Virus-Like Particles for Drug Delivery: A Review of Methods and Applications

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HIGHLIGHTS

- VLPs are highly versatile structures, adaptable for many drug delivery applications
- Mutations to VLP structural proteins can improve VLP drug delivery
- VLP cargo loading, release and activation are key traits for drug delivery

ABSTRACT

Virus-like Particles (VLPs) are self-assembling protein nanoparticles that have great promise as vectors for drug delivery. VLPs are derived from viruses but retain none of their infection or replication capabilities. These protein particles have defined surface chemistries, uniform sizes, and stability properties that make them attractive starting points for drug delivery scaffolds. Here, we review recent advances in tailoring VLPs for drug delivery applications, including VLP platform engineering approaches as well as methods for cargo loading, activation, and release. Finally, we highlight several successes using VLPs for drug delivery in model systems.

KEYWORDS: Virus-like Particles; Drug delivery; stimuli-responsive; protein library; genetic engineering; protein fusion

ABBREVIATIONS: VLP, virus-like particle; MS2, Male Specific Bacteriophage 2; TMV, Tobacco Mosaic Virus; Dox, doxorubicin; EPR, enhanced permeability and retention; SyMAPS, Systematic Mutagenesis and Assembled Particle Selection; PEG, Polyethylene Glycol; HBV, Hepatitis B Virus; CCMV, Cowpea Chlorotic Mosaic Virus; PhMV, Physalis Mottle Virus; MLV, Murine Leukemia Virus; Magnetic Resonance Imaging, MRI; Polydopamine, PDA

INTRODUCTION

Virus-like particles (VLPs) are self-assembling protein nanoparticles, typically derived from the capsid protein of non-enveloped viruses that infect bacterial, insect, plant, and mammalian cells as seen in Figure 1. VLPs are empty viruses without any infection, maturation, or replication components, an important feature for their biotechnological applications [1]. Engineers are repurposing VLPs for a variety of applications from imaging agents [2] to vaccine scaffolds [3–5], but we devote this review to exploring advances in engineering VLPs as drug delivery vehicles.

VLPs have multiple properties that make them desirable for drug delivery applications. VLPs are safe and biocompatible delivery vehicles. These particles are uniform in size when compared to other inorganic core nanoparticles such as gold core nanoparticles, which is valuable for drug delivery studies where the uniform size will increase the consistency of the accumulation of the nanoparticles in the tissues of interest. VLPs can be expressed in large quantities with ease in bacterial, plant, or mammalian systems through recombinant expression. The defined chemistry of these particles enables chemical modification of the interior and exterior by taking advantage of reactive amino acid residues such as lysine (N-hydroxysuccinimide ester modification) or cysteine residues (thiol-maleimide chemistry) [6]. Specifically, VLPs can be engineered with exterior targeting groups, enhancing the specificity of these delivery vehicles [7,8]. Many VLPs can also dissemble into coat protein monomers and dimers and then reassemble around cargo to easily load imaging agents, enzymes, and therapeutics into their interior [9]. This review highlights the recent developments towards engineering and using VLPs for drug delivery applications as shown in Figure 2, focusing on the VLP platform, VLP cargo loading, and cargo release or activation from VLPs.

VLP Platform Physical Property Engineering

VLPs are versatile drug delivery platforms due to the ease with which their physical properties can be modified to suit specific application needs via genetic manipulation of the sequences encoding coat proteins. For example, VLP pH and temperature stability can be altered to create particles that remain stable in the bloodstream and preferentially disassemble in the low pH endosomes of cells to release their therapeutic cargo. This platform tuneability is a unique aspect of the technology, creating the potential to use model VLP drug delivery systems for new targets. Some notable studies have shown that novel viruses can be identified with unique properties, such as increased thermal [10] and pH [11] stabilities, which can be adapted into new VLP scaffolds. A second approach, which is commonly employed, is to use rational or directed evolution strategies to create variants of existing VLPs that have optimal size, shape, thermal stability, pH stability, or surface charge properties.

VLP size and/or shape can be shifted via genetic-level point mutations to improve their utility as a drug delivery vehicle. Single amino acid changes to the protein sequence that can create large changes in protein function. For example, a smaller variant of the Male specific Bacteriophage 2 (MS2) VLP (17 nm vs. 27 nm for wild type) was identified with a mutation at position 37 from serine to proline (S37P) [12]. A second study identified a nanodisk assembly of the Tobacco Mosaic Virus (TMV) VLP conferred by two mutations from lysine to arginine (K53R/K68R) [13]. In both cases, point mutations conferred the formation of uniquely sized and shaped particles [12,13] compared to their wild-type counterparts. For VLP drug delivery to tumors, point mutations that shift the size and/or shape can lead to particles with enhanced permeability and retention (EPR) effect [7,8], the effect that defines the ability of nanoparticles to enter tumors via the leaky vasculature that forms as a tumor grows. A key result of this effect is that engineers must optimize the balance of drug carrying capacity and tumor penetration. While larger diameter or aspect ratio particles can carry a larger therapeutic payload, the engineered VLPs accumulate less into tumor environments in accordance with the EPR effect. For example,

performed biodistribution studies were performed with novel 18 nm diameter TMV nanodisk VLPs, 27 nm MS2 icosahedral VLPs, and 50 nm nanophages in a U87-Luc tumor bearing mice model[14]. The results demonstrated that mice survival improved the most with TMV nanodisk VLPs modified on the interior with chemotherapeutic doxorubicin (Dox) and on the exterior with polyethylene glycol (PEG). The delivery vehicles were each loaded with the same amount of Dox and compared to delivery of free Dox at the same concentration. Drug delivery with TMV nanodisk VLPs produced an advantageous result that would not be discovered without the use of the smaller-sized, disk-shaped TMV VLP variant. Importantly, VLP size and shape can be adjusted while retaining specific disassembly and chemical modification properties, making these nanoparticles attractive because a VLP can be tailored to match a specific drug delivery application without compromising its other preferred features.

VLPs with non-standard surface charge, thermal stability, or pH stability can also be identified through directed evolution. In this approach, researchers create a collection of variants of the coat protein, termed a protein library, and then screen or select for the particles in the library that have the physical properties of interest. The library can consist of a few variants or millions of variants, depending on the expected likelihood of finding a beneficial change. Protein libraries composed of point mutations have proven useful in tuning the properties of VLP drug delivery vehicles. For example, a 2,688 member library was generated for all single amino acid mutations of the MS2 VLP and selected for assembled particles [15]. High-throughput sequencing was used to quantify each VLP mutant's "fitness", which in this case was assessed by enrichment during the purification of MS2 VLP assembled particles. The entire process was named Systematic Mutagenesis and Assembled Particle Selection (SyMAPS). Following this seminal work, the SyMAPS process was applied to design a library of proteins with two point mutations targeted to a loop region of MS2 and uncovered VLPs with different pH and thermal stability properties than wild-type MS2 [16]. Three double mutants were discovered that were competent for assembly,

disassembled around the early endosome pH of 4.6, and were thermally stable at temperatures greater than 50 °C. These VLPs are candidates for commercial drug delivery applications because they are shelf stable and designed to disassemble under conditions that mimic those of endosomes. Researchers also have created smaller targeted libraries to test hypotheses about the importance of specific residues. For example, the Finn group created a library of 14 Qβ variants in which exterior lysines were mutated to other residues [17]. They hypothesized that one or more of these lysines played a role in mammalian cell binding. After selecting for the ability to bind cell membranes, the researchers identified a Qβ variant with a native lysine at position 46 mutated to glutamine (Qβ K46Q), which increased the positive charge of the particle and decreased binding of VLP to the cell membrane when compared to the wild-type particle. Creating VLP libraries, whether for pre-identified residues of interest or in a broad search for impactful mutants, is a powerful way to genetically modify the protein particle to improve drug delivery capabilities.

Engineering the Cargo Loading of VLPs

The ideal drug delivery scaffold should have flexibility in loading cargo into the interior or onto the exterior [18,19] in order to easily create scaffolds for a variety of use cases. This remains true for VLPs. Cargo in this review is defined as any therapeutic agent that the VLP is delivering to target cells. Targeting ligands are attached to nanoparticles in order to increase the likelihood that particles are delivered to the specific targeted cell, and the targeting groups are designed to bind specific cell receptors. Passive targeting experiments often incorporate a non-reactive spacer on the exterior such as PEG [20], while active targeting experiments incorporate cell-specific targeting ligands such as antibodies on their exterior [21,22]. These additions, while necessary to facilitate delivery, are not defined as cargo in this review. Cargo can be chemically conjugated to, loaded onto the exterior or interior of, or encapsulated within VLPs.

Inserting domains into VLP-encoding coat protein genes can give rise to new VLPs with novel handles to attach cargo to the interior or exterior. Protein domains are commonly added to the N- terminus[23], C-terminus[24], or both [25] because altering these regions typically does not compromise the structures of either the protein or VLP. VLPs with robust tolerance to domain insertions, whether at the termini or within the polypeptide, are particularly desirable because these particles can serve as models for inserting chemically reactive handles, adjuvants, or binding moieties into the VLP. These insertions can be rationally designed with specific peptide sequences or be discovered through a library-based approach. The SyMAPS approach was used to create a loop insertion library in which all possible three-amino-acid peptides were encoded for insertion within the MS2 VLP at the FG loop [26], a location known to tolerate alterations. This approach yielded novel assembly-competent MS2 VLP variants and informed some design rules for the inserted peptide sequences. Additionally, a modified SyMAPS approach was implemented to genetically modify the N-terminus of MS2 VLPs to create particles with new chemically reactive handles [27]. To do so, they created a library of every possible three-amino-acid peptide preceded by a proline and inserted at the N-terminus to take advantage of N-terminal proline conjugation chemistry [28]. After the selections for assembly and chemical reactivity, PNYR-MS2 and PYQR-MS2 mutant VLPs were identified as promising variants that permitted the desired modification at high yield. This study demonstrates that domain insertions in the VLP gene offer a viable method for cargo attachment on the assembled particle.

Engineered protein-protein interactions offer an additional method, often in tandem with a domain insertion, to associate cargo with a VLP. For example, the SpyCatcher/SpyTag system creates isopeptide bonds between their appended proteins and has been explored in cargo attachment studies with VLPs [29]. Using this system, researchers demonstrated cargo attachment to both the interior and exterior of VLPs [21,30]. In a recent study, the SpyCatcher domain was inserted into an exterior loop region of the Hepatitis B Virus (HBV) VLP and the

SpyTag domain to the cargo protein, yeast cytosine deaminase [30]. The researchers also simultaneously appended targeting peptides to the exterior of their cargo-loaded VLP via the same SpyCatcher/SpyTag, and performed cellular delivery experiments that demonstrated the effectiveness of these modified HBV VLPs at killing cancer cells. Additionally, the P22 scaffolding protein was engineered to create a protein-protein interaction with therapeutic enzyme cargo that catalyze important steps in the GSH pathway [31]. These enzymes were genetically fused to the N-terminus of the scaffolding protein that is used to form the P22 VLP and were encapsulated by co-expression of the capsid protein and the enzyme scaffold fusion. The resulting enzyme-encapsulating P22 VLPs were effective in the treatment for GSH-deficient cancer cells in *in vitro* studies. These examples illustrate the utility of protein-protein interactions in loading VLPs with relevant cargo or targeting groups.

In addition to engineered protein-protein interactions, researchers can leverage a VLP's natural affinity for negatively charged molecules to encapsulate and deliver nucleic acids [32]. VLPs are derived from viruses that use a protein exterior to encase their nucleic acid genomes, so there is an inherent affinity for negatively charged nucleic acids within every VLP. Taking advantage of this affinity typically requires incubating disassembled capsid proteins with therapeutic nucleic acids and stimulating re-assembly of the VLPs with encapsulated nucleic acid cargo. Cowpea Chlorotic Mosaic Virus (CCMV) VLPs were designed to encapsulate silencing RNA and anti-sense oligonucleotides in this way [33,34]. Cellular delivery experiments with CCMV VLPs loaded with RNA showed that treatment efficacy improved when using a VLP as compared to free RNA. Electrostatic interactions were leveraged to form theranostic, therapeutic and diagnostic Qβ VLPs in which the therapeutic cargo included RNA and the imaging components were fluorescent proteins [35,36]. These VLPs conferred delivery of RNAi downregulating expression of DNA repair mechanisms that made treatment with a chemotherapeutic more effective in mice brain tumor xenograft models. As these examples show, leveraging electrostatic

interactions of the VLP interior is an efficient way to load nucleic acids into VLPs, and can be used in conjunction with other methods to alter VLP disassembly and cargo delivery.

Engineering Cargo Release and Activation from VLPs

A key feature of VLPs is the programmability of their cargo release or activation. Cargo is sometimes modified to become compatible with other nanoparticle delivery vehicles, but these modifications can adversely affect the cargo's therapeutic function after entering the diseased cells. Additionally, cargo can be effective in killing cells, but are too dangerous for widespread delivery into the body. Due to the options for cargo association, VLPs overcome both challenges, offering a method to deliver toxic cargo specifically to cells while retaining therapeutic efficacy.

VLP-cargo interactions can be engineered so that drug payloads are released in response to certain stimuli (*e.g.,* pH, proteolytic enzyme presence) in a local environment of interest. For example, plant-derived Physalis Mottle Virus (PhMV) VLPs were investigated for pH mediated release of chemotherapeutic drugs [37,38]. A recent study focused on chemically conjugating a prodrug derivative of doxorubicin to the interior of PhMV VLPs, forming a pH responsive hydrazone bond to the VLP [37]. In a separate experiment, the same research group conjugated the drug cisplatin to the VLP [38]. Both experiments showed cargo release after incubation at pH 5.2 for multiple hours. Tumor xenograft mice injected with loaded PhMV VLPs had significantly improved survival when compared to free doxorubicin or cisplatin. VLPs can also be used to deliver cargo that is activated upon enzyme-mediated release. For example, VLPs that deliver Cas9-sgRNA ribonucleoproteins include a protease-cleavable linker between the Cas9 cargo and Gag structural protein [39,40]. Cleaving the Cas9 from the structural protein used to anchor Cas9 to the VLP interior enables the Cas9-sgRNA ribonucleoprotein to edit DNA. Murine Leukemia Virus (MLV) VLPs were shown to effectively deliver Cas9 proteins N-terminally fused to an MLV protease site on the C-terminus of a Gag protein and that these Cas9 fusions still effectively modify genomic DNA in primary cells, embryo cells, and in mice upon proteolysis [39]. In a

similarly novel way, human immunodeficiency virus (HIV) VLPs were able to deliver Cas9 cargo proteins N-terminally fused to an HIV protease site on the C-terminus of a Gag protein to localize the Cas9 into the VLP interior. The loaded HIV VLPs successfully transport Cas9 to edit the genomes of human T-cells effectively without the need for electroporation [40].

VLPs are also compatible with light activatable drug delivery systems. These cargo are most effective at facilitating photodynamic or photothermal therapy [41]. The therapies use small molecule photosensitizers that react to light in the 600-800 nm wavelengths to generate reactive oxygen species which then kill tumor cells. Qβ VLPs were engineered for photothermal therapy by chemically conjugating the near infrared (NIR) dye Croc (thiophene-croconaine dye) to the exterior of the particle [42]. Injecting these loaded VLPs into mice with 4T1 murine breast cancer tumor xenografts led to suppression of 70% of the tumors in mice 4T1 tumor xenograft models compared to free photosensitizer dye alone. Furthermore, Qβ VLP photothermal therapy prolonged survival time and reduced lung metastasis by 85% in mice compared to the control. In another example, TMV theranostic VLPs were engineered by chemically conjugating a Gd-dodecane tetraacetic acid contrast agent to the interior of the VLP for magnetic resonance imaging (MRI), photoacoustic imaging, and photothermal therapy [43]. These VLPs were appended with the photothermal agent polydopamine (PDA), and the Gd-TMV-PDA VLPs showed improved MRI imaging capabilities and killed prostate cancer cells after irradiation at 808 nm for as little as 3 minutes. Collectively, these studies show how VLPs loaded with cargo can be delivered to areas of interest and activated with light.

CONCLUSION

There have been several exciting advances in VLP engineering for drug delivery applications over the past five years. Researchers demonstrated how to modify VLPs with both genetic and chemical methods to tune platform and cargo parameters for specific delivery goals, and established platforms for engineering VLPs to have favorable size, shape, surface charge,

pH and thermal stability properties. By taking advantage of the inherent genotype-to-phenotype link associated with viruses, recent progress has demonstrated the power of generating libraries of VLPs to identify VLP properties with improved drug delivery properties. Moreover, whether through genetic, chemical, or electrostatic methods, VLPs can be loaded with therapeutic cargo, and the VLP–cargo interactions can be modified for responsive release or therapeutic activation Recent advances in genome editing are giving rise to a promising new application for VLPs: the delivery of Cas9 proteins for gene and cell therapy. With ever-expanding toolkits for the engineering of VLPs, we expect to see a continued shift of the field of nanoparticle drug delivery towards a more functional and effective future.

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DECLARATION OF INTEREST

The authors declare no conflicts of interest.

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