prolines was recently applied for functionalization with a 5 kDa polyethylene glycol (PEG) polymer for improved particle stability and *in vivo* stealth.[37,40] Any surface-exposed tyrosine phenol can couple with diazonium salts at pH ∼ 9 via electrophilic aromatic substitution (examples provided in later section), representing higher flexibility relative to proline-based conjugation. Tyrosine residues can also chelate metal ions, and contributed to the loading of ∼3500 paramagnetic gadolinium ions per particle for high-relaxivity magnetic resonance imaging.[41]

INDIRECT CHEMICAL APPROACHES

The direct chemical conjugation approaches described above are significantly limited by the availability of surface-exposed, reactive residues. While genetic insertion of desired amino acids at the surface can often overcome this issue, as demonstrated by established lysine and cysteine mutants for TMV, this may not be feasible for all viral particles.[36] Highly reactive functional groups can instead be introduced onto existing residues chemically, without genetic engineering, to enable subsequent click reactions with various chemicals of interest. For example, surface exposed lysine amines can be first reacted with a maleimide-functional protected thiol. Subsequent deprotection by hydroxylamine produced new thiol groups on the rod VLP surface, which were clicked with the anticancer drug vcMMAE.[42] Indirect chemical approaches have also been used to enhance delivery specificity of VLPs to microenvironments such as tumors. For example, a TMV lysine mutant was decorated with cancer-associated nucleolin-targeting F3 peptide via a two-step strategy.[43] The surface amines were first reacted with homobifunctional maleimides followed by reaction with cysteine containing F3. This approach is simpler than the protected thiol-based example above because it eliminates the need for a deprotection step. However, using such bifunctional linkers may risk self-conjugation of cysteine-containing viral proteins within or between particles. The latter issue can result in particle aggregation, which is consistent with the TEM images shown for these products. While thiol-based click chemistry holds undeniable value for efficient surface decoration, the widespread presence of thiols in peptides and proteins can thus compromise its selectivity, simplicity, and/or stability.

https://doi.org/10.1021/acsabm.1c01204
ACS Appl. Bio Mater. XXXX, XXX, XXX−XXX

Since all peptides and proteins are constructed from a relatively small pool of amino acid ingredients, there is high potential for redundancy leading to cross-reactivity and aggregation. For example, the amine and thiol groups of lysine and cysteine can both react with acid groups of aspartate and glutamate in the presence of carbodiimide catalysts, leading to undesired linkages. Amine, acid, thiol, and alcohol groups are also ubiquitous in many nonpeptidic structures of biomedical interest, and undesirable reactions at these groups may occur during particle functionalization in these cases. The incorporation of non-natural chemistry into existing VLP platforms is a promising strategy to allow selective conjugations when needed. Bioorthogonal click chemistries, which do not involve naturally occurring chemical moieties, are a very attractive solution to this concern due to their speed, yield, and selectivity in biological environments.[30] Through established, chemical manipulations, naturally occurring residues can be converted to the highly reactive, bioorthogonally clickable functional groups desired (Table 1). The result is a modular viral particle that can efficiently and selectively decorated with diverse structures via bioorthogonal click chemistry.[30]

Tyrosine phenols present on the surface of TMV have been recently coupled to diazonium salts to form ketones.[44] This was followed by oxime formation for coupling with PEG and PEG-polyethylene block copolymers.[44,45] In a similar approach, tyrosine phenols were converted to alkynes to allow the highly efficient, copper-catalyzed alkyne−azide cycloaddition (CuAAC).[46] The resulting particles underwent CuAAC in the presence of bis-azidomethylbenzene to produce azide-functional rod VLPs.[34] The subsequent products were readily clicked with complementarily reactive tumor-associated carbohydrate antigens. This roundabout, three-step procedure was necessary to attain azide functionality because reactions between tyrosine and the less reactive azido-benzenediazonium were unsuccessful.

Various chemical reactions were used to convert standard residues into bioorthognally clickable groups, each with distinct challenges. The general strategy involves standard coupling of a surface-exposed residue to a heterobifunctional structure which contains an extra functional group for subsequent click reactions. Azides and alkynes are the most common examples of these groups, as they can undergo a highly efficient, copper-catalyzed cycloaddition. The conversion of cysteine thiols to alkynes via Michael addition with maleimide-alkynes was recently used for functionalization of TMV with tumor-associated carbohydrate antigens.[47] This route offered unusually high speed and conversion, but required an extra preparation step since cysteine is not naturally present on the particle surface and must be chemically or genetically inserted. Functional groups that are naturally found on the surface of popular viral templates include amines, which are present on PVX, and the acids and phenols on the surface of BSMV and TMV. Acid groups can be converted via carbodiimide-based amide coupling, as was recently demonstrated with propargyl amine. However, this involved a huge, 120-fold excess of reagent due to poor reaction efficiency. Amine groups were similarly converted to alkynes and azides via reactions with the corresponding NHS-esters.[48,49] This reaction also requires excess reagent, however, and suffers from the poor stability of NHS-esters, which are spontaneously hydrolyzed in aqueous media. One advantage is that many heterobifunctional NHS-esters are now commercially available, including benzaldehydes that were used to adapt lysine amines on PVX for the highly efficient hydrazone ligation reaction.[49] This advantage does not hold for the coupling of tyrosine phenols to heterobifunctional diazonium salts, which are dangerous reagents that typically need to be synthesized *in situ*. Nonetheless, this coupling was recently used to convert tyrosine phenols into alkynes and ketones, the latter of which allowed subsequent functionalization via oxime formation.[44−46] This strategy did not extend to the introduction of azides, however, due to the low reactivity of azide-functional diazonium salt.[34] The efficiency, modularity, and selectivity of bioorthogonal click-chemistry represents a major advancement in viral particle functionalization, but incorporating the unnatural chemical structures required for this technology can be challenging for some particles and functional groups of interest. Furthermore, this approach is not amenable to functionalization with all ligands; for example, the incorporation of complementary, unnatural chemistries into protein-based ligands can be challenging and prohibitively expensive at commercial scales.

INDIRECT BIOCONJUGATION

Growing interest in particle functionalization with proteins led to the development of advanced bioconjugation methods that overcome the size limitation of direct fusion while retaining selectivity and scalability. One innovative approach to accomplish this is to decouple the self-assembly process and surface modification, similar to chemical coupling-based approaches. This entails a two-step preparation in which functional peptides or proteins are added postassembly so that they cannot block any native coat protein contacts necessary for VLP assembly. Multiple reversible and irreversible biomolecule-mediated linkages are known to enable such a scheme. Although this strategy adds additional steps, it promises to greatly improve on the modularity of direct fusion approaches without compromising selectivity or scalability.

Several two-step, site-selective bioconjugation strategies that were recently implemented for rod-shaped VLP decoration employ reversible protein interactions. (Figure 2e). The first such approach took advantage of the exceptionally strong interaction between streptavidin and biotin. Streptavidin is a 66 kDa protein whose tetrameric form binds tightly to the small vitamin biotin.[50] Biotin with a short hydrophilic poly(ethylene oxide) linker and NHS reactive group was successfully bonded to mutant TMV with surface exposed lysine residues (Table 1). The resulting, fully biotinylated VLP was readily decorated with various, streptavidin-fused proteins of interest (Figure 2e).[51] These fusions include green fluorescent protein (GFP) and the canine oral papillomavirus L2 protein, both of which elicited antigen-specific humoral immune responses in mice and guinea pigs when delivered as VLP conjugates.[52] While biotin−streptavidin binding is an important capability for modular VLP functionalization, it suffers from several challenges. First, the protein binding event is reversible, as no chemical bonds are formed in the process. While this may be favorable in some cases such as on-site cargo release in proteolytic conditions, it can also hinder stability and characterization methods involving protein denaturation. Second, streptavidin is nearly three times larger than most rod-shaped VLP subunits, and its fusion to some proteins of interest may disrupt expression, structure, and activity. Third, streptavidin classically functions as a tetramer with multiple binding pockets for biotin. This can lead to imprecise conjugation with risk of VLP cross-linking and aggregation.

https://doi.org/10.1021/acsabm.1c01204
ACS Appl. Bio Mater. XXXX, XXX, XXX−XXX

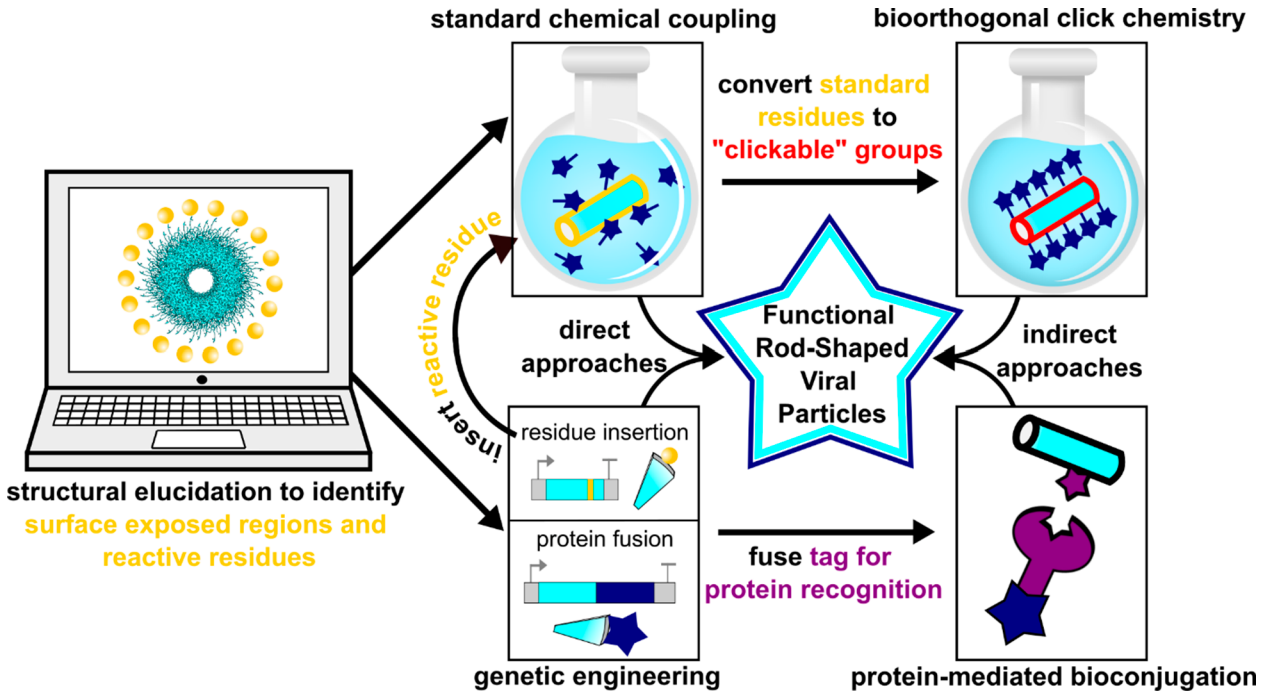


Figure 3. Engineering workflow for the chemical and biological functionalization of rod-shaped viral particles via direct and indirect approaches. The depicted protein structure was adapted from ref 74.

Another highly versatile two-step conjugation method was recently established by expressing a short LPXTG peptide at the surface of the papaya mosaic virus, enabling recognition by the bacterial transpeptidase, sortase A.[53] This platform allowed efficient coupling to long, 26−39 amino acid peptides for flu vaccine development. Nonetheless, sortase-mediated coupling is also reversible and suffers from low yields and wasted reagents.[54] While such reversible interactions are useful for modular functionalization with proteins, they thus compromise conjugation stability and/or efficiency.

Irreversible conjugation is typically superior to reversible strategies, such as those based on biotin−streptavidin binding or sortase activity, due to improved efficiency and stability. SpyCatcher/Tag technology has received enormous interest in recent years as a site-selective, *irreversible* bioconjugation approach with comparable orthogonality and efficiency to "click" chemistry.[55] SpyCatcher is a 116 amino acid protein that spontaneously forms a covalent isopeptide bond with the 13 amino acid SpyTag peptide.[56] Recent optimization yielded fully functional variants as small as 9 kDa.[57] SpyTagging rod VLPs at their C-termini allows robust, site-selective functionalization with a variety of SpyCatcher fusions (Figure 2d). This approach was recently applied to TMV and the PVX for conjugation with a variety of catalytic enzymes.[58,59] A similar scheme was used for coupling proteins to SpyCatcher-conjugated gold, upconverting nanoparticles, and quantum dots.[60] The SpyTagged VLP platform thus shows great promise, although its medical applications have not yet been investigated. While irreversibility is a key advantage of this approach, other reversible functionalization methods offer unique advantages.

STIMULI-RESPONSIVE SURFACE FUNCTIONALIZATION

When reversible interactions can be controlled, they become a very powerful tool for environmentally-responsive particle functionalization. Numerous noncovalent linkages can reversibly form and disassemble in response to various stimuli, including light, heat, solvents, mechanical force, pH, or chemicals, endowing bulk materials with advanced, shear-thinning and tough mechanical properties.[61,62] Even at the nanoscale, this stimuli-responsive behavior can be very useful for applications such as controlled drug delivery.[62] One of the most widely studied classes of supramolecular structures are the cyclodextrins (CDs). CDs are carbohydrate rings of glucose monomers with excellent biocompatibility, aqueous solubility, and ease of preparation that can reversibly bind multiple hydrophobic small molecules such as adamantanes, azobenzenes, and ferrocenes.[63] For example, CD enabled VLP decoration with adamantane-linked folic acid, rhodamine, doxorubicin, and PEG.[64] The folic acid improved cellular uptake via folate receptor-mediated endocytosis for enhanced cancer cell killing *in vitro* by doxorubicin. Azobenzene is particularly interesting due to its reversible, light-responsive conformational change, which can allow spatiotemporal control over material structure.[65] Cyclodextrin-based viral particle functionalization thus holds tremendous promise for the design of highly responsive materials.

Nucleic acid hybridization is another type of selective, reversible, and stimuli-responsive interaction with unique advantages that can be leveraged for functionalization. This involves the sequence-specific binding of RNA or DNA to the single-stranded RNA template of rod-shaped plant viral particles and is thus highly selective. This approach was recently used to link TMV rods with hydrogel microparticles and chitosan supports in a controlled, longitudinal orientation.[66−68] Since nucleic acid hybridization does not involve coat proteins, it creates a unique opportunity for orthogonal functionalization at the protein surface with various ligands such as fluorescent dyes.[66] Furthermore, hybridizing DNAs are attached with precise, one-to-one stoichiometry and can offer their own functional properties. With the advent of DNA

https://doi.org/10.1021/acsabm.1c01204
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origami, nucleic acids are no longer seen as passive templates but instead appreciated as active nanostructures with responsive, tunable, and predictable hierarchical structure and function.[69] These advanced materials have already shown tremendous promise in medicine, for example as targeting agents. By hybridizing viral RNA templates to DNA origami, TMV disks were recently encapsulated in reversibly foldable DNA cages.[70] The cages switched between open and closed configurations corresponding to unfolded and folded hairpins, respectively, by the addition of complementary strands. Through a similar approach, partially disassembled rod VLPs were hybridized to tubular and triangular DNA nanostructures whose assembly was readily controlled through toehold-displacement.[71,72]

FUTURE DIRECTION AND CONCLUSION

Various conjugation strategies have been implemented for rod-shaped VLP functionalization with diverse structures ranging from small molecules to (bio)polymers. Synthetic and biological ligands can be introduced via chemical procedures, but the latter allow alternative, bioconjugation strategies. For example, rod-shaped plant viral particles can be functionalized with nucleic acids via hybridization to their encapsidated RNA templates.[73] Peptides and proteins can also be directly fused to viral coat proteins through genetic engineering, although this process is sometimes tedious and unsuccessful for large fusions.[23] However, viral particles are readily decorated with short peptide or chemical ligands that can bind or react with receptor proteins for facile, site-selective functionalization.[51,52,59] A similar two-step strategy exists for chemical coupling, in which standard residues on the viral particle surface are converted to unnatural structures with enhanced reactivity.[46] These "clickable" functional groups can be coupled to diverse modifications of interest via bioorthogonal click chemistry.[30] The choice between direct and indirect chemical coupling and bioconjugation ultimately depends on viral particle structure, available chemistries, and the degree of modularity and selectivity required for a given study or application (Figure 3).

Bioorthogonal click chemistry and receptor protein-mediated coupling (i.e., SpyCatcher/SpyTag and biotin/streptavidin) are powerful techniques that exhibit speed, efficiency, yield, modularity, and selectivity. However, important challenges and opportunities remain. Thus far, click reactions, omitting the relatively promiscuous thiol-based chemistries, are only possible by converting standard amino acid residues to clickable moieties, followed by purification and subsequent functionalization. The analogous, two-step bioconjugations using streptavidin or SpyCatcher require fusing these proteins to functional structures, which may alter expression, folded structure, and function. Future efforts should focus on streamlining these two-step chemical and biological conjugations. For example, the relatively small SpyCatcher protein should be fused directly to rod VLP coat proteins, as was accomplished with other VLPs.[10] Developing a SpyCatcher-fused, rod-shaped VLP will facilitate facile functionalization with SpyTagged peptides and proteins of interest, which can be more easily expressed or synthesized than their SpyCatcher analogs. Such unprecedented level of modularity would greatly accelerate the conjugation of peptides and proteins to rod VLPs for many applications.

Similar opportunities exist for click chemistry due to significant recent progress in genetic code expansion. Future efforts should aim to directly incorporate nonstandard amino acids (NSAAs), including click-reactive groups, into rod VLPs by heterologous expression in yeast or bacteria. These advances would greatly benefit the field of rod VLP functionalization for medical and various other applications. Their high efficiency and near-quantitative yields may support decoration with multiple groups in stoichiometric amounts for improved, multifunctional constructs. For example, targeting antibodies, cell-penetrating peptides, stealth polymers, small molecule drugs, and fluorescent, radioactive, or magnetically active reporters may all be integrated into a single, advanced delivery system. Such a combination is likely to have clinical value but is rarely studied because its preparation is difficult or impossible for most particles. Further control in this regard might be achieved by the incorporation of multiple, orthogonally reactive NSAAs. Similarly, the orthogonally selective Spy and Snoop Catcher/Tag pairs may be applied here for increased modularity.[59] These dually orthogonal strategies would be particularly useful for the spatial patterning of functional moieties along a VLP, as described below.

The self-assembly mechanism of rod-shaped plant viruses offers a unique, elegant opportunity to control the spatial organization of functional ligands on their surface. Ligand proximity and patterning can greatly enhance interactions with biomolecules and cells but are not readily achieved by other particle platforms.[75] Studies on TMV suggest that viral growth begins at a short, origin of assembly sequence (OAS) and proceeds to the 5′ template end through the stacking of multimeric disk intermediates.[76,77] Such directional self-assembly creates a unique opportunity for nanoscale surface patterning that is otherwise very difficult to achieve with icosahedral viruses and nonviral nanoparticles. Janus-type particles have already been prepared, with mutant, functionalized coat proteins clustered onto one side of the TMV VLP.[36] However, only low-throughput, TEM characterization was used to validate this nanoscale spatial control. Higher-throughput methods, based on Förster resonance energy transfer for example, are needed for further validation. Furthermore, the disk-by-disk assembly mechanism is speculated to allow the clustering of functional groups into striped domains along the VLP (Figure 4).[78] This would represent an

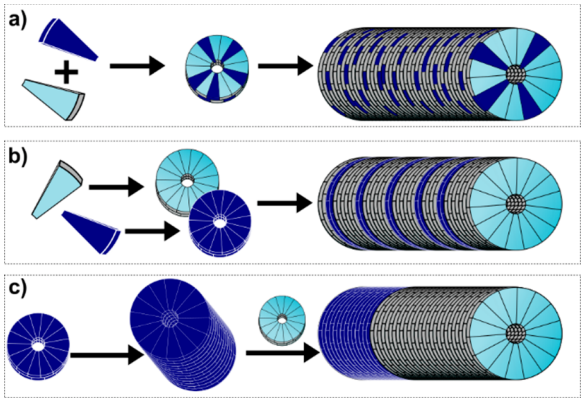

Figure 4. Self-assembly routes for spatial patterning of functional ligands (dark blue) on the viral particle surface: (a) mixing coat proteins for random ligand display, (b) mixing preassembled disk intermediates for striped patterning, and (c) sequential assembly for block patterning (Adapted with permission from ref 78. Copyright 2018 Springer Nature).

https://doi.org/10.1021/acsabm.1c01204
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incredible achievement, as such precise surface patterning is impossible with most established particle platforms. However, this has not yet been experimentally demonstrated. It is important to note that the spatial control here is highly sensitive to assembly mechanism and kinetics and transport limitations may interfere and increase dispersity. For example, poor mixing might result in imbalanced VLP growth, where some VLPs are completely formed while others are truncated. However, hybridizing DNA oligomers have been shown to reversibly and responsively stop VLP assembly at selected regions along the RNA template, which can overcome these kinetic constraints.[73] Future work should aim to validate and tightly control spatial patterning of VLPs. Once this technology is established it will help elucidate new relationships between particle surface organization and functionality.

Rod-shaped plant VLPs have many inherent advantages and demonstrated potential for medical applications including imaging, drug delivery, and vaccination. Future efforts to streamline and accelerate VLP functionalization and establish nanoscale organization of functional groups will augment the potential clinical applicability of this powerful particle platform.

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A.J.V. and K.V.S. wrote and edited the manuscript.

Funding

This publication was made possible by the Delaware INBRE program, supported by a grant from the National Institute of General Medical Sciences (NIGMS, P20 GM103446) from the National Institutes of Health and the State of Delaware and a grant from the National Science Foundation, no. 2028618.

Notes

The authors declare no competing financial interest.

ABBREVIATIONS

CD = cyclodextrin
cryo-EM = cryogenic electronic microscopy
CuAAC = copper-catalyzed alkyne−azide cycloaddition
DUPA = (((*S*)-5-amino-1-carboxypentyl)carbamoyl)-L-glutamic acid
GFP = green fluorescent protein
HIV-1 = human immunodeficiency virus 1
iLOV2 = improved light, oxygen or voltage sensing domain of *Arabidopsis thaliana* phototropin 2
LPXTG = leucine-proline-variable residue-threonine-glycine
MRI = magnetic resonance imaging
NHS = *N*-hydroxy succinimide
NSAA = nonstandard amino acid
OAS = origin of assembly sequence
PEG = poly(ethylene glycol)
POI = peptide/protein of interest
PVX = potato virus X
RGD = arginyl-glycyl-aspartic acid
TEM = transmission electron microscopy
TMV = tobacco mosaic virus
vcMMAE = maleimidocaproyl-valine-citrulline-*p*-aminobenzyloxycarbonyl monomethylauristatin E
VLP = virus-like particle

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https://doi.org/10.1021/acsabm.1c01204
ACS Appl. Bio Mater. XXXX, XXX, XXX−XXX