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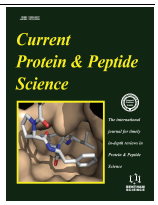
REVIEW ARTICLE

Engineering Virus-like Particles for Antigen and Drug Delivery



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Abstract: Virus-like particles (VLPs) are nanoscale biological structures consisting of viral proteins assembled in a morphology that mimic the native virion but do not contain the viral genetic material. The possibility of chemically and genetically modifying the proteins contained within VLPs makes them an attractive system for numerous applications. As viruses are potent immune activators as well as natural delivery vehicles of genetic materials to their host cells, VLPs are especially well suited for antigen and drug delivery applications. Despite the great potential, very few VLP designs have made it through clinical trials. In this review, we will discuss the challenges of developing VLPs for antigen and drug delivery, strategies being explored to address these challenges, and the genetic and chemical approaches available for VLP engineering.

Keywords: Virus-like particles, drug delivery, vaccines, epitope, immunity, protein engineering, chemistry, nanoparticles.

1. INTRODUCTION

In the mid 1960s, pathologists first observed the formation of nanoscale particles from virus-infected human and animal tissue samples that resembled the virus in morphology but were non-infectious [1-3]. These virus-like particles (VLPs) were soon discovered to consist of viral proteins but do not contain the viral genetic material (Fig. **1**). Further studies showed that VLPs have the ability to present viral epitopes via authentic, repetitive, highly organized structures similar to those in a native virion but without the risk of infection. These features have attracted significant interest in developing VLPs as novel immunogens in prophylactic vaccines. More importantly, the ability of VLP to self-assemble into a virion-mimicking structure in the absence of the viral genetic material has led to a major departure of its development as vaccines from the previously adopted "isolate-inactivate-inject" approach, which had spawned the successful influenza, polio, and MMR vaccines [4]. The first VLP vaccine was approved in 1981 and was composed of purified hepatitis B virus surface antigen (HBsAg) VLPs purified from the plasma of chronically infected individuals [5]. Although the vaccine was costly to produce and was sourced from a limited supply of donors [6], it was very effective and proved the efficacy of VLP for use as prophylactic vaccines. With the advent of recombinant DNA technology, it was discovered that HBsAg VLPs could be produced in the yeast *Saccharomyces cerevisiae*, and these VLPs were formulated into the Recombinvax HB vaccine licensed in 1986 [6]. The use of recombinant methods to produce VLP marked the second generation in VLP production, leading to higher production yields and the ability to produce VLPs derived from viruses of diverse genetic backgrounds (Fig. **1**). The high tailorability of VLP proteins has since been the subject of genetic and chemical engineering to expand their applications beyond the simple presentation of native antigenic epitopes. These third-generation chimeric VLPs have been developed for various purposes including imaging reagents [7-10], template synthesis [11, 12], and catalysts [13-15] (Fig. **1**). Nevertheless, arguably the most studied applications of chimeric VLP lies in their use as targeted antigen and/or drug delivery vehicles. This is not surprising as viruses (and therefore VLPs) are natural nanoparticles that have perfected the art of cellular delivery of biomolecules over eons of evolution and present many advantages over synthetic nanoparticle systems. For example, VLPs are biocompatible [16, 17], less toxic [18], and functionally tunable through genetic engineering. In the past four decades, much progress has been made in the field of VLP research with many recombinant production systems developed for a wide range of applications extensively reviewed elsewhere [19-24]. In this paper, we focus on the design rationale for developing VLP to suit various antigen and drug delivery applications as well as the corresponding engineering strategies to introduce these modifications.

2. DESIGN CONSIDERATION

Antigen and drug delivery using VLPs often require distinct and opposing design considerations. The goal of antigen delivery is to achieve a sufficiently strong and long-lasting adaptive immune response against the antigen, which may entail the selective activation of humoral or cellular immunity. This often requires enhancement of immune activation and/or fine-tuning of the selection and presentation of the

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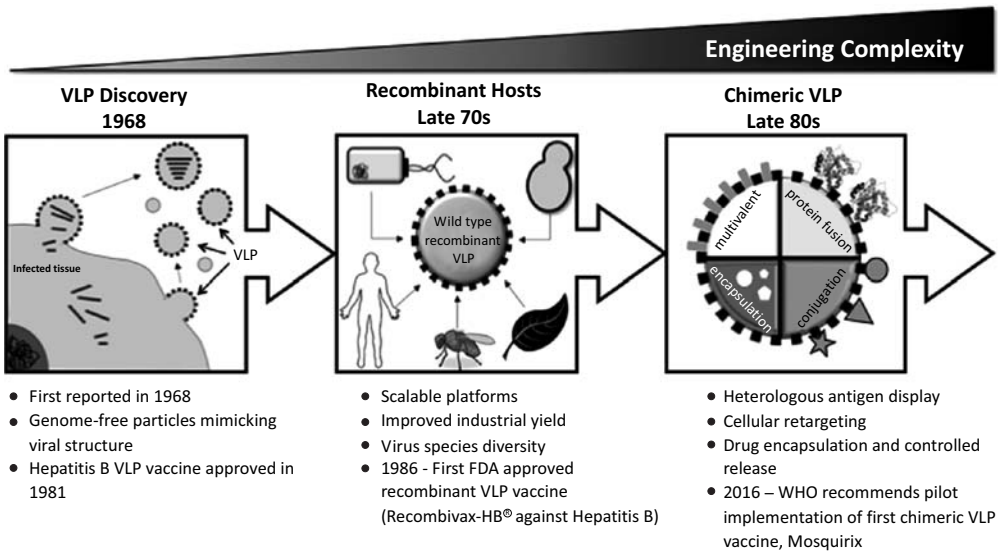

Fig. (1). An overview of the evolutionary stages in the development of VLPs. Wild-type VLPs were first discovered in virus infected tissue samples, but advances in recombinant engineering enabled the production of engineered VLPs in a variety of recombinant host systems. More recently, additional functionalities not present in VLPs composed of native viral proteins have been introduced by chemical and genetic engineering approaches. These modifications have allowed increased tunability and greatly expanded applications including their use as antigen and drug delivery vehicles.

antigenic epitopes. On the other hand, the goal of targeted drug delivery is to maximize delivery efficiency. To this end, the design of VLPs as drug delivery vehicles should aim to evade immune detection to prolong systemic circulation, recognize and accumulate at the target site, and exhibit controlled drug release functionalities. In this section, we will put forward VLP design considerations to achieve the goals for antigen and drug delivery (Fig. **2**).

2.1. Antigen Delivery

As viruses often display many copies of only a few proteins [25], it has been postulated that the co-evolution of viruses and vertebrates has led to the ability of the immune system to rapidly detect, discriminate, and respond to the repeated and ordered structure of a virus [20]. Therefore, antigen delivery on the organized and repetitive structure of a VLP often provides enhanced immunogenicity compared to other delivery system designs, such as subunit vaccines (a comparison of antigen delivery systems has been reviewed elsewhere [26, 27]). In fact, VLPs are such potent immune stimulators of B cells that self-antigen displayed on VLP has been shown to break B-cell self-tolerance and induce the production of autoreactive antibodies [28-31]. The particulate nature of VLPs also leads to efficient uptake by antigen presenting cells (APCs) to activate both $CD8^+$ and $CD4^+$ T cells, which can further enhance the antibody response [32, 33]. However, unlike viruses, VLPs often elicit weak T-cell responses due to the lack of additional stimuli presented by viral replication [20, 34]. Yet, recent studies have suggested the necessity of targeting T cells in developing effective vaccines. For example, the conventional influenza vaccine design relying on antibody responses is inadequate; instead, pre-existing influenza-specific T-cells correlate with protection [35-37]. Therefore, additional design considerations are required to enhance the capability of VLPs to induce T-cell immune responses.

2.1.1. Enhancing T-cell Activation

Adjuvants like alum [38] and oil-water emulsions [39] have been routinely used in vaccine formulations for almost a century to enhance the immune response. Not surprisingly, VLP-based vaccines such as Gardasil® and Cervarix® co-administer human papillomavirus-like particles with aluminum salts [40] and aluminum hydroxide/monophosphoryl lipid A (MPLA) [41] adjuvants, respectively. Due to the recombinant and synthetic nature of VLPs, the functionalization of immunostimulatory molecules on the VLP has emerged as a new vaccine strategy to enhance their immunogenicity [42]. These self-adjuvanting VLPs have been shown to generate similar immunostimulatory effects as co-administration with significantly less quantities of the same adjuvant [43], thus minimizing adverse immunotoxicity. Below, we will present various classes of immunostimulatory molecules that can be used in self-adjuvanting VLP vaccine designs.

Although not fully understood, studies have suggested that adjuvants improve the vaccine efficacy partly by enhancing the activation and maturation of APCs, such as dendritic cells (DCs) and macrophages [44]. These activated APCs then induce the maturation of T cells and B cells that are crucial in adaptive immune responses and immunological memory [45, 46]. It is well established that APC activation is frequently triggered by the recognition of pathogen-associated molecular patterns (PAMPs) by toll-like receptors (TLRs) expressed on the APC surface [47]. In addition to upregulating the expression of major histocompatibility complex (MHC) for enhanced antigen presentation to T cells, activated APCs provide a cytokine and chemokine microenvironment that supports strong antigen-specific cellular and humoral immune responses [48]. Therefore, TLR ligands and their synthetic analogs represent an attractive class of adjuvants that can enhance APC maturation, which

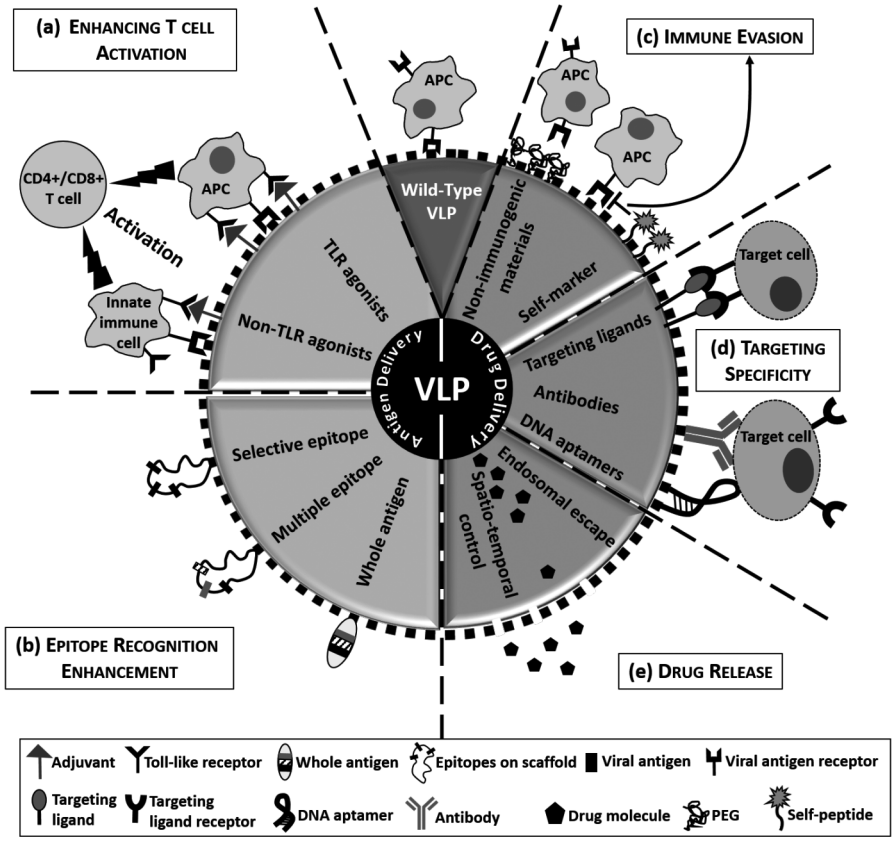

Fig. (2). Design considerations for developing VLPs for antigen and drug delivery. The efficient delivery of antigen by VLPs can be engineered by **(a)** enhancement of T cell activation via the use of immunostimulating adjuvants and **(b)** the rational design of whole antigen or epitopes. Efficient drug delivery using VLPs can be achieved by **(c)** surface modification of the VLPs to shield the viral antigens from the immune system, **(d)** introduction of cell-specific targeting biomolecules on the surface of the VLPs and **(e)** implementing strategies for the effective and controlled release of the drug.

in turn improves the activation of antigen-specific T cells [49, 50].

TLRs are activated by specifc ligands (listed in [51]), which can be exploited as potential VLP adjuvants. Oligonucleotides (CpG DNA, ssRNA), lipopeptides (E_8Pam_2Cys), and peptides (flaggelin) are TLR agonists that have been introduced into VLPs to significantly increase specific T-cell responses (Table **1**). Although T cell activation through TLRs is thought to primarily occur indirectly through the activation of APCs, there is increasing evidence that a range of T-cell subsets including $CD8^+$ T cells [52-54], $CD45RO^+$ memory $CD4^+$ T cells [55], $CD4^+CD25^+$ T_{reg} cells [56, 57], and Th17 cells [58] also express TLR2 and/or TLR5 on their surface, thus can be activated directly. The relative contribution of direct (T-cell TLR activation) and indirect (APC TLR activation) mechanisms is unknown and warrants further investigation. It should also be noted that molecules activating other immune signaling pathways that are independent of TLRs can also be displayed on VLPs to enhance T-cell activation [59, 60]. For instance, the activation of natural killer T cells by α-galactosylceramide (α-GalCer), the activation of DCs by CD40L, and VLP directly functionalized with supporting cytokines have been shown to increase antigen-specific T-cell responses (Table **1**).

Table 1. Immune stimulators utilized in self-adjuvanting VLPs.

Stimulant	Receptor	VLP
CpG DNA	TLR9	Hepatitis B virus [61], Qβ [61-63]
ssRNA	TLR7/8	Papaya mosaic [64]
E_8Pam_2Cys	TLR2	Hepatitis C virus [65]
Flagellin	TLR5	Influenza [66], Rabies [67]
α-GalCer	NKT TCR	Rabbit hemorrhagic disease [43]
CD40L	CD40	HIV [68], SIV [69]
GM-CSF	CD116	Rabies [70], SIV [71]

2.1.2. Epitope Recognition Enhancement

The adaptive immune response is predicated on the ability of B cells and T cells to recognize antigenic protein fragments known as epitopes. The identification of B-cell and T-cell epitopes that can be exploited for vaccination is a major research field that presents many challenges [72-76]. In cases where epitopes are unknown for a given antigenic protein, the whole or nearly whole protein can be delivered in its

native form for immune recognition. Indeed, by means of genetic fusion [67, 77-86], conjugation [87, 88], or pseudotyping [78, 89-92], large antigenic proteins have been successfully incorporated in VLPs for vaccination, eliciting effective antigen-specific humoral and/or cellular immune responses. In cases where the epitopes have been identified, the delivery of selected epitopes presents several advantages over that of large antigens. First, epitopes are often easier to be incorporated into a VLP than larger antigens thanks to their short sequences imposing minimal interference with VLP protein function and assembly (see Section 3.1 for details). Many sites in a range of VLP proteins have been successfully used for epitope insertion (Table **2**). Second, the small size of epitopes also entails the possibility of incorporating multiple different epitopes or multiple copies of the same epitope into one or more sites in a VLP protein to elicit an improved immune response. For example, multiple epitopes have been inserted into HBcAg VLPs to improve cytotoxic T-cell responses against hepatitis B virus [93] and *Plasmodium falciparum* [94] as well as to increase the breadth of antibody response against influenza virus [95]. Other studies have demonstrated that increasing the number of repeats of an influenza M2 epitope displayed on HBcAg [96] or nodavirus [97] VLPs increases M2-specific antibody titers in mice. Finally, epitopes that are specific for certain immune cells such as B cells, $CD4^+$ T cells or $CD8^+$ T cells have been incorporated into VLPs to selectively activate different arms of the adaptive immune system [98] providing further control over the desired immune response.

Despite these advantages, there are several challenges associated with the design of epitope-based vaccines. In the case of B-cell epitopes, their location in a VLP carrier protein can greatly affect the magnitude of the resulting antibody response. It has been shown that the region around amino acid 80 of the HBcAg protein is the major antigenic determinant of the HBcAg VLP [99], and the placement of heterologous epitopes in this immunodominant region results in a stronger epitope-specific antibody response than in other locations of the HBcAg [100]. Another challenge is associated with the conformational structure of a significant fraction of B-cell epitopes [101], making it more difficult to present them on the VLP surface in an antigenic form than linear epitopes. Yet, vaccines targeting conformational B-cell epitopes provide a means of generating broadly neutralizing antibodies for viruses that have proven difficult to vaccinate against such as influenza [102] and RSV [103, 104]. One class of the most common conformational epitopes are those with α helical structure found in coiled-coil motifs of many enveloped viral glycoproteins [105]. Antibodies targeting these α helix epitopes have been shown to inhibit cellular entry of HIV [106], Ebola [107], and influenza [108] viruses. To achieve conformational presentation of these α helix epitopes, the leucine zipper domain of the yeast transcription factor GCN4 has been used as the flanking sequence to stabilize their conformation [106, 109-111]. Presentation of epitopes with more complex conformations, such as a helix-turn-helix structure, is not as straightforward and may require computational design of specialized scaffolds to obtain the correct epitope conformation (reviewed in [112]).

T-cell epitopes, although linear, also present unique challenges. The sequences flanking T-cell epitopes have been implicated in the efficiency of antigen processing and presentation [113, 114], thus present an engineering opportunity for modulating epitope-specific T-cell activation. Analysis of the flanking sequences of highly presented T-cell epitopes has led to the identification of both natural and synthetic sequences that can either promote or inhibit the epitope presentation to T cells [115, 116]. Additionally, oligoalanine residues flanking an epitope have been shown to increase the epitope presentation efficiency, possibly by providing a buffer from nearby interfering sequences [114]. Rueda *et al.* applied this concept when designing parvovirus-like particles to present an immunodominant $CD8^+$ T-cell epitope of chicken ovalbumin (OVA). By inserting an additional 3-5 aa of the natural OVA sequence flanking the epitope, the resulting parvovirus-like particles demonstrated considerable improvement of the epitope presentation compared to the OVA epitope alone [117]. In addition to epitope flanking sequences, the selection of T-cell epitopes themselves is challenging due to their dynamic nature. It is believed that the long-lasting protective memory T cells respond to only a few peptides derived from the pathogen, termed immunodominant epitopes [118-120]. However, the immunodominant epitopes can change depending on an individual's disease state [121-123], and subdominant T-cell epitopes are also shown to be important in controlling viral replication [124]. To address this challenge, further cooperative advancements in high throughput T-cell epitope mapping [125, 126], disease pathology [127, 128], and personalized approaches in vaccine development [129] are required.

2.2. Drug Delivery

Effective drug delivery vehicles should ideally be safe, encapsulate cargo, evade the immune system, specifically target cells or tissues, and release the cargo at the destination in a controlled manner. VLPs lend themselves well to these objectives, as viruses are essentially delivery vehicles for nucleic acids. In addition, VLPs are versatile structures amenable to chemical and genetic modifications that result in predictable, highly defined, and homogenous alterations. These traits have generated numerous interests in using VLPs as vehicles for drug delivery [130, 131]; however repurposing VLPs as drug delivery vehicles still presents challenges such as their inherent immunogenicity, their cell/tissue targeting specificity, as well as the cargo release. We will discuss design strategies and recent progress in addressing these challenges.

2.2.1. Immune Evasion

As discussed in section 2.1, VLPs are inherently immunogenic. Furthermore, recall activation of the immune response caused by pre-existing immunity or repetitive doses can result in rapid clearance of the VLPs from the host, leading to reduced circulation time and reduced drug delivery efficiency. Therefore, the intrinsic immunogenicity of VLPs presents a significant hurdle to their use as drug delivery vehicles, and necessitates modifications to facilitate immune evasion. Surface attachment of polyethylene glycol (PEG), i.e. PEGylation, is the most established approach for immune stealthing of delivery vehicles and has been widely employed to protect synthetic nanoparticles [132-134], viruses [135-138] and VLP [18, 139] from host immune re-

sponses. However, alternatives to PEGylation are being sought due to drawbacks such as reduced binding affinity to cellular receptors [140], unexpected pharmacokinetics due to changes in the VLP physiochemical properties [141] and particularly its non-biodegradability [142]. Polyketal shells, formed by photopolymerized cross-linking of amino ketal methacrylamide monomers and ketal bismethacrylamide, have been found to be effective in encapsulating and shielding adenovirus vectors designed for the targeted delivery of DNA and siRNA [143] while being completely biodegradable and non-toxic. Polyketal shells, as well as other natural [144-148] and synthetic [149] non-immunogenic polymers that have provided immune stealthing of synthetic nanoparticles, could potentially be employed to impart immune evasion properties to VLPs. In addition to physical masking with non-immunogenic materials, recent studies have investigated an "active stealthing" approach that disguises synthetic nanoparticles and VLPs with the "self" marker CD47, a glycoprotein present in all mammalian cell membranes used by macrophages and leukocytes as a marker to distinguish self from non-self structures [150]. Nanobeads [151] or P22 VLPs [152] displaying this computationally derived CD47 self-peptide significantly reduced immune activation and immune clearance in mouse models [152]. Active stealthing with self-peptides may present several advantages in that these short peptide sequences introduce minimal alteration in the structure thus the function of VLP proteins and targeting ligands, as well as the size of the modified VLPs is minimally affected.

2.2.2. Targeting Specificity

VLPs often retain the natural tropism of the wild-type virus, which can be exploited to deliver therapeutic cargoes to specific tissues or organs. For example, JC polyomavirus and rotavirus VLPs have been shown to target xenografted human bladder tumor nodules or intestinal cells [153, 154] in mice, respectively. However, the natural tropism of VLPs presents a disadvantage when targeting other sites is desirable. In these cases, the VLPs can be retargeted by the display of cell-specific targeting ligands. Cancer cells, one of the most common targets for drug delivery, often overexpress receptors that help promote their growth such as folate, epidermal growth factor, and transferrin receptors [155]. Therefore, VLPs presenting respective ligands have been widely used for targeted delivery and uptake by various cancer cells [156-159]. However, it should be noted that these receptors are also expressed, to a lesser extent, on healthy cells, resulting in cytotoxicity associated with off-target delivery and reduced delivery efficiency due to competion with natural ligands found in circulation [160]. To improve targeting specificity and delivery efficiency, other targeting ligands have been utilized.

Antibodies are a classic example of targeting ligand with exquisite specificity and have been used to retarget VLPs for drug delivery [77, 90]. Full-length antibodies (~150 KDa) are potentially immunogenic and their large size may limit tissue penetration [161]. These drawbacks can be partially addressed by smaller antibody derivatives such as scFv (~30KDa) and Fab (~50KDa). However, antibodies and their derivatives are generally expensive and too big to be genetically fused to VLP proteins (with some exceptions [90, 162]). Due to these limitations, VLPs have been conjugated with other smaller (<20KDa) and less expensive targeting ligands in the form of DNA aptamers [163-165] and peptides [166, 167], which can achieve similar binding specificity and affinity as antibodies [155]. While DNA aptamers still require conjugation to the VLP surface, targeting peptides have the option of being genetically fused to viral capsid proteins [168, 169], greatly simplifying the VLP production. More importantly, large combinatorial libraries of DNA aptamers and peptides are easily created and screened for desired binding affinity and targeting specificity, even when the cell receptor is unknown [170, 171]. This high-throughput engineering approach not only provides the flexibility in the choices of targeting ligands, but also enables the tuning of their binding affinity to cell surface receptors, which is critical in overcoming the "binding barrier" that inhibits the tissue penetration of nanoparticles and VLPs [172, 173]. To this end, lower affinity ligands might be useful for the penetration of solid tumors while high affinity ligands may be more useful for applications such as haematological cancers where tissue penetration is not an issue [160].

2.2.3. Drug Release

The cellular entry and transport of VLPs proceeds according to the life cycle of the native viruses. Initial cell entry by enveloped viruses typically proceeds by fusion of the virus lipid membrane with either the plasma membrane or endosomal membrane [174]. Regardless of the mechanism, the release of the cargo by enveloped viruses occurs almost instantly and directly into the cytoplasm. Non-enveloped (i.e. capsid) viruses generally gain entry into the cell by receptor-mediated endocytosis but cannot escape the endosome by membrane fusion and therefore must rely on non-fusion strategies for release of their molecular payload into the cell [175]. Consequently, capsid VLPs have been engineered to take advantage of the unique endosomal environment for triggered drug release in acidic [176, 177] and reducing conditions [178, 179] typically found within an endosome. In another strategy, fusogenic peptides can be displayed on the capsid VLP surface to insert themselves into the lipid bilayer of the host cell membrane, bypassing endocytosis to achieve direct entry into the cytosol [180, 181]. This approach may be useful for sensitive cargos such as siRNA that are not stable in the harsh conditions of the endosomal environment [182].

Activation strategies encompassing *in situ* stimuli including endosomal pH change, proteases and redox molecules provide limited control over drug release. There has been recent interest in photosensitive chemistries where controlled irradiation of light can enable better and precise spatio-temporal control over the activation and/or release of the drug molecules from nanoparticles [183]. The deep tissue penetration, low cellular toxicity, and highly tunable nature of light therapy (wavelength, intensity, beam diameter, location and duration) make it well-suited for biomedical applications [184]. Therefore, light activated release of molecular cargo from synthetic nanoparticles [185-187], viruses [163, 188] and VLPs [164, 189-191] has been reported as an attractive approach for controlled drug release. In addition to initiating drug release, photo-activation is another effective strategy for the activation of photosensitive therapeutics.

Bacteriophage Qβ [190] and MS2 VLPs [164] have been reported as vehicles for the targeted delivery of light activated porphyrins, which upon photo-induction release singlet oxygen radicals into the cells for photodynamic therapy. Various other photochemical mechanisms have demonstrated controlled drug release from nanoparticles [184, 192, 193], and therefore represent valuable approaches to design VLPs for improved spatio-temporal control of drug release.

3. VLP ENGINEERING APPROACHES

The extent to which VLPs can be engineered to improve antigen and drug delivery is often dictated by the methods available for their synthesis. Therefore, much effort has been devoted to developing novel and increasingly refined approaches for VLP modification with the aim to expand their functional capability. As the main molecular determinant of VLP assembly, targeting, and immune recognition, the protein component of VLPs often serves as the engineering target. By means of genetic fusion, conjugation, and non-covalent interactions, the VLP proteins can be modified with functional entities such as peptides, proteins, drugs or other bioactive molecules. Depending on the engineering objectives, characteristics of the protein(s) present in the VLPs should be evaluated to select the most suitable approach.

3.1. Genetic Fusion

Proteins and peptides can perform diverse functions in a delivery system such as acting as the targeting entity, the stealth reagent, the antigen to be delivered, or the therapeutic drug [23, 130]. Therefore, genetic fusion of the gene encoding the protein/peptides in frame with the gene encoding a VLP protein is widely used to expand the VLP functional capability. Genetic fusion allows for the production of homogeneous VLPs without the additional downstream processing steps that are often required for conjugation (see section 3.2). In the meantime, it presents numerous challenges for researchers. The genomes of viruses are often small and encode few proteins [20], suggesting that viral proteins may have evolved over millennia to embody the optimal sequences necessary for carrying out the viral lifecycle. Therefore, modifying the protein sequences by inserting even short sequences can cause issues with protein folding, VLP assembly, and VLP stability. Even if the resulting fusion protein can assemble into a particle, this does not guarantee that it will present epitopes in the intended conformation [194]. The factors affecting the success of a genetic fusion are discussed below.

Although internally-displayed fusions are useful for T-cell antigen delivery, they are rarely utilized due their inability to interact with the external environment or to allow release of peptide drugs in a bioactive form (with exception [195]). When acting as targeting entities, stealth reagents, or extracellular antigens for immune cells (*e.g.* B cells), the protein/peptide fusions have to be exposed on the VLP surface to interact with the external environment. Surface exposed fusions are commonly inserted into the surface loops in VLP proteins as opposed to the N or C terminus. Compared to terminal fusions, which only constrain the inserted poplypeptide at one end, a surface loop insertion imposes constraints on both ends of the polypeptide. This is usually not a problem for the display of small fusions such as linear epitopes [196], but can cause difficulties for inserting larger proteins, which may be required for applications such as antigen presentation when epitopes have not been identified from the antigenic protein sequence [225].

A survey of the literature suggests that VLPs derived from different viruses exhibit varying degree of tolerance for the size of fusions they can incorporate (Table **2**). In addition, the choice of virus can be further facilitated by careful evaluation of geometries of the fusion sites in the VLP protein to match the distance between the N and C termini of the insert. This is exemplified by the ability of hepatitis B core antigen (HBcAg) VLPs to display a green fluorescence protein consisting of 238 amino acids (aa) on its surface utilizing only a short linker [225, 226], whereas prior studies had only succeeded in inserting short sequences (less than 50 aa) [227]. The same strategy has enabled the functional insertion of the outer surface protein C of *Borrelia burgdorferi* (188 aa) [228], mature IL-33 (158 aa) [80] and envelope domain III of dengue virus (104 aa) [229] into the major immunodominant region of HBcAg with or without short linkers. If the polypeptide fusion to be inserted and the target VLP carrier protein have incompatible geometries, creative engineered linkers can be employed to successfully display the insert. For example, a 256 aa protein with N and C termini at opposing ends could be displayed on the surface of HBcAg using either long, flexible, glycine-rich linkers [230] or a digestible linker that was cleaved *in situ* after coexspression of the corresponding protease [231]. Therefore, rational design of protein linkers aided by detailed crystal structures can circumvent issues of insert conformation. Various flexible, rigid, or cleavable linkers that can be potentially used to enable the insertion of large proteins in a VLP protein have been well reviewed elsewhere [232].

Another factor affecting the success of genetic fusions is the physical properties of the insert and VLP proteins such as charge, isoelectric point, and hydrophobicity. Abidin *et al.* reported that hydrophobic peptide insertions in the VP1 capsid protein caused severe aggregation of the resulting polyomavirus VLPs, and that the introduction of charged aspartic acid residues flanking the insert improved the solubility and recovery [233]. In another study, Bendahmane *et al.* showed that the coat protein of tobacco mosaic virus (TMV) is highly sensitive to the insertion of short peptide sequences that increase the positive charge and isoelectric point of the coat protein, ultimately leading to assembly inhibition and impaired function [234]. Lu *et al.* also reported poor attachment of negatively charged antigens and nucleic acids to the anionic surface of HBcAg VLPs that could be overcome by decreasing the negative charge of the capsid [235]. Although the last study does not involve genetic fusion, these examples illustrate the possibility of augmenting the physical properties of either the insert or the VLP proteins to overcome unfavorable interactions.

Compared to capsid VLPs, enveloped VLPs generally offer a number of advantages for the presentation of large surface fusions. First, at least one terminus of a glycoprotein is often exposed on the surface of enveloped VLPs allowing surface-displayed insertions to be introduced at a terminus rather than a position in the middle of the protein, as is often

Table 2. Common genetic fusion sites in VLP proteins.

Virus	Fusion Protein	Insert Site (aa)	Surface Exposure	Insert Size (aa)	References
Bacteriophage MS2	CP	12-17	High	6-26	[197-200]
Bacteriophage P22	SP_{141}	N Terminus	Internal	163-370	[201-203]
Hepatitis B	Core Antigen	C Terminus	Low	45-64	[93, 204]
		74-83	High	20-256	[93, 205-207]
		N Terminus	Low	36-41	[100, 208]
Human Papilloma Virus	L1	C Terminus	High	8-150	[209, 210]
		133-137	High	13-20	[211, 212]
		281-287	High	8	[213]
Johnson Grass Mosaic Virus	CP	C Terminus	High	8-98	[214-216]
Parvovirus	VP2	N Terminus	High	9-147	[117, 217]
		226-228	High	11	[98]
Potato Virus X	CP	N Terminus	High	9-27	[218-221]
Tobacco Mosaic Virus	CP	C Terminus	High	11-133	[222-224]

the case with surface insertions into proteins of non-enveloped VLPs. Additionally, the mobility of surface proteins in enveloped VLPs creates a more forgiving environment for the incorporation of large fusions and full-length proteins than semi-crystalline, capsid-based VLPs. This is exemplified by the ability of enveloped viruses and their VLPs to form pseudotypes that contain viral proteins from diverse genetic backgrounds co-localized into a single particle [89]. Furthermore, the ecto- and transmembrane domains of viral glycoproteins are often easily interchangable [78, 79, 81-83, 86]. Indeed, large full-length proteins such as murine GM-CSF [70], *Salmonella* flagella [66, 67], murine CD40L [68], and *Escherichia coli* heat-labile enterotoxin B subunit [67] have been genetically fused to the transmembrane domains of HIV-1 [68], baculovirus [68], rabies [67, 70], and influenza [66] proteins.

3.2. Conjugation and Non-Covalent Interactions

New functional capabilities can also be engineered into VLP delivery vehicles by means of conjugation or non-covalent coupling of drugs, targeting ligands, or antigens. Both conjugation and non-covalent interactions circumvent some of the drawbacks associated with genetic fusions such as issues with VLP assembly, VLP stability and undesirable alterations of protein structure [230, 236]. In addition, they both allow for the attachment of multiple proteins (*e.g.* peptide libraries) or non-proteins (*e.g.* haptens [237] or chemical pharmaceuticals [238]). On the other hand, as functionalization via conjugation and non-covalent interactions is often performed *in vitro* after the VLP synthesis, they often add greater process complexity such as lengthy reaction steps [239] and multiple purification rounds [240] that can result in significant losses of VLPs [179]. Compared to genetic fusion, these strategies also produce modified VLPs that are less homogeneous due to chemical linkage and non-covalent coupling processes not being 100% efficient [241, 242]. Methods for VLP functionalization via conjugation and non-covalent interactions have been reviewed elsewhere [243]. The discussion below will place these methods in the context of engineering VLPs for delivery applications.

3.2.1. Conjugation

Conjugation has commonly explored the functional groups of the natural amino acids in the VLP proteins. Lysine and cysteine are the most common residues utilized for covalent modification among others [179, 239, 244, 245]. Lysine, which is statistically about 6 times more common than cysteine in a given protein [246], provides a high degree of modification using N-hydroxysuccinimide (NHS) esters [243]. Through chemical linkage to lysine residues, various VLPs have been modified to maximize drug doses [179], targeting ligand density [240] and antigen density [87]. By modulating the chemical reaction conditions (*e.g.* the ratio of VLPs to desired conjugate), it is possible to achieve some degree of control over the molecular loading/density [247]. Cysteine, on the other hand, provides a more targeted approach due to its relative rarity compared to lysine. For this reason, cysteine is often genetically introduced into protein sites of interest to enable site-specific functionalization [164, 248] using maleimide linkers. However, care must be taken not to introduce unwanted disulfide bonds, in which case the removal of reactable cysteine residues may be necessary [247].

The recent introduction of unnatural amino acids (uAA) into the genetic code allows for site-specific modifications, minimal sequence interference, and the use of reactive moieties that are completely bio-orthogonal (*i.e.* lack cross-reactivity) to the natural amino acids. In addition, conjuga-

tion with uAA can proceed through much more efficient chemical reactions to produce a more homogeneous product than conjugation with the canonical amino acids [241]. For example, uAAs presenting alkyne or azide motifs are especially popular and have been incorporated into Qβ [249, 250], TMV [251], MS2 [250], and HBcAg [235] VLPs to enable the cycloaddition click chemistry. The synthesis of VLPs containing uAA is outside the scope of this review but methods are described elsewhere [252, 253].

Although chemical conjugation has been used for encapsulation [164, 239, 254], the permanence of covalent bonds makes it more suitable for VLP surface functionalization for targeting [159, 165, 240], antigen presentation [247], or exterior drug loading [179], especially in the case of non-protein conjugates. Notably, VLP displaying folate [240] or nucleic acid aptamers [163, 165] have been used to target cancerous cells while other applications have included nicotine conjugates for the treatment of nicotine dependence [237] and the stealthing of VLPs by PEGylation [240]. Reversible chemistries can also be employed to achieve different release kinetics. For example, Aljabali *et al.* showed that CPMV VLPs conjugated to the chemotherapeutic drug doxorubicin by a stable amide bond were taken up by HeLa cells *in vitro* and released the drug after proteolytic degradation associated with the endolysosomal pathway. On the other hand, conjugation of doxorubicin by a labile disulfide bond resulted in most of the drug being released into the culture medium after 24 hours, essentially acting as free doxorubicin [179].

3.2.2. Non-covalent Interactions

Methods of non-covalent functionalization of VLPs have simpler implementation than conjugation, as no chemical reactions are required. A wide range of materials can take part in non-covalent interactions with VLP proteins such as proteins [255], nucleotides [256-258], metals [10, 259], and other bioactive molecules [260]. In addition, the membrane of enveloped VLPs can interact with lipoproteins [71, 90, 261]. The varying strengths of different non-covalent interactions affect their utility for engineering various aspects of VLP delivery vehicles. Relatively strong non-covalent interactions, such as biotin/streptavidin [31] or glycosylphosphatidylinositol-anchored (GPI-anchored) proteins, have been used for surface presentation of targeting [90] or immunostimulatory [71, 261] motifs. However, being weak in nature, most non-covalent interactions do not provide the stability of covalent bonds, making them more suitable for delivery of encapsulated cargos, especially larger molecules that cannot diffuse through the VLP capsid once assembled [159].

The inherent affinities present in VLPs can be explored to encapsulate desirable materials. For instance, the inner surface of the viral capsid is often positively charged and thus amenable to encapsulating genomic materials, therapeutic nucleotides [256-258], and other negatively-charged substrates such as polymers [189] , nanoparticles [262], and enzymes [255]. In addition, the pre-existing mechanisms that viruses use to bind nucleic acids can be repurposed to incorporate diverse cargos in VLPs. Ashley *et al.* took advantage of the ability of MS2 viral capsid to internally bind an RNA sequence known as the *pac* site [263]. Through conjugation of this *pac* site to quantum dots, siRNA, doxorubicin, or ricin toxin-A chain, these cargos were encapsulated in the MS2 capsids by disassembly and reassembly *in vitro* [166]. For a more simplified production strategy, intracellular VLP assembly and cargo encapsulation was accomplished utilizing bi-functional RNA aptamers that bind both Qβ coat protein and an arginine rich peptide (Rev) fused to fluorescent proteins [264] or luciferase and peptidase E enzymes [265].

Native VLP affinities may prove insufficient for the encapsulation of some materials, and therefore some investigators have introduced their own mechanisms to enable new non-covalent interactions for improved encapsulation efficiency. In one study, an internal hydrophobic binding pocket was created by genetically manipulating protein cages derived from the pyruvate of dehydrogenase E2 subunit to enable efficient encapsulation doxorubicin [260]. The genetic introduction of polyhistadine tags provides another strategy for introducing specific interactions for encapsulation of metal-containing cargos such as magnetic nanoparticles [10], quantum dots [266], and nitrilotriacetic-acid (NTA) conjugated compounds [176]. Protein/protein interactions can also be introduced as demonstrated in one study, where the CPMV capsid protein was fused to one strand of a coiled coil motif to enable encapsulation of fluorescent proteins fused to a complimentary coiled coil strand in the CCMV VLPs [267]. These examples demonstrate a rational design approach to introduce non-covalent interactions for efficient cargo encapsulation. When multiple, complex, non-covalent interactions are involved, directed evolution has been employed successfully for additional functional enhancement. In an elegant study, Wörsdörfer *et al.* developed an intracellular encapsulation system in *E. coli* driven by electrostatic interactions between lumazine synthase capsid and a polyarginine tag appended to the C terminus of the cytotoxic HIV protease [268]. When the tagged protease was co-expressed with the capsid proteins, a growth advantage was conferred to cells producing capsids that could better sequester the protease, thereby providing a selective pressure that led to the identification of a mutant VLP with encapsulation capacity increased by 5 to 10 fold [268].

CONCLUSION

VLPs exhibit properties such as self-assembly, natural tropism, and intrinsic immunogenicity that make them an attractive technology platform for antigen and drug delivery. To achieve a satisfactory clinical outcome, however, these properties often need to be augmented, altered, or even masked, which is a challenging task from the design and synthesis perspectives. For antigen delivery, VLPs have demonstrated potential as witnessed by the FDA approval of VLP-based hepatitis B and HPV vaccines. However, outside of these examples, VLPs have seen very limited success often due to their inability to elicit effective immune responses, especially T-cell responses that are critical for long-lasting immune memories against many pathogens and cancers. Many recent studies have focused on enhancing T-cell responses by the inclusion of immune activators found in viral replication processes or other pathogens in the VLP design. In addition, mutation-prone viruses such as influenza and HIV as well as non-viral targets such as malaria and cancers require the immunogenic presentation of cross-protective

epitopes. Rational epitope design aided by identification of immunogenic insertion sites, epitope flanking sequences, and multivalent epitope display has increased the versatility of VLPs and has allowed them to initiate tailored and specific immune responses to a variety of targets. In the case of drug delivery, VLPs still face largely the same challenges of synthetic delivery vehicles in that they must evade the immune system, specifically target the cells of interest, and deliver the therapeutic cargo. Therefore, most of the recent progresses in engineering VLPs for drug delivery have taken advantage of the innovations in the field of synthetic nanoparticle-based delivery. Additionally, the proteinaceous nature and self-assembly of VLPs gives them a distinct advantage over synthetic nanoparticles in that they are very homogeneous, biodegradable, and easily subjected to predictable alterations through genetic manipulations.

Despite these recent advancements, VLPs still present a few drawbacks compared to other delivery systems, most notably their synthesis and modification. VLP synthesis involves several complex variables such as the host of choice, the complexity of the VLP proteins, and purification (reviewed elsewhere [24, 269, 270]). Further, VLP modification can be achieved by genetic alteration; however, its success is largely unpredictable and highly dependent on the choice of VLP, the site of modification, and the characteristics of the fused peptides (size, hydrophobicity, isoelectric point, *etc.*). Although conjugation is a relatively robust alternative to genetic manipulation for VLP modification, it is usually limited to chemistries based on the canonical amino acids. The introduction of non-natural amino acids into VLPs allows for simplified production, increased homogeneity and site-specific modification but requires specialized production capabilities.

Regardless of the specific application, recent VLP designs have benefited significantly from an ever-increasing understanding of the immune system, pathogen-host interactions, and cancer biology. Likewise, without the advancements in protein engineering, materials science, and chemistry, the synthesis of these VLP designs would not be possible. Therefore, only continued breakthroughs at these interdisciplinary interfaces will enable VLPs to achieve their clinical potential.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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