



Rip it, stitch it, click it: A Chemist's guide to VLP manipulation

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ARTICLE INFO

Keywords:

Virus
Virus-like particles
Self-assembly
Disassembly
Non-infectious
Drug delivery

ABSTRACT

Viruses are some of nature's most ubiquitous self-assembled molecular containers. Evolutionary pressures have created some incredibly robust, thermally, and enzymatically resistant carriers to transport delicate genetic information safely. Virus-like particles (VLPs) are human-engineered non-infectious systems that inherit the parent virus' ability to self-assemble under controlled conditions while being non-infectious. VLPs and plant-based viral nanoparticles are becoming increasingly popular in medicine as their self-assembly properties are exploitable for applications ranging from diagnostic tools to targeted drug delivery. Understanding the basic structure and principles underlying the assembly of higher-order structures has allowed researchers to disassemble (rip it), reassemble (stitch it), and functionalize (click it) these systems on demand. This review focuses on the current toolbox of strategies developed to manipulate these systems by ripping, stitching, and clicking to create new technologies in the biomedical space.

1. Introduction

Supramolecular self-assembly is a common approach for formulating nanomaterials using (Yadav et al., 2020) diverse molecular building blocks such as lipids, (Felice et al., 2014; Lee, 2008; Xing and Zhao, 2016) metals, (Katayama et al., 2019) proteins, (McManus et al., 2016; Sun et al., 2020; Wang et al., 2020) and polymers. (George et al., 2019; Nitta and Numata, 2013; Sofi et al., 2019; Wang et al., 2017a) It is possible to synthesize varied and functional nanostructures with these building blocks that bear unique physicochemical properties. The higher-order structure and emergent function obtained from these nano-assemblies have opened the door for addressing complicated problems in drug delivery, (Vilar et al., 2012; Wang et al., 2016, 2018) catalysis, (Brea et al., 2010) and targeting of biomaterials for therapeutic applications. (Cheng et al., 2020) Generally, self-assembly is a spontaneous process where thermodynamically favorable interactions occur amongst unorganized subunits ultimately giving rise to ordered structures. While synthetic systems—particularly in organic solvents—are enthalpically driven by forces like hydrogen bonding or π -stacking, biological systems typically are driven entropically, as self-assembly in aqueous systems often results in the release of ordered

water around hydrophobic residues. (Jiou et al., 2014; Mendes et al., 2013; Schmit et al., 2010) Nature has exploited these driving forces for billions of years to create living beings—beings that have begun to create new materials using inspiration from the elegance of biological self-assembly. (Pochan and Scherman, 2021) Development of the cell membrane through the interaction of phospholipid bilayers, (Ng et al., 2004) protein folding, (Levin et al., 2020) and DNA arrangement (Krissanaprasit et al., 2021) are just a few examples of how self-assembly governs the emergence and maintenance of life within biological systems. (Wang et al., 2017b) Further, self-assembly is pivotal in acquiring structured materials such as phase-separated polymers, liquid crystals, and crystalline polymers. (Yadav et al., 2020)

Self-assembled proteinaceous structures have been extensively investigated for the development of bio-functional materials. The rational design of protein-protein interactions is very complex and predicting how proteins assemble is still a developing field. As a result, proteinaceous nanomaterials are engineered mainly based on existing self-assembled systems like viruses, (Bai et al., 2008) chaperones, (Geitner and Schmid, 2012) amyloids, (Díaz-Caballero et al., 2021) elastin, (Le et al., 2013) resilin, (Renner et al., 2012) and silk-like proteins. (Kluge et al., 2008) By using naturally occurring self-assembling

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<https://doi.org/10.1016/j.virol.2022.10.008>

Received 19 July 2022; Received in revised form 9 October 2022; Accepted 19 October 2022

Available online 31 October 2022

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proteins as a starting point, it has become possible for proteinaceous materials to be used as scaffolds to construct various supramolecular interfaces. (Solomonov and Shimanovich, 2020) For instance, protein-based nanomaterials display structural features with great potential for the fabrication of drug delivery systems, (Zdanowicz and Chroboczek, 2016) imaging agents, (Schwarz and Douglas, 2015) targeting agents, (Suffian and Al-Jamal, 2022) and vaccines. (Roldão et al., 2010)

Viruses are some of nature's most resilient self-assembled nanostructures that have been exploited in their non-infectious forms for various applications. Recent work on controlling their assembly, disassembly, and chemical functionalization have created a new field—Chemical Virology—that seeks to understand the complexities of self-assembly with proteins and exploit these structures for application in medicine and materials. In this review, we focus specifically on virus-like particles (VLPs) and plant viruses, a family of proteinaceous materials whose cargo-loading ability and biocompatibility have piqued the interest of researchers involved in the development of therapeutics and prophylactics.

2. Virus-like particles

VLPs are proteinaceous materials derived from viruses that can self-assemble but lack the genetic material needed for their replication within the host cell. (Zhao et al., 2019) Genetic engineering and self-assembly are two of the most common methods employed to fabricate these virus-like particles from their parental virions. The viral genetic code is responsible for determining the protein composition of the viral capsid, and researchers have been using genetic engineering and bioinformatics to modify the protein capsids and create biodegradable and non-toxic nanomaterials. (Jeevanandam et al., 2019) Today, CRISPR/Cas9-based gene editing is one of the most widely used genetic engineering techniques to produce VLPs from viruses. (Jeevanandam et al., 2019; Teng et al., 2021) When genetic engineering cannot introduce the desired function, chemical bioconjugation is used to modify or target both natural and synthetic amino acid residues on the protein capsid of the VLPs. (Biabanikhankahdani et al., 2018) In this way, VLPs have emerged as a semi-synthetic biological material that has shown promise in areas as diverse as enzymatic reactors to imaging agents. (Herbert et al., 2020; Pokorski et al., 2011a,b; Waghvani et al., 2020) VLP expression and *in vitro* self-assembly can be achieved through various host systems such as bacterial, plant, yeast, mammalian, and insect cells. (Liu et al., 2016) The capsid self-assembly happens when VLP subunits and other viral components interact to minimize the free energy required to construct high-order structures. (Garmann et al., 2019) Capsid formation is a multistep process that begins with a nucleation phase, where a capsid oligomer is formed. (Le and Müller, 2021) This is followed by the growth phase, where VLP subunits interact. (Hagan, 2014; Perlmutter and Hagan, 2015) Finally, the capsid is created via the interaction of smaller building blocks resulting in a highly ordered structure. (Hagan and Elrad, 2010) Though favorable, this assembly process can be complicated and vary from VLP to VLP. (Chi et al., 2003)

VLPs have recently gained popularity in applications ranging from diagnostic tools in non-invasive imaging (Herbert et al., 2020) to drug delivery. (Lee et al., 2018) They are also popular vaccine candidates, as many of these proteinaceous materials are intrinsically immunogenic and can elicit potent humoral and cell-mediated immune responses. (Luzuriaga et al., 2021; Shahrivarkevishahi et al., 2022; Welch et al., 2018) Compared to other commonly used nanoparticles such as liposomes, lipid-based nanoparticles, and dendrimers, many VLPs allow orthogonal surface modifications to install more than one new chemical moiety. (Benjamin et al., 2020b; Shahrivarkevishahi et al., 2021) For instance, drug delivery components can be installed via a single set of reactions, and stealth PEG coatings that promote solubility can orthogonally be attached to the same VLP via a second reaction. VLPs are also

structurally rigid enough that their tertiary and quaternary structures can actively template the formation of new nanomaterials, such as gold nanoparticles and Metal-Organic Frameworks (MOFs). (Benjamin et al., 2018) Additionally, VLPs can be disassembled/reassembled *in vitro*, allowing foreign materials to be encapsulated within their capsid. (de Ruiter et al., 2019) The encapsulated material is protected from harsh conditions (e.g., nuclease degradation), and its cellular delivery is controlled through elegant surface modification of the capsid. (Rhee et al., 2011)

3. Structural diversity of VLPs

The self-assembly of VLPs is a diverse, synergetic, and multiprotein process. Though highly complex, much has been gleaned from biophysical studies, such as isothermal titration calorimetry (ITC), (Jeevaeva et al., 2010; Nebel et al., 1995) to monitor heat dissipation upon association of different VLP subunits. Studies performed by Maassen et al. quantitatively and qualitatively investigated the impact of associations between coat proteins (CPs) and cargo (e.g., single-stranded DNA) on the stability of VLPs. (Maassen et al., 2019) Their work shows that self-assembly results from protein-protein associations, which are primarily dependent on hydrophobic interactions and the electrostatic interactions between CPs and different cargo. When hydrophobic surfaces interact, they release large amounts of highly structured water. Consequently, even though the capsid becomes more ordered, the overall system has higher entropy. Interestingly, these weak hydrophobic interactions are non-directional—unlike non-covalent supramolecular bonds like hydrogen bonding—yet these forces drive the formation of VLPs with different geometries, including rod-like, icosahedral, or spherical. (Zeltins, 2013) This review will mainly focus on seven very simple viruses—illustrated in Fig. 1. These systems have been utilized broadly and are relatively simple—they lack a lipid membrane (*i.e.*, non-enveloped), and few proteins are involved in their formation. We will cover their laboratory purification and characterization techniques and post-synthetic modifications (e.g., self-assembly, disassembly, and bioconjugation).

4. Commonly reported virus-like particles

The architectural diversity of various VLPs provide researchers with different options for designing hybrid materials. (Chung et al., 2020) Here, we summarize commonly used VLPs that have been extensively exploited to develop bio-functional nanomaterials.

4.1. Bacteriophage Q β (Q β)

is a porous icosahedral VLP with a 28–30 nm diameter. (Benjamin et al., 2018) Q β is a member of the *Leviviridae* family, (Herbert et al., 2020) a group of phages that display a 4.2 kilobase single-stranded RNA (ssRNA) genome, (Cui et al., 2017) a capsid built from 180 copies of a 14 kDa CP, (Sungsuwan et al., 2017), (Lee et al., 2018) Bacteriophages belonging to the *Leviviridae* family are characterized by a capsid comprising a major CP and one or two species of minor structural proteins responsible for genome packing and recognition. These supporting structural proteins are essential for absorbing the virion to cell receptors and transporting the RNA genome into the gram-negative bacterial cells; however, they are not required for the assembly or structural integrity of the viral capsid. As a result, recombinant expression of a cloned version of the major CP results in the production of Q β VLPs. (Kozlovskaya et al., 1993) The resulting VLP only has 180 copies of a single CP, as opposed to the native (or wild-type) virus, which has additional proteins used to infect its host, *E. coli*. The positively charged inner surface of this CP plays a pivotal role in its self-assembly into the Q β capsid, as it binds bacterial RNA, driving the formation of intact capsids during recombinant expression in *E. coli*. (Herbert et al., 2020) Q β has been extensively investigated as a drug delivery vehicle, (Chen et al., 2017) cancer

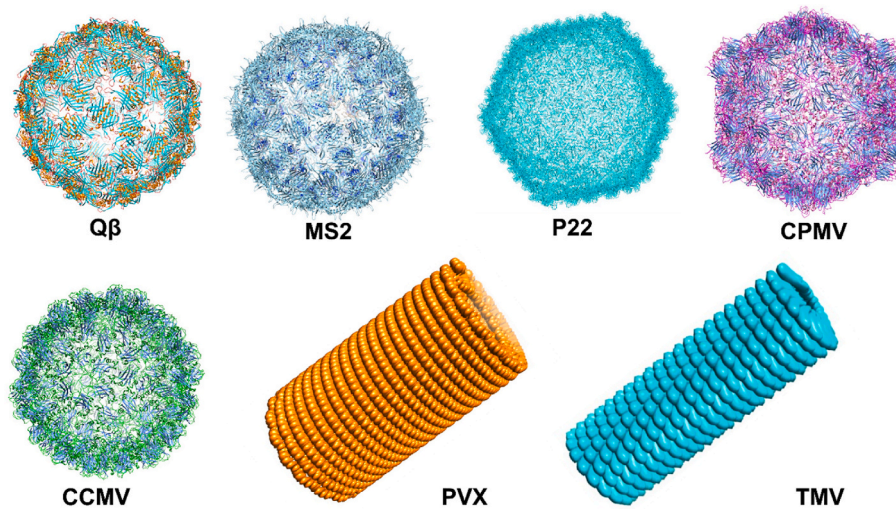


Fig. 1. Structural diversity of commonly reported icosahedral and rod-shaped virus-like particles and viruses.

therapeutic, (Shahrivarkevishahi et al., 2021) and has performed well in clinical trials when used with immunoadjuvants. (Lemke-Miltner et al., 2020) Q β displays three surface-exposed lysine residues and two surface-exposed cysteine residues on each CP, (Au - Chen et al., 2018; Chen et al., 2016; Shahrivarkevishahi et al., 2021) which have facilitated multiple orthogonal surface modifications.

4.2. P22

is a bacteriophage whose native host is *Salmonella typhimurium*. It was found that its assembly and maturation depend on two major components – a 47 kDa CP which is responsible for the formation of the capsid shell, and a 33.6 kDa scaffolding protein (SP) which acts as a template and direct CPs for proper assembly into a VLP when recombinantly expressed in *E. coli*. (Kutter, 2001) (Patterson, 2018) The P22 capsid was the first bacteriophage shown to perform generalized transduction – a process where the virus transfers its genetic material into a bacteria. This finding opened various research avenues for developing phage-mediated transfer of genetic material. (Casjens and Grose, 2016) In 1973, Lobban and Kaiser demonstrated the first use of bacteriophages as DNA cloning vectors using P22. (Petes et al., 1978) The structure of the P22 VLP is first built by the assembly of 250 SP units to form a procapsid, which is subsequently disassembled for the DNA to enter. These SPs are the precursors for the self-assembly of the 420 CPs, forming the VLP. It is worth noting that only the last few C-terminal residues of the SP are required for facilitating capsid formation. (Moore and Prevelige, 2002) As a result, many groups have taken advantage of this to fuse enzymes or proteins to P22 capsids. (Seul et al., 2014) These stylish genetic alterations have allowed P22 to be used in cargo loading (Sharma et al., 2017) and imaging applications. (Lucon et al., 2012).

4.3. Bacteriophage MS2

is another icosahedral RNA phage member of the *Leviviridae* family. (Salas and de Vega, 2008) MS2 is known to infect *E. coli*, (Salas and de Vega, 2008) and hence found its original use as an indicator virus at waste management plants for determining *E. coli* contamination levels. (Farkas et al., 2020) Self-assembly of MS2 affords icosahedral particles of 29 nm in diameter. (Biela et al., 2022) The genome of MS2 is composed of a positive-sense ssRNA with 3,569 nucleotides which encode four proteins: the major CP, the replicase, the lysis, and the maturation protein. (Fu and Li, 2016) Further, recombinant expression of cloned CPs will promote the self-assembly of MS2 VLP. (Mastico et al.,

1993) The resulting capsid is known for its outstanding stability, making it an excellent model for studying factors affecting capsid integrity (e.g., pH, temperature, and ionic strength). (Caldeira and Peabody, 2011; Furiga et al., 2011; Hashemi et al., 2021) This structural feature has been exploited for *in vitro* capsid disassembly/assembly, which has found use in drug delivery. (Ashley et al., 2011) MS2 is also suitable for genetic modification through the insertion of peptides into the N terminus of the CP. (Tumban et al., 2012) This strategy, in particular, has been exploited for multiple epitope presentations in the field of vaccine research. (Peabody et al., 2008)

4.4. Commonly reported plant viral nanoparticles

Like VLPs, plant viral nanoparticles (VNPs) have emerged as self-assembling nanostructures. (Martí et al., 2022) VNPs are just as structurally diverse as engineered and non-infectious VLPs; some display structural similarities (e.g., size and shape) to icosahedral bacteriophages, whereas others are helical-shaped. (Chung et al., 2020) VNPs are widespread in crops across the globe, including fruits and vegetables, and can seriously threaten agricultural production. (Rubio et al., 2020) However, these are only infectious to plants, making their application in mammalian-based medicine possible. (Steinmetz, 2010) It is worth pointing out that literature often uses the two terms interchangeably, though the distinction that VNPs are infectious is essential.

4.5. Cowpea mosaic virus (CPMV)

is a picorna-like virus of the order *Picornavirales*, family *Secoviridae*, and genus *Comoviridae* that naturally infects the black-eyed pea plant *Vigna unguiculata*. (Hesketh et al., 2017) CPMV is a 30 nm isometric, icosahedral lattice capsid with a net negative surface charge. (Patel et al., 2020) The capsid shape provides a large surface area-to-volume ratio, which is advantageous for enhanced multifunctional group display and cargo-loading capacity. CPMV has a bipartite, positive-sense RNA genome comprised of RNA-1 (5.89 kbp) and RNA-2 (3.48 kbp) encapsulated separately. (Rae et al., 2008) The native tropism of CPMV for cell surface displayed vimentin—a type III filament upregulated in cancer progression—and the enhanced permeability and retention effect allows them to extravasate from the tumor neovasculature and efficiently penetrate them preferentially. (Beatty and Lewis, 2019)

4.6. Cowpea chlorotic mottle virus (CCMV)

is an icosahedral, non-enveloped member of the Bromoviral family of plant viruses that infects cowpea plants. (Wilts et al., 2015) The CCMV capsid is 28 nm in diameter, icosahedral in shape, and self-assembles from the interaction of 180 CPs. (Konecny et al., 2006) Capsid particles are stabilized through RNA-protein interactions between the anionic RNA and the inner positive charge of the capsid proteins. (Konecny et al., 2006) CCMV is notorious for its structural dynamism – a structural feature that makes it a very attractive VNP for post-expression modifications. (Liepold et al., 2005) For example, the capsid can undergo pH-dependent swelling, allowing for entrapment of foreign material within CCMV without the need for *in vitro* disassembly/reassembly. (Wilts et al., 2015) The capsid also displays several functional handles used for conjugation approaches. (Vervoort et al., 2021)

4.7. Tobacco mosaic virus (TMV)

Was the first virus to be discovered and was instrumental in shaping virology as a field. (Van Regenmortel, 2008) It has a helical rod shape of 300 nm in length and 18 nm in diameter. (Hema et al., 2019) Despite its stability, the virus has been known to mutate in the laboratory environment, though mutations are easily detected via electrospray ionization mass spectrometry (ESI-MS). (Lumata et al., 2021) The three-dimensional structure of TMV has been extensively characterized using X-ray crystallography and cryo-electron microscopy (Cryo-EM). (Muller, 1999) The outer surface of TMV is made of 2,130 CPs, each having 158 amino acids. (Hema et al., 2019) The inner part has a diameter of 4 nm, and a viral ssRNA made of 6,400 nucleotides is located at the center of the helix, giving TMV its ability to replicate in host cells. (Lomonosoff and Wege, 2018) Further, the biodegradability and biocompatibility of TMV plus its structural features have been widely exploited for MRI imaging applications and other modalities. (Bruckman et al., 2013; Niehl et al., 2016; Wang et al., 2019)

4.8. Potato virus X (PVX)

Belongs to the family *Alphaflexiviridae* and is a member of the genus *Potexvirus*. It is a flexuous filamentous rod having a length of 515 nm, a diameter of 13 nm, and monopartite positive-sense ssRNA. It has 1,270 identical CPs with a molecular weight of 25 kDa arranged around the 6.4 kbp ssRNA. (Lico et al., 2015) The C-terminus of each CP subunit is located within the particle. In contrast, the N-terminus is exposed and can be easily modified at the genetic level to display amino acids or small peptides. It displays a helical array (3.6 nm pitch) with the viral RNA packed between the helix turns. There are 8.9 CP subunits (each consisting of 236 amino acid residues) per turn of the primary helix.

5. Commonly used expression systems to produce icosahedral VLPs and rod-like viruses

Expression systems of eukaryotic and prokaryotic origin are commonly employed to produce VLPs. (Arevalo et al., 2016) The two standard eukaryotic systems used to produce VLPs are plant and mammalian cell cultures. The most used prokaryotic system is bacteria, specifically *E. coli*. VLPs are generally resistant to proteases and are thermally robust. Hence, they can be prepared and purified at room temperature in most cases with minimal or no chromatography. Depending on the type of VLP or VNP used, different purification and characterization techniques are employed following the expression of these materials (see Fig. 2 and Fig. 3). While some of these techniques overlap between systems, others differ significantly. In this section we discuss some of the commonly used expression systems and have gone in detail with the most popular approach for each VLP/VNP.

5.1. Mammalian cell culture

Though this review does not cover VLPs or VNPs typically produced in mammalian cell culture, it is an essential method for making many types of VLPs/VNPs used in vaccine and gene transfection. These platforms are attractive because they can accommodate post-translational modifications needed within the cell (e.g., methylation, acetylation, hydroxylation, and phosphorylation) for proper capsid formation.

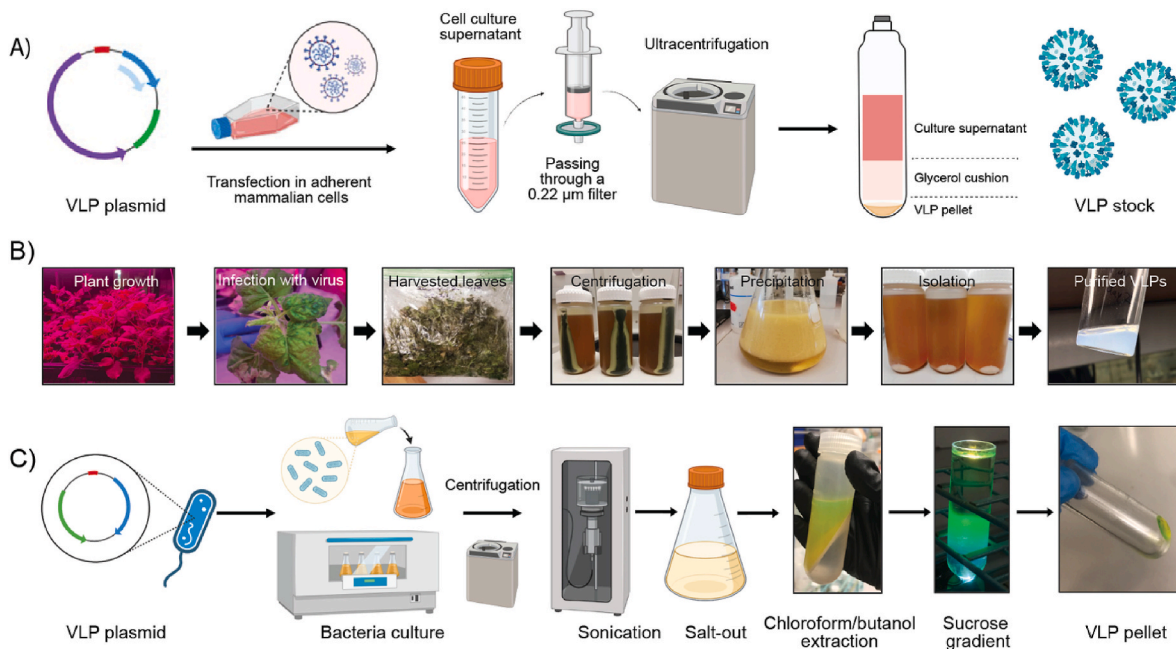


Fig. 2. Common platforms for the expression of VLPs and viruses. A) Mammalian VLP and viral expression. B) Plant-based expression of viruses (e.g., TMV). C) Bacterial culture expression using BL21 (DE3) cells.

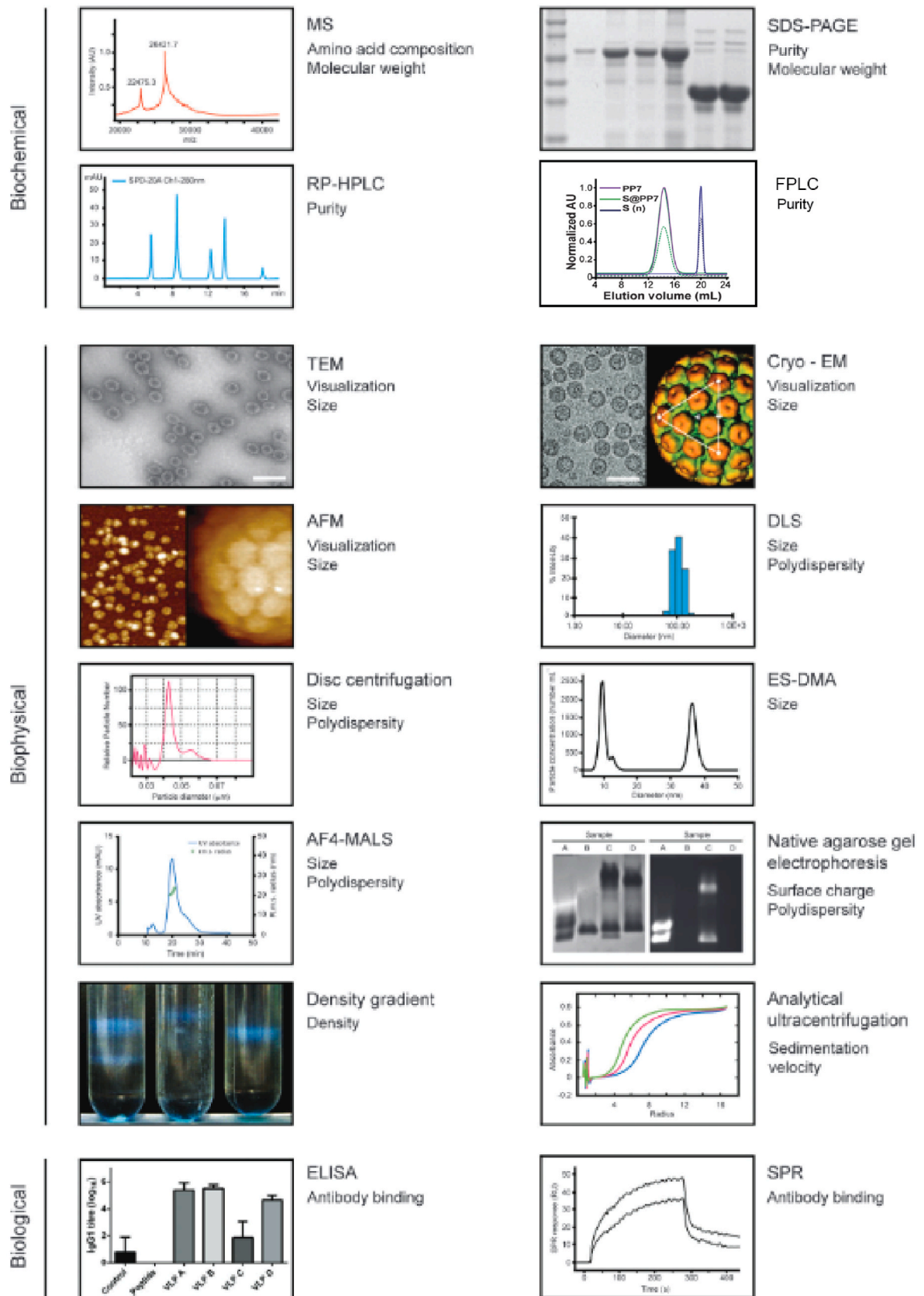


Fig. 3. Post-expression characterization techniques for VLPs and viruses. (Lua et al., 2014). Copyright from © John Wiley and Sons and modified with (Herbert et al., 2020).

Additionally, this method of expression is the most suitable for creating complex enveloped VLPs comprising of multiple structural proteins. (Fuenmayor et al., 2017) However, this expression system is notorious for lower VLP yields compared to other platforms such as *E. coli*. (Fuenmayor et al., 2017) Some popular cell lines used for VLP expression include human embryonic kidney 293 (HEK293), Chinese hamster ovary (CHO), and baby hamster kidney-21 (BHK-21). (Nooraei et al., 2021) These cell lines have been widely employed for VLPs such as influenza, HIV, and rabies. (Cervera et al., 2013; Fontana et al., 2015; Thompson et al., 2013)

5.2. Plant culture

Plant-based VLP production systems are a popular choice for many VLPs since they offer high protein expression levels, cheap maintenance, and simple purification. (Nooraei et al., 2021) The most popular plants used for expressions of VLPs and VNs are *Solanum lycopersicum*, (Saldaña et al., 2006) *Solanum tuberosum*, (Warzecha et al., 2003) *Arabidopsis thaliana*, and *Nicotiana tabacum*. (Greco et al., 2007) In particular, *Nicotiana benthamiana*—a relative of the tobacco plant from Australia—is a common plant utilized in laboratory setting due to its ability to host expression of heterologous gene sequences and a fast growth rate. (Goulet et al., 2019) The typical laboratory strain has retained a loss-of-function mutation in Rdr1 (RNA-dependent RNA polymerase 1), making it a popular platform for expressing numerous VLPs.

5.3. Bacterial culture

Bacterial expression systems are commonly employed to produce recombinant proteins, and VLPs are expressed using a similar approach. These expression systems are popular for their low cost and high protein expression yields. (Nooraei et al., 2021) However, this method is unsuitable for VLPs that could require post-translational modifications. (Fuenmayor et al., 2017) Protein solubility and incomplete disulfide bond formation are other factors that challenge bacterial expression systems. (Naskalska and Pyrc, 2015) The most commonly used bacterial strains for VLP expression include DH5 α , BL21 (DE3), and clear *E. coli*. (Au - Chen et al., 2018; Shahrivarkevishahi et al., 2021). Further, these strains tend to be transformed via heat shock or electroporation (Rahimzadeh et al., 2016) and can be grown using different media such as Lysogeny Broth (LB), Super Optimal Broth (SOB), or Terrific Broth (TB).

6. Pre-synthetic modifications (point mutations, unnatural amino acids, fusion protein expressions)

One of the main advantages of VLPs is their amenability to incorporating functional surface modifications at the genetic level. In most cases, peptide or protein sequences of interest can be directly added to the primary amino acid sequence of the coat protein subunits pre-synthetically by modifying the plasmid directly. These insertions or extensions allow the presentation of these groups on either interior or exterior surfaces. (Peyret et al., 2015; Rohovie et al., 2017) Additionally, point mutations on amino acid residues have been explored to fine-tune a variety of cellular and tissue targeting properties as well as the structure and stability of VLPs. (Fiedler et al., 2012) As an example, lysine residues on Q β were found to mediate non-specific cellular interactions by protonation and interactions with negatively charged membrane lipids and cell surface polysaccharides in mammalian cells. Single point mutations of lysine residues on Q β with negatively charged glutamic acid at position 13 (K13E) drastically increased the cellular binding. Conversely, replacing the lysine at position 46 (K46Q) with a neutrally charged glutamine residue resulted in a reduction of cellular binding of Q β to A431 cells. These mutations affected the van der Waals interactions, subsequently altering the VLP conformation and particle

binding. (Martino et al., 2021) Such point mutations have been applied to silence the undesired non-specific background interactions of VLPs with mammalian cells for a better starting point for cell and tissue targeting applications.

Beyond just changes to a single amino acid, fusion of foreign proteins onto capsid proteins has been used to design chimeric VLPs for various *in vivo* applications. These exogenous epitopes can be incorporated into VLPs pre-synthetically by genetic fusion of the sequences encoding the foreign epitopes at the C- or N-terminus or internal sites of the coat proteins. (Crisci et al., 2012; Yoshikawa et al., 1993) For instance, Q β VLP has been used to display human epidermal growth factor (EGF) on its exterior surface by C-terminal genetic fusion, resulting in structurally homogenous particles displaying 5–12 EGF copies on its surface. (Pokorski et al., 2011a,b) There are some benefits to this approach as well; for instance, it was shown that gp33 peptide derived from lymphocytic choriomeningitis virus glycoprotein 33 expressed on RHDV VLP by genetic fusion has a greater cytotoxicity towards gp33 coated target cells *in vivo* than the RHDV that expressed gp33 by chemical conjugation to its surface. (Li et al., 2013; Zepeda-Cervantes et al., 2020) It has been noted that stable VLPs with fusion proteins have limits to how large and charged these new epitopes can be. (Zepeda-Cervantes et al., 2020) Finally, genetic incorporation of unnatural amino acids containing functional groups like azides that can be orthogonally modified have been employed frequently to design polyvalent particles. These mutants on several VLPs as well as some of their products are discussed below. The genetic incorporation followed by chemical addressing of these functional groups eases the bioconjugation of the resulting particles without concerns of cross-reactivity or the need for complicated protecting groups. (Strable et al., 2008).

7. Post-synthetic modifications

A notable characteristic of viruses and VLPs is their ability to be post-synthetically modified. Often these reactions are straightforward and very high yielding. For example, these proteinaceous materials display a wide range of surface-exposed amino acids which can be used for bioconjugation reactions. (Benjamin et al., 2020a) Bioconjugation strategies have recently gained much attention, as they can be used for tailoring VLPs and VNs to perform specific functions such as imaging, cellular trafficking, and targeted drug delivery. (Shahrivarkevishahi et al., 2022) Though convenient, these reactions are not simple to execute in cases where the availability of exposed amino acids is limited, resulting in reduced efficiency of bioconjugation. (Hermanson, 2013) Other challenges include functionalization with hydrophobic molecules, which can occasionally cause precipitation. Strategies to overcome this include orthogonally attaching solubilizing PEG linkers or reducing the number of equivalents used to functionalize the surface. Fig. 4 highlights some approaches to decorate the surface-exposed amino acids found in VLPs and viruses. (Benjamin et al., 2020a) A variety of bioconjugation products for previously discussed VLPs and their mutants have been reported; to keep the discussion concise, we have included tables of a wide variety of products reported for each respective VLP in the subsequent sections (see Tables 1–7).

8. VLP-specific purification, characterizations, pre- and post-synthetic modifications

8.1. Q β

Recombinant expression of Q β begins with transforming BL21 (DE3) or clear *E. coli* (for endotoxin-free product) with a plasmid. (Brown et al., 2009) The bacterial culture is grown at 37 °C and 0% relative humidity under shaking conditions and protein production is induced using isopropyl β -D-1 thiogalactopyranoside (IPTG). Following expression and cell lysis, the protein is freed from the bacterial pellet with the help of either a microfluidizer or via sonication, and cell debris is removed via

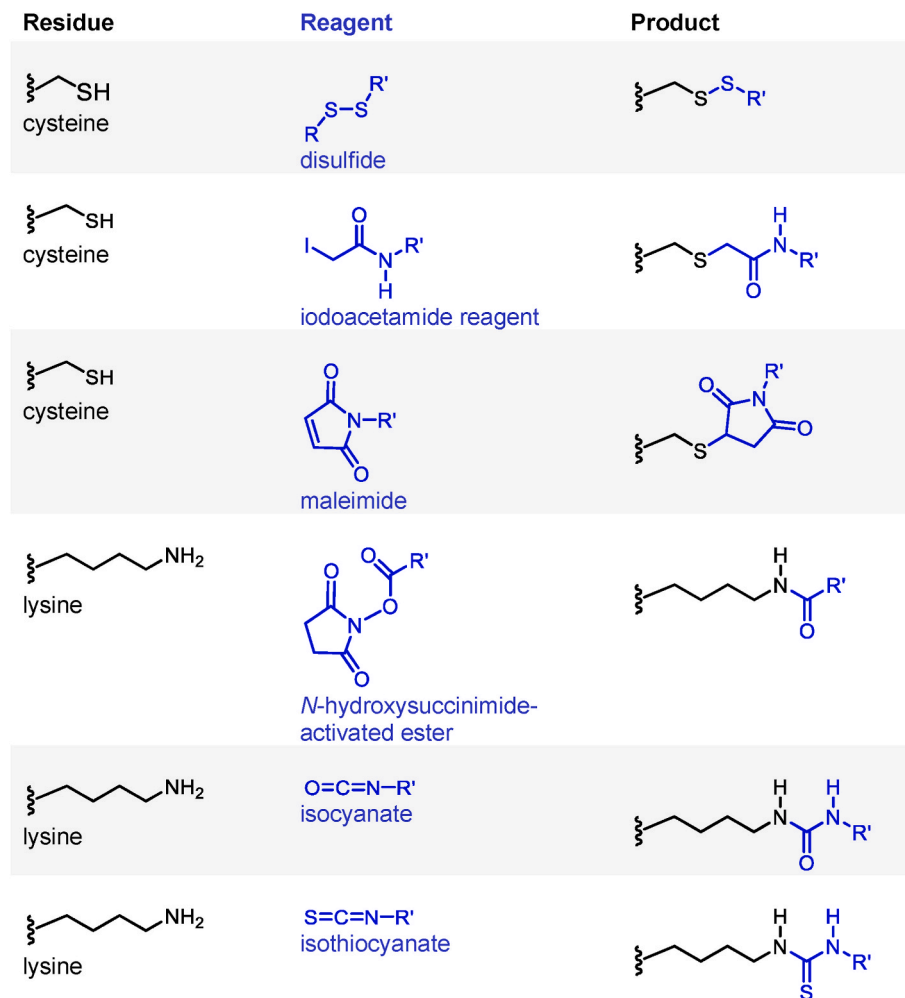


Fig. 4. Common amino acids targeted for bioconjugation techniques in VLPs and VNP.

centrifugation.

The protein in the supernatant is salted-out from the solution using an excess of ammonium sulfate. The precipitate is then incubated with equal volumes of chloroform/n-butanol, where excess lipids and membrane-bound proteins are removed from the solution. The extracted aqueous layer is then purified via fast-protein liquid chromatography (FPLC) or sucrose gradients, depending on the scale of the expression. The first step after purification is concentration determination, where different colorimetric assays such as Bradford, Lowry, or BCA can be employed. Confirmation of Q β 's monodispersity is quantitatively measured via dynamic light scattering (DLS) and qualitatively via transmission-electron microscopy (TEM). The purity of the final product can be assessed through reducing and non-reducing sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) and agarose gel electrophoresis. (Brown et al., 2009; Cui et al., 2017)

Q β can be modified at lysine residues K2, K13, and K16, as well as the N-terminus; several of these mutants already exist. The VLP also has disulfides at C74 and C80 that can be modified individually, which reduces the thermal stability of the VLP. Another alternative is to employ a cross-linking reaction using dibromomaleimide chemistry optimized by Chen et al., which does not alter the thermal stability of the final VLP. (Chen et al., 2016)

8.2. P22

Recombinant expression of P22 is commonly achieved using a pET vector containing the scaffold P22 protein and CP. Bacterial cultures are

grown at 37 °C under shaking conditions, and protein expression is induced using IPTG in their mid-log phase. Bacterial pellets are then incubated in lysis buffer consisting of sodium chloride and sodium phosphate, RNase, DNase, and lysozyme. The protein is released from the lysed cells via sonication, and the clarified supernatant post centrifugation is run through a sucrose gradient. Final purification is carried out through FPLC and fractions containing intact P22 capsids are collected. Characterization of the VLP is carried out using SDS, agarose, and TEM. (McCoy and Douglas, 2018; Patterson et al., 2012; Schwarz et al., 2015)

8.3. MS2

Bacteriophage MS2 is recombinantly produced using a pBAD plasmid and protein production is induced with arabinose. For protein isolation, bacterial culture pellets are resuspended in basic taurine buffer supplemented with DTT, MgCl₂, DNase, and RNase. Incubation in taurine buffer is followed by sonication and collection of cells via centrifugation. Supernatants are purified through Sephadex and then through FPLC. Final purified products are concentrated using 100 kDa MWCO protein concentrators and characterized through size exclusion chromatography (SEC), DLS, SDS-PAGE, agarose, and TEM. (Biela et al., 2022; Plevka et al., 2008)

8.4. CPMV

CPMV plant virus is expressed in black-eyed peas (*Vigna unguiculata*).

Table 1

Q β examples of bioconjugation reactions. In the figures below, amino acid residues containing carboxylates are shown in green, primary amines in blue, and thiols are shown in yellow.

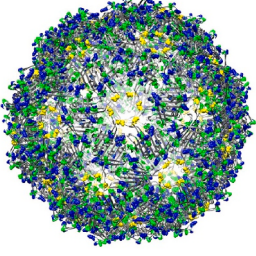
VLP	Residue	Incorporated material	
Q β		K2, K13, K16, N-terminus	Tn antigen,(Yin et al., 2013) bovine serum albumin,(Hong et al., 2009) fluorescein,(Hong et al., 2009) triple-sulfated ligand,(Mead et al., 2014) AF 488,(Pokorski et al., 2011) AF 568,(Banerjee et al., 2010) human holo-transferrin,(Banerjee et al., 2010) poly (2-oxazoline),(Manzenrieder et al., 2011) PEG-C60,(Steinmetz et al., 2009) oligomannosides,(Astronomo et al., 2010) LacNAc, 50 BPC sialic acid,(Kaltgrad et al., 2008)Gd (DOTA)(Prasuhn et al., 2007)
		M16HPG	Oligomannosides(Astronomo et al., 2010)
		T93AHA	RGD-PEG, biotin(Hovlid et al.)
		C74, C80	Fluorescent PEG, MPEG, polymers and multimeric protein-polymers (Poly(N-isopropylacrylamide [AKNM1]),(Leung et al., 2017 ; Tao et al., 2009) low and nonimmunogenic antigens (Nicotine[AKNM2])(Maurer et al., 2005)

Table 2

P22 examples of bioconjugation reactions.

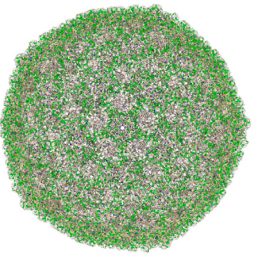
VLP	Residue	Incorporated material	
P22		S39C	Poly (2-aminoethyl methacrylate), (Lucon et al., 2012a) poly(tris (hydroxymethyl) methacrylamide)(Lucon et al., 2013)
		K110C	Streptavidin (Kang et al., 2010)
		K118C	Poly(tris(hydroxymethyl) methacrylamide), (Lucon et al., 2013)Gd (III)-DTPA-DAA, (Qazi et al., 2013) Gd(III)-DOTA, Gd(III)-DTPA, (Min et al., 2013) streptavidin (Kang et al., 2010)
		V119C	Streptavidin (Kang et al., 2010)
		S133C	Gd(III)-DOTA, Gd(III)-DTPA (Min et al., 2013)
		C-terminal LPTEG tag	Polyglycine GFP, polyglycine hemagglutinin head (Patterson et al., 2017)
		C-terminal SpyTag	EGFRAfb, HER2Afb, (Kim et al., 2019) hemagglutinin head (Sharma and Douglas, 2020)
		C-terminal Cysteine tag	Fluorescin-5-maleimide (Servid et al., 2013)

Table 3

MS2 examples of bioconjugation reactions.

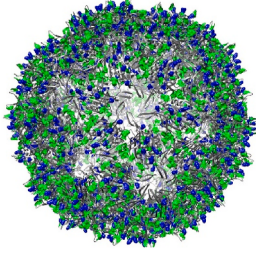
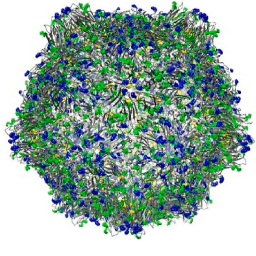
VLP	Residue	Incorporated Material	
MS2		Y85	Diazonium salt, (Hooker et al., 2004) FAM-SE, (Kovacs et al., 2007) [¹⁸ F]-benzaldehyde, (Hooker et al., 2008) Gd(III)complex (Datta et al., 2008) Gold NPs(Capehart et al., 2014)
		T19pAF	AF488, DNA, (Capehart et al., 2013) DNA aptamers, (Tong et al., 2009) fibrin, (Obermeyer et al., 2014) peptides, (Carrico et al., 2008) PEG, (Farkas et al., 2013) porphyrin (Stephanopoulos et al., 2009) DNA (El Muslemay et al., 2014)
		N87C	AF 488, AF 680, (Obermeyer et al., 2014) DOTA, (Farkas et al., 2013) AF 350, Oregon Green 488, (Stephanopoulos et al., 2009) Gd(III) complex, (Garimella et al., 2011) Taxol, (Wu et al., 2009) Small peptides (α -endorphin) (Lobba et al., 2020)
		K106, K113, N-terminus	PEG (Kovacs et al., 2007) TREN-bis-HOPO-TAM ligand (Datta et al., 2008)

Table 4

CPMV examples of bioconjugation reactions.

VLP	Residue	Incorporated material	
CPMV		Lysine	Anti-PD-1 peptide (SNTSESF)(Gautam et al., 2021) Bifunctional DBCO-PEG4-NHS ester to enable the chemical conjugation Azide-modified antibodies (IgG, hlgG)(Park et al., 2020) NHS-Sulfo-Cy5, PEG 2000 (Shukla et al., 2020) Pluronic F127 coated VLPs
		Lysine and cysteine	Peptides for vaccines: Myostatin 1 SARS-CoV-2 peptides CH401R (peptides) Fluorophore: Cy5.5 NHS ester Therapeutic peptides: ApoAI-4FN(Shin et al., 2021) Human HLA-A2 restricted peptide antigen NY-ESO-1 (Patel et al., 2020) Azide alkyne-functionalized generation 3 dendron with Carboxylic group (Wen et al., 2016) Complex carbohydrates, peptides, polymers, and proteins (Gupta et al., 2005). polyarginine (R5) cell-penetrating peptides (CPPs)(Wu et al., 2012) CPMV-azides/alkynes with dye alkynes (Wang et al., 2003). Water-solubilized quantum dots of red and green emissions (Portney et al., 2007)

Plant leaves are inoculated with CPMV in a solution of potassium phosphate (KP) buffer and incubated for 18–20 days. ([Wen et al., 2012](#)) The infected leaves are mixed and homogenized in phosphate buffer, and the leaf debris are pelleted out. The supernatant is used for

Table 5
CCMV examples of bioconjugation reactions.

VLP	Residue	Incorporated material
CCMV	Lysine	H6/G3 peptides (Chung et al., 2021) M-lycotoxin peptide L17E (Lam and Steinmetz, 2019) DTSSP (3,3' - dithiobis -(sulfo succinimidylpropionate) (Pretto and van Hest, 2019)
	C-terminus	Oligo-ethylene glycol (OEG) short-chain and an ArgGly-Asp (RGD) peptide (Wu et al., 2014)
	N-terminus	Small molecules and proteins, bearing a C-terminal LPETG-tag (Schoonen et al., 2015)
	Cysteine	24 amino acid peptide (Gillitzer et al., 2002) Photosensitizer (Ru (bpy) ₂ phen-IA) (Suci et al., 2007)

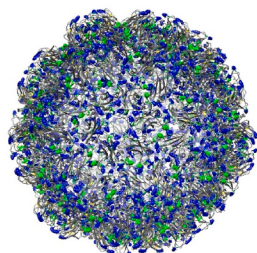


Table 6
TMV examples of bioconjugation reactions.

VLP	Residue	Incorporated material
TMV	Y129	Superoxide sensors, (Lee et al., 2020) high relaxivity MRI contrast agents (Bruckman et al., 2013)
	E97, E106	Texas Red maleimide, (Yi et al., 2005) RBITC, (Liu et al., 2016) FITC, Cy5, (Dharmarwardana et al., 2018) Mitoxantrone, (Lin and Steinmetz, 2018) Phenanthriplatin (Lin and Steinmetz, 2018; Liu et al., 2016; Shukla et al., 2021; Yi et al., 2005)
	T153K	Prostate specific antigen, (Shukla et al., 2021) thrombolytic therapy (Geiger et al., 2013; Shukla et al., 2021)
	S123C	Maleimide PEG conjugation (Koch et al., 2015; Zhou et al., 2013)
	K53, K68	Doxorubicin (Finbloom et al., 2016)
	E50Q, D77 N	Disassembly of TMV (Lu et al., 1996)
	T103C	Au nanowires (Zhou et al., 2013)
	MBP	Gold nanoparticles (Love et al., 2015)
	S3C + T153K	Dual functionalization (Wege and Geiger, 2018)

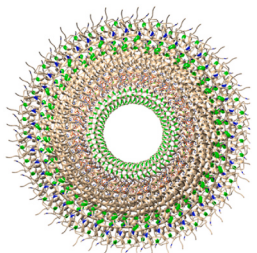
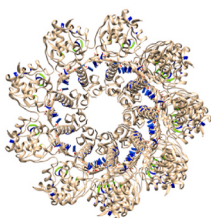


Table 7
PVX examples of bioconjugation reactions.

VLP	Residue	Incorporated Material
PVX	Lysine	Oregon Green 488, Biotin, PEG (Steinmetz et al., 2010). Fluorescein (Roder et al., 2019)
	N-terminus	Peptides (Lee et al., 2017)
	Cysteine	Oregon Green 488 (Roder et al., 2019)



chloroform-butanol extraction, following which the CPMV is pelleted out after incubation with PEG and NaCl. The pellet is resuspended in phosphate buffer and run through a sucrose gradient for further purification. Purified CPMV is preserved in phosphate buffer at pH 7.0 to preserve its infectivity if needed. The concentration of the purified virus is determined using UV-Vis spectroscopy and by measuring the OD at 260 nm. (Wang et al., 2019; Wellink, 1998)

8.5. CCMV

CCMV virus is expressed and propagated in cowpea leaves. (Bancroft and Hiebert, 1967; Zhao et al., 1995) For the isolation and purification, infected cowpea leaves are blended with an acidic sodium acetate buffer. The VLP is salted out using PEG and NaCl, and the centrifuged pellet is resuspended in sodium acetate buffer. Final steps include running CCMV through a sucrose gradient and resuspending the purified, centrifuge CCMV in sodium acetate buffer. Characterization is carried out through DLS, TEM, agarose, and SDS-PAGE. (Ali and Roos-sinck, 2007)

8.6. TMV

Tobacco (*Nicotiana benthamiana*) plants are used to express and propagate TMV virus. (Geiger et al., 2013; Shukla et al., 2021) Leaves of the plants infected with TMV solution are harvested after two weeks and stored at -80 °C until required. In the purification process, infected leaves are blended with potassium phosphate (KP) buffer supplemented with 2-mercaptoethanol. The slurry is filtered or strained, and supernatant clarified through centrifugation undergoes a chloroform-butanol extraction. The TMV is pelleted out post centrifugation using treatments of NaCl, PEG, and Triton X-100. The pellet is resuspended in KP buffer and separated on a sucrose gradient, of which the light blue band is extracted with the help of an LED light shining from the bottom of the centrifuge tube. Excess sucrose is washed off after centrifuging the extracted colloidal band and resuspending again in KP buffer and stored at 4 °C until further use. (Lumata et al., 2021) Characterization is typically carried out by TEM, SDS PAGE, agarose, SEC, and intact protein mass spectrometry. Liquid chromatography-mass spectrometry (LC-MS) is another technique used to analyze the integrity of TMV. (Dharmarwardana et al., 2018; Lee et al., 2020)

Native TMV is often modified via diazonium coupling to a surface-exposed tyrosine residue on the outside of the virus. In addition, the inner channel of the virus can be modified at one of several glutamic acid residues. However, diazonium coupling on TMV is tricky; therefore, several mutants of TMV exist that add functional handles that are much easier to use in such bioconjugations.

8.7. PVX

PVX virus is usually produced using tobacco (*Nicotiana benthamiana*) plants. (Shukla et al., 2014) First, the PVX-derived plasmid vector is obtained, which consists of a cDNA copy of the PVX viral genome and the 35S promoter. Four-week-old tobacco plant leaves are treated with Celite 545, and then inoculated with the plasmid. After 14–21 days post-inoculation, the leaves are harvested and homogenized in phosphate buffer, filtered and centrifuged to remove plant debris. An additional round of supernatant clarification can be achieved by treating with Triton X-100. Subsequent treatment with NaCl/PEG helps in pelleting out PVX, and after adding 2-mercaptoethanol and urea, purification is carried out on a sucrose gradient. The light scattering band is collected and dialyzed against a borate buffer. Finally, the virus is characterized by DLS, TEM, SEC, and SDS-PAGE and the concentration is determined by UV-Vis spectroscopy at 260 nm. (Shukla et al., 2013)

9. Disassembly-reassembly of VLPs and viruses

Disassembly and reassembly of VLPs is an area of research allowing for the removal of native DNA or RNA and non-covalent loading and encapsulation of cargo. (Stupka and Heddle, 2020) These techniques developed from an increased understanding of the protein capsid unit structures and their native assembly. Advancements in biochemical and biophysical techniques provided insights into which protein interactions helped form and stabilize the capsid. This knowledge was exploited to devise *in vitro* techniques to disassemble the capsid down to their CP units and then recombine them (see Fig. 5). Foreign material can be incorporated in the second half of the process to help facilitate reassembly, encapsulating said foreign material as the VLP comes together. This method provides a way to incorporate molecules and particles that cannot be conjugated or cannot fit through a pore. Additionally, nucleic acids and other delicate materials can be protected inside these VLPs from harsh environments that could otherwise impact their structural and functional integrity. Below we discuss VLP- or VNP-specific disassembly-reassembly processes and their applicability toward loading the capsid in a ‘supramolecular’ approach. We note that with some VLPs and VNPs, it is also possible to load the capsid “*in vivo*” during expression. For example, the Finn group developed a system to load fluorescent proteins into Q β all in one step during expression. (Rhee et al., 2011) The below methods differ in how the expressed capsid is ‘opened up,’ and cargo is then encapsulated around the reassembled virus, and the flexibilities and limitations that come with particular VLPs.

9.1. Q β

The assembly of the Q β capsid occurs via nonspecific electrostatic interactions between the genomic RNA and the CPs. (Twarock et al., 2018) The cargo-loading process can be achieved both *in vivo* and *in vitro*. *In vivo*, the helices from multiple CPs interlink into dimers to directly bind to the RNA, encapsulating it in an icosahedral capsid. (Cui et al., 2017) The packaging is promoted by RNA hairpins, which selectively bind to the Q β CPs during assembly. Some *in vivo* systems have been optimized to express RNA of interest, around which the CPs assemble and yield the desired encapsulation product. (Fang et al., 2018) This process can be performed *in vitro* with a much larger variety of cargo materials such as DNA, RNA, or even small proteins, as long as the proper conditions are met. *In vitro* assembly is less efficient than *in*

in vivo, primarily because of the inefficient encapsulation of the genetic material and low yields of reassembled Q β with the desired genetic material packed inside. (Fang et al., 2018) However, continuous efforts of researchers in the field have helped develop some successful protocols.

The disassembly of Q β *in vitro* starts in a dithiothreitol (DTT)-rich environment to reduce the disulfide bonds. The disassembly buffer also contains a high salt concentration to disrupt the interactions between CPs by decreasing the propensity for CP-CP hydrogen bonding interactions. The RNA is precipitated out with a Lewis base such as MgCl₂. The Q β is then reassembled in a solution containing a new “guest” material to be encapsulated in an appropriate buffer of interest. (Cui et al., 2017) Purification steps to get rid of the excess salts following disassembly often include dialysis and/or running the system through a Sephadex, Sepharose, or Sephacryl column. (C. Gomes et al., 2019)

Herbert et al. further optimized the conditions to include urea along with DTT and NaCl to induce complete disassembly. (Fang et al., 2018; Herbert et al., 2020) The group then encapsulated smURFP—a small ultra-red fluorescent protein—into the Q β capsid to create an *in vivo* imaging tool. The optimized reassembly conditions allowed the fluorescent protein to act as the “nucleating center” for capsid assembly, instead of the native genetic material. When the same conditions were used without any cargo, the capsid proteins could not reassemble. Since Q β 's stability is improved by the disulfide bonds between the cysteines in the CPs, it is wise to induce disulfide bond formation with H₂O₂ again to ensure the cargo does not leave the pores. To ensure the original RNA inside the Q β is eliminated and only cargo of choice remains, the disassembled CPs may be treated with RNAses prior to reassembly if required. Using nucleases not only facilitate the elimination of the native RNA; Storni et al. follow up their encapsulation of the DNA-based immunoadjuvant CpG in Q β with a DNase incubation to eliminate the unencapsulated DNA while the protein shell protects the encapsulated DNA. (Storni et al., 2004)

9.2. P22

In the case of P22, fundamental studies focused on the conformational changes that the CPs undergo to facilitate the exit of SPs and make room for DNA packaging. Based on circular dichroism and Raman spectroscopy, Tuma et al. proposed that the assembly of P22 is first initiated by the interactions between the α -helical SP subunit (gp8) and

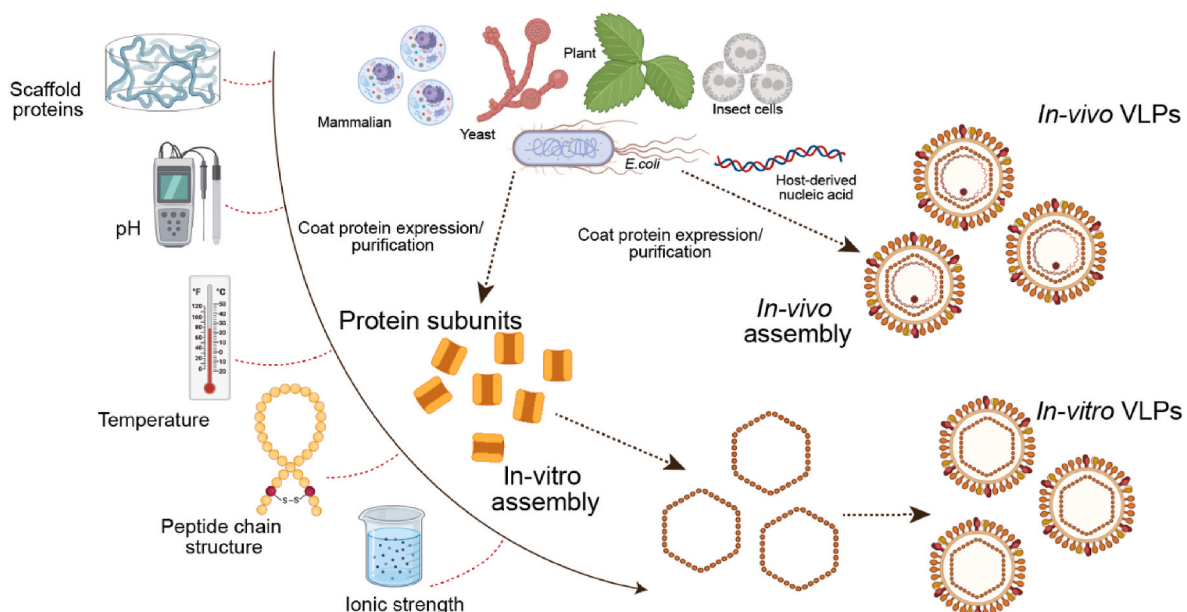


Fig. 5. Schematic representation of self-assembly and factors affecting the design and production of VLPs.

β -stranded CP subunit (gp5). (Tuma et al., 1996) These interactions drive the reproducibility of the assembly and thermostability of gp8 in helical form. When unbound, gp8 unfolds, and as the equilibrium shifts towards having more unfolded gp8, assembly of gp5 is initiated. Unfolded gp8 becomes available for further assembly of gp5 units, indicating that the SP acts as a catalyst. The process allows the equilibrium steps enough time to incorporate the dsDNA and is assumed to be the event that drives gp8 unfolding. This is because the packaged genome competes for the internal volume and binding sites previously occupied by the SP. When replicated *in vitro*, the authors propose using guanidine hydrochloride or heating to disturb the SP, facilitating the unfolding event.

Disassembly via heat expansion is one of the most straightforward approaches for the breakdown of the P22 procapsid, as it undergoes irreversible morphological changes upon heating. At around 65 °C, the capsid's maturation occurs where its size increases from 58 to 64 nm, with the expansion helping release the SPs. Upon further heating to 75 °C, the capsid expands more, and twelve gp5 subunits on the protein shell are irreversibly released, creating 10 nm “holes” in the capsid, an assembly referred to as the “wiffleball” assembly. (Putri et al., 2015) A standard protocol for this approach is incubating the VLP in NaPO₄ and MgCl₂ and heating till the complete expansion of the procapsid, resulting in the wiffleball conformation. It was found that 50% of the expansion from regular to wiffleball conformation was achieved when heated to 65 °C, 100% expansion was achieved at 70 °C, and wiffleball conformation was observed upon heating to 75 °C. (Morris and Prevelige, 2014)

Modern research continues to seek disassembly methods that can be employed in physiological conditions or within ranges of temperature and pH that are suitable for delicate biomaterials to be encapsulated within these VLPs. Kelly et al. demonstrated the use of ring-opening metathesis polymerization (ROMP) for capsid disassembly. (Kelly et al., 2021) First, the P22His₆GFP was functionalized on the surface-exposed lysine residues with 5-norbornene-2-carboxylic acid using EDC and sulfo-NHS coupling. Aqua-Met—a ruthenium-based ROMP catalyst—was added for ROMP initiation and subsequent capsid disassembly. This method was compared with the more traditional and previously discussed method of heating till 100% expansion was achieved. The breakdown of the capsid was demonstrated by native gel electrophoresis, TEM and DLS.

Even though disassembly for controlled release of fused proteins (to the SP) has found considerable success, re-assembly of P22 remains a challenge for researchers even today. This is due to the inability of CPs to form the uniform icosahedral structures in the absence of SPs, instead forming complex spirals and closed shells of varying sizes. (Earnshaw and King, 1978) More recent advancements demonstrate the unique applications of reassembly techniques, where the SP and CP are individually purified and combined subsequently. Sharma and Douglas vary the stoichiometric ratios of two variations of SP (wild-type and fusion protein with alcohol dehydrogenase) to act as the center for the reassembly of the purified CPs. (Sharma and Douglas, 2020) The assembled P22s of varying types were studied for their trends in enzymatic activity, which was concluded to have an inverse relationship to the fraction of wild-type SP. Such a system is an example of a finely tuned nanoreactor that can yield a precise amount of enzymatic activity desired for a system.

9.3. MS2

Natural assembly of the MS2 capsid occurs through dimerization of CP monomers into the C/C subunit as shown in Fig. 6. This is followed by the binding of an RNA hairpin, causing a conformational change in the FG loop region turning the C/C dimers into A/B dimers. The dimers form the icosahedral VLPs through interactions facilitated by the FG loop of 60 A/B dimers and 30 C/C dimers. (Fu and Li, 2016) This assembly method leads to the formation of robust particles causing disassembly to

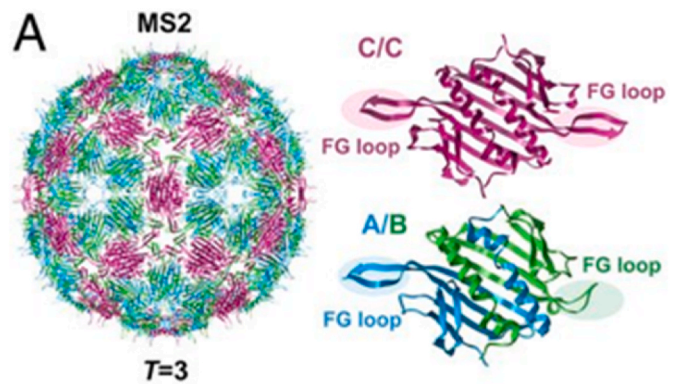


Fig. 6. Structure of the C/C and A/B subunits and their assembly into the MS2 icosahedral VLP. (Borodavka et al., 2012). Copyright from © 2012 National Academy of Sciences.

require relatively harsh methods. (Li et al., 2019)

In general, wild-type MS2 protein cages disassemble when lowering the pH and then reassemble in the presence of different types of oligonucleotides. The capsid can be disassembled into CP dimers by treatment with 33%–66% acetic acid while being incubated on ice. Centrifugation is used to remove precipitated RNA and maturase enzymes. The capsid proteins are then desalted using dialysis against an acetic acid gradient or a desalting column. Agarose and native PAGE can be used to confirm disassembly as CP dimers have a molecular weight of 28 kDa. FPLC elution profiles can also be used to confirm the disassembly while UV-Vis spectroscopy can be used to find the concentration of CPs before reassembly.

One of the common methods of reassembly is RNA-driven, in which cargo can be modified with a pac-site RNA. (Ashley et al., 2011) MS2 VLPs reassemble around cargo when CP dimers and RNA-modified cargo are combined in pH 8.5 Tris-HCl buffer at room temperature in a spontaneous process that takes 1 h. This solution can be purified by passing through a Sephadex column to remove free cargo and CP dimers, and/or by running fractions on 1% agarose. This method allows for various types of materials to be encapsulated, such as nanoparticles (e.g., quantum dots), protein toxins (e.g., ricin toxin A-chain), and small-molecule drugs (e.g., doxorubicin). (Ashley et al., 2011) TEM and DLS can be used to confirm the size and structure of the reassembled VLPs. Encapsulation efficiency is commonly done through the disassembly of the particles to release the cargo. UV-Vis is then used to quantify the released RNA, and SDS-PAGE is used for protein quantification; additionally, fluorimetry can be used for fluorescent cargo. Reassembly can also be done by an osmolyte-mediated method where either DNA-modified or charge-modified proteins can induce capsid assembly without the native RNA. (Glasgow et al., 2012) An assembly method driven by capsid concentration using neutral pH buffer was recently developed by Li et al. This method can encapsulate negatively charged cargo such as GFP or modified metal nanoparticles through electrostatic interactions. The cargo is mixed with CPs and subsequently dialyzed to increase the concentration to a point where the VLPs self-assemble around the material. (Li et al., 2019)

9.4. CPMV

CPMV's genome consists of bipartite, positive-sense ssRNA. Therefore, encapsulation of the viral genome—a crucial step in virus assembly—is challenging as the capsid proteins must preferentially select the genome segments of interest from a high background of cellular mRNA. The two segments of interest are RNA-1, which contains non-structural genes, and RNA-2, which consists of sequences encoding the large (L subunit) and small (S subunit) CPs. (Cheung et al., 2010; Hesketh et al., 2015) Usually, the components of CPMV will be resolved

as three distinct bands on a sucrose gradient. The top layer contains the artificial top component (AT) – the protein capsid with no detectable RNA, the middle layer has the protein capsid with RNA-2, and the bottom layer has the protein capsid with RNA-1. Though the capsid assembly process of CPMV is not well elucidated in the literature, some suggested mechanisms exist. The C-terminal extension to the S subunit is implicated in capsid assembly and RNA packaging, but understanding their roles have been challenging because the normal maturation of the RNA-filled capsid involves its cleavage and dissociation. Hesketh et al. have reported detailed characterization and insight on CPMV biogenesis through high-resolution cryo-EM of a CPMV empty VLP (eVLP) collected on an electron microscope. Cryo-EM reveals the C-terminal extension to the S subunit down to 3.0 Å resolution. Also, the structure for wild-type CPMV contains its larger genomic RNA at 3.4 Å resolution, where the density of the genomic RNA is also resolved. This data, along with the “*de novo*” model built using the high-resolution map obtained from cryo-EM are helpful in understanding which residues are involved in VLP formation and which residues are involved in RNA packaging. (Hesketh et al., 2017)

In case of CPMV, the predominant use of disassembly and reassembly is for eliminating any present genomic RNA and subsequent production of eCPMV (empty CPMV) shells. The traditional method to disassemble, remove genomic RNA, and reassemble CPMV is through alkaline/basic treatment, which degrades genomic RNA leaving behind only the AT band in the sucrose gradient. (Ochoa et al., 2006) An improved protocol was reported by Lee et al. and Zheng et al. via freeze-drying to remove RNA from wild-type CPMV. The resultant genome-free CPMV VLPs retain their capsid structure, which was confirmed through cryo-EM. (Zheng et al., 2019) The limitation of current chemical methods of RNA removal from the capsid is that trace amounts of RNA are still left behind with most techniques. Also, the necessity of fractionation to obtain mostly empty particles and harsh conditions that often lead to denaturation of the capsid over time can be avoided using the lyophilization technique, which was followed by a simple RNase treatment. (Lee et al., 2017)

An alternative to *in vitro* disassembly-reassembly is production of genetically modified eCPMV, is produced from the agrobacterium-mediated infiltration of *Nicotiana benthamiana* leaves with a plasmid construct expressing the precursor of the L- and S-CPs (VP60) and the virus-derived proteinase (24K) required for its processing. The eCPMV particles produced from this method using a purification protocol similar to that of native CPMV were confirmed to be free from RNA of both the virus and host origin. (Wen et al., 2012)

9.5. CCMV

CCMV is considered a model system for viral assembly, as it was the first icosahedral virus to be reassembled *in vitro*. Virulence of the system is still preserved when assembled from the purified capsid protein dimers and viral RNA of the wild-type CCMV. (Bancroft and Hiebert, 1967) A remarkable property of reassembled wild-type CCMV is that it can adopt a wide variety of polymorphic shapes by merely varying the chemical and physical conditions of the reassembly process. (Lavelle et al., 2007)

Wild-type CCMV is stable at pH 3.0–6.0 under low ionic strength (<0.5 M) solutions. Therefore, basic, high salt conditions are used for disassembly of the capsid arrangement. DTT is added to the disassembly buffer to prevent the formation of disulfide bonds, and PMSF is added to inhibit proteases during the disassembly process. The RNA is pelleted out through ultracentrifugation and supernatant is collected to obtain disassembled CP.

It was found that lowering the ionic strength and pH below 5.0 can reassemble the capsid proteins to form empty CCMV particles of similar structure without the viral RNA genome. (Fox et al., 1998) CCMV can be reassembled in the absence of genetic material or other encapsulating cargo. Lavelle et al. have reported several reassembly conditions, with

Table 8

Different reassembly conditions explored for CCMV.

	pH	Salt concentration	Divalent cation/chelating agent	Product obtained
1	4.8	High	Mg ²⁺	Single layered spherical CCMV-like capsids with 28 nm
2	4.8	High	None/EDTA	CCMV-like particles of 28 nm size. Additional presence of multi-layered, non-uniform spheres.
3	4.8	High, with DTT	Mg ²⁺	Both intact and partially assembled CCMV-like particles of similar size
4	4.8	Low	With/out Mg ²⁺	Mixture of assembly products (multi-layered and partially assembled)
5	4.8	High	Ba ²⁺ (poor divalent cation)	Low yield of intact CCMV-like particles

varied buffer composition in both ionic strength and pH, whose findings are briefly summarized in Table 8. (Lavelle et al., 2007)

They concluded that the optimal protein-only reassembly of CCMV from its purified CP is most efficient in buffer conditions of pH 4.8, high salt concentration, and in the presence of divalent cations. (Bancroft and Hiebert, 1967)

CCMV capsid reassembly in the presence of viral RNA genome differs from capsid reassembly with CP only, depending on the CP-CP and CP-RNA interactions and on the order in which the above interactions are “turned on”. To evaluate how the CP-CP and CP-RNA interaction strength affects the overall reassembly process, Garmann et al. reported an RNA-mediated assembly protocol for CCMV. They tested dialysis conditions that minimized both CP-CP and CP-RNA interactions and subsequently turned on these interactions by selectively controlling the pH and ionic strength of the buffers used for the dialysis. Depending on the number of dialysis steps used in the assembly process, they can be carried out as a “one-step” or “two-step” reassembly. (Garmann et al., 2019)

9.6. TMV

Once the TMV enters the plant cells, the virus disassembles, releases its RNA, and initiates the infection to create new viruses. (Holmes, 1979) The mechanism proposed by Caspar et al. includes repulsion between carboxylate-carboxylate functional groups on glutamic and aspartic acids, which resides at the interface between TMV CP subunits. If TMV virus particles are in the extracellular environment, the repulsive forces arising from the negative charges of the carboxylate groups are stabilized by cations like Ca²⁺ and H⁺. When TMV enters the plant cells, the drop in pH, loss of cations, and repulsion between carboxylate groups destabilize the virus and initiate the disassembly process. The first interaction involved in this process is between E50 from the subunit on the axial interface and D77. (Culver, 2002) Subsequent interactions include repulsive forces between E95 and E156 from the neighboring subunit, followed by repulsion between D116 and phosphate groups of the RNA. (Weis et al., 2019)

The first step of destabilizing and disassembling the virions is initiated by removing the CP from the viral RNA. Wilson et al. suggest a mechanism for *in vivo* disassembly that involves attaching ribosomes of the plant cells to the start codon, which get exposed after the removal of the first 23 CPs. The ribosomes bind to the 5' end and start translating the replicase open reading frame leading to the removal of CPs and other component proteins. (Weis et al., 2019) Disassembly of TMV in laboratory conditions can be done by treating purified TMV with glacial acetic acid. The RNA and CPs can be separated through centrifugation, purified through dialysis, and confirmed by PAGE and UV-Vis for further use.

In the reassembly process, it was found that different conditions can

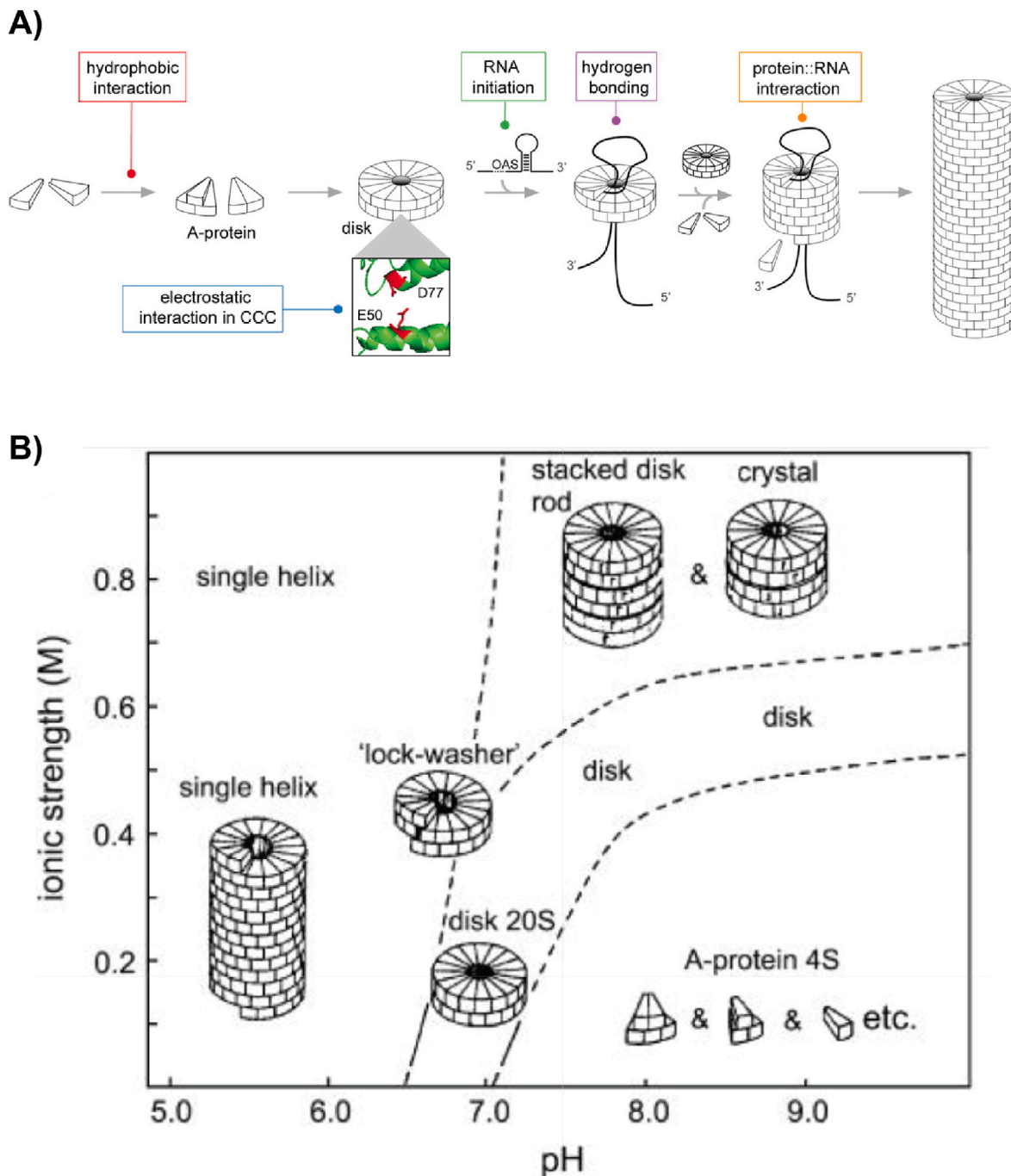


Fig. 7. A) Process of TMV assembly highlighting various interactions between CPs-CPs and RNA-CPs at different stages of reassembly. (Lee et al., 2021), copyright from © 2020 Wiley-VCH GmbH. B) Stability and states of TMV polymeric protein subunits in aqueous medium in the absence of RNA genome at various ionic strengths at RT, where pH ranging from 5.0 to 7.0 forms nanorods of TMV. (Kegel and van der Schoot, 2006), copyright from © 2006, Biophysical Society.

result in different structural assemblies of the helical aggregates. At pH 7.4, the TMV CPs assemble into 20S disks made of 17 molecules per ring. X-ray studies have shown the protein disks to be arranged in a manner where they have gaps that can accommodate the RNA. As shown in Fig. 7, the nucleation process starts by inserting a hairpin loop between two layers of the subunit. The loop binds to the turn and then opens the base-paired stem-loop, transforming the full disk into a short helix. The disk then entraps the RNA, and more disks are incorporated, elongating the helix. While this helix is elongated, the more it elongates, the more RNA is pulled out of the central cavity. In this fashion, RNA connects with the subunit proteins, starting the virus' nucleation. The symmetrical rod shape is achieved through non-covalent interactions between

subunit proteins. (Zhou et al., 2013) Reassembly in laboratory conditions is quite straightforward; assembly of individual CPs with the RNA cargo of choice is performed simply by co-incubating the constituents at 30 °C for 16–20 h in phosphate buffer. (Lam et al., 2016)

9.7. PVX

There are partially overlapping genes in the PVX RNA genome called the triple gene block that consists of TGBp1, TGBp2, and TGBp3, which are responsible for the cell-to-cell movement of PVX. It was found that even though encapsidated RNA of PVX is non-translatable in cell-free translational systems, interaction with TGBp1 will eventually convert

it to a completely translatable form. (ATABEKOV et al., 2007) It is reported that the selective binding of TGBp1 to one end of the PVX CP will create a metastable structure by destabilizing the whole helical PVX particle. Even though the binding of this TGBp1 to one end of the PVX plays an essential role in virus disassembly, it was found to be insufficient to achieve complete disassembly. Kiselyova et al. demonstrated that co-incubation of TGBp1 with RNase-treated PVX followed by centrifugation resulted in complete disassembly of PVX, which was characterized by AFM. (Kiselyova et al., 2003)

Goodman et al. previously described a straightforward method for the disassembly of PVX by freeze-thawing in the presence of LiCl. Ultracentrifugation, pellets down the residual intact VNPs and aggregated CPs, leaving free CPs in the supernatant ready for reassembly. (Goodman et al., 1976) In the reassembly process, the viral RNA was combined with the CPs in LiCl at room temperature; the optimum pH of the reassembly buffer was found to be 6.0–6.2. The assembly rate can be measured turbidimetrically, but a more direct indication of rapid assembly can be obtained by pipetting RNA and protein in the appropriate stoichiometric mixtures directly into a basic sodium EDTA buffer. The reassembly does not occur when the experiment is carried out at 4 °C – indicating that PVX reassembly is an entropy-driven reaction. Reassembled PVX has been reported to be less infective than its native counterpart; the ribonuclease-resistant specific infectivity of reassembled PVX varied from 1 to 14% compared to native PVX as measured by local lesions. The observed low infectivity is due to partial hydrolysis of the RNA after mixing with the CP, which has been previously quantified using PAGE. Dissociated CP subunits do not cross-react with appropriately prepared antiserum made to the virus. However, the reconstituted virus cross-reacted strongly against such antiserum, showing that virus-specific determinant groups were generated upon assembly, which can be used as another test to determine the extent of reassembly when appropriate. Despite the functional differences, structural features observed in the electron micrographs of native and reassembled PVX and their subsequent analysis by optical diffraction do not indicate any distinct differences between both samples. (ATABEKOV et al., 2007)

10. Conclusion

VLPs and VNPs are self-assembled nanomaterials that have been extensively investigated in diverse applications. These proteinaceous materials are ideal for imaging and vaccine development investigations, and many groups have exploited this potential. Most VLPs and VNPs are amenable to surface functionalization, disassembly, and reassembly as means to manipulate them. In this review, we have extensively detailed possible methodologies to load cargo and add directing moieties on the surface of these particles, allowing them to be used for various biomedical applications. The architectural diversity of VNPs and VLPs affords researchers numerous options to pick from when tailoring their experimental designs for creating hybrid materials. As the field grows exponentially, the number of VLPs and VNPs currently being tested as immunoadjuvants, vaccines, and imaging agents will continue to flourish and propagate. Finally, we hope to see these emerging technologies transition into clinics soon.

CRedit authorship contribution statement

Yalini H. Wijesundara: Writing – original draft, Writing – review & editing. **Fabian C. Herbert:** Writing – original draft, Writing – review & editing. **Sneha Kumari:** Writing – original draft, Writing – review & editing. **Thomas Howlett:** Writing – original draft, Writing – review & editing. **Shailendra Koirala:** Writing – original draft. **Orikedha Trashi:** Writing – original draft. **Ikeda Trashi:** Writing – original draft. **Noora M. Al-Kharji:** Writing – original draft. **Jeremiah J. Gassensmith:** Conceptualization, Writing – review & editing.

Declaration of competing interest

The authors have declared that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

J.J.G acknowledges National Science Foundation (DMR-2003534) and the Welch Foundation (AT-1989- 20190330).

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