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Affinity selection of epitope-based vaccines using a bacteriophage virus-like particle platform

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Display of epitopes on virus-like particles (VLPs) is a highly effective technique for enhancing the immunogenicity of antigens that are poorly immunogenic in their native context. VLP-based vaccines can be used to elicit long-lasting, high-titer antibody responses against diverse target antigens, even self-antigens. Most VLP platform-based vaccines are rationally engineered; specific target epitopes or domains are arrayed so that they are displayed at high-valency on the surface of VLPs. In this review, we describe an alternate technique for vaccine discovery using VLPs. This strategy, analogous to filamentous phage display, allows bacteriophage VLP-based vaccines to be identified from a vast library of potential vaccines by affinity selection. This technology integrates epitope discovery and immunization functions into a single platform.

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Introduction

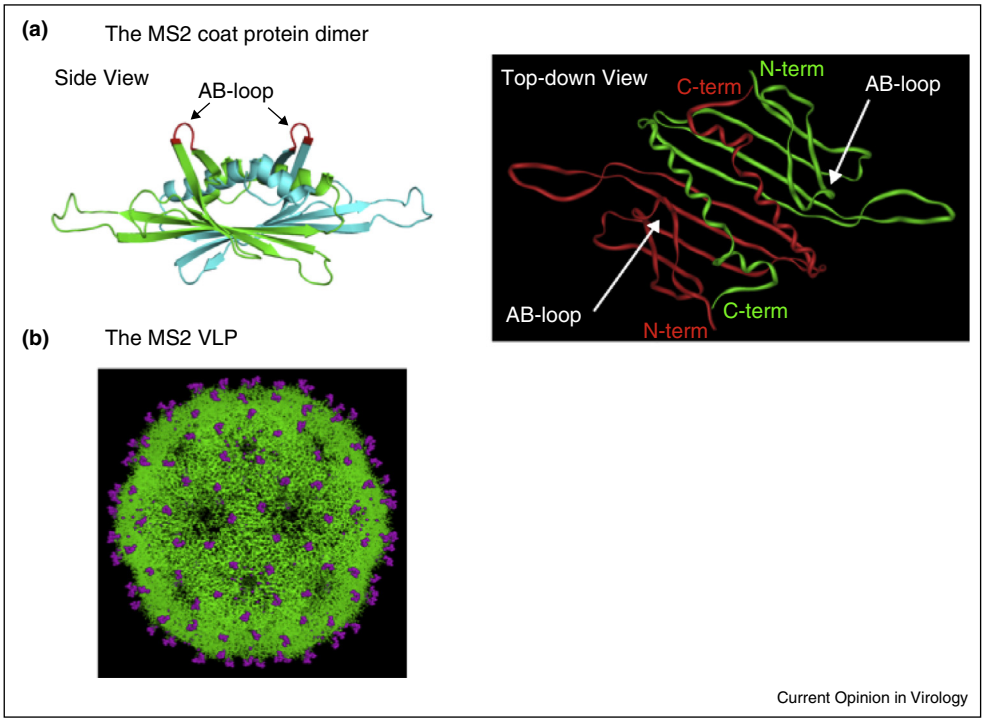
Many viral structural proteins have an intrinsic ability to self-assemble into virus-like particles (VLPs). VLPs can be used to produce vaccines against the viruses from which they are derived, but they also show great promise for presentation of epitopes from other sources, even when those targets are normally poorly immunogenic. VLPs make effective vaccine platforms because they possess a number of physical features that enhance their immunogenicity (reviewed by [1]). Antigens need to traffic to lymphoid organs in order to initiate immune responses, and the size of VLPs (usually between 20 and 200 nm in diameter) is optimal for transport to the subcapsular region of lymph nodes where antigen initially encounters B cells [2•]. Once they traffic to the lymph node, the multivalent, highly repetitive structure of VLPs is particularly important for enhancing interactions with B cells. Antibody production is initiated by interactions between antigen and its cognate B cell receptor (BCR) on the surface of naïve B cells. Antigens that have highly dense, multivalent structures, such as VLPs, can activate B cells and induce antibody responses at much lower concentrations than monomeric antigens, and without the requirement for exogenous adjuvants [3–6]. In particular, antigens that contain repeated epitopes with a spacing of 50–100 Å, which includes most virus particles, optimally induce B cell responses [7•,8•,9], although the precise contributions of antigen spacing and particle size to immunogenicity have not carefully been quantified. These highly multivalent antigens provoke extensive cross-linking of the BCR and stimulate B cell proliferation, migration, and upregulation of the expression of molecules that permit subsequent interactions with T helper cells [6], the generation of memory B cells, and the long-lived plasma cells that produce high-titer antibody responses for years to decades after vaccination [10].

VLPs, such as those derived from Human Papillomavirus (HPV), can be used to produce vaccines against the viruses from which they are derived [11•]. However, this review will focus on the use of VLPs as platforms to display heterologous target antigens. An impressive accumulation of data demonstrates the effectiveness of VLP presentation as a method for boosting antibody responses to diverse molecules, including epitopes derived from pathogens, chemical agents, and even self-antigens [1,12]. Although VLPs derived from diverse virus types can serve as effective platforms for antigen display [13], here we will focus on the use of bacteriophage VLPs; in particular a family of related spherical RNA bacteriophages in the Leviviridae family. These bacteriophage VLPs have certain engineering advantages for antigen display and have already been shown, in human clinical trials, to effectively serve as highly immunogenic platform for boosting antibody responses against diverse targets.

Bacteriophage VLPs as a vaccine platform

Leviviruses, including MS2, PP7, AP205, and Qß, are small icosahedral bacteriophages with short (<4 kb) single-stranded (+)-sense RNA genomes encoding just four proteins. These viruses encode a single structural protein, coat protein, which forms a homodimer and then self-assembles into a 27 nm-diameter icosahedral particle consisting of 90 dimers (Figure 1). These VLPs can be produced rapidly and in large amounts in bacteria, and are

Figure 1



The structure of the MS2 coat protein shown from two views. The left panel shows an edge-on view of coat protein. The two polypeptide chains are colored blue and green, with the three amino acids of each of the AB-loops shown in red. The panel on the right shows a top–down view of the dimer. The locations of the AB-loop, N-terminus, and C-terminus are indicated. We can generate VLP libraries by inserted random peptides at either the N-terminus or within the AB-loop of the coat protein single-chain dimer.

easily purified. Bacteriophage VLPs also naturally encapsidate single-stranded RNA, which can serve as a molecular adjuvant through engagement of toll-like receptors 7/8 [14,15]. In many cases the VLP structures are understood in great detail, and their molecular biology and genetics have been extensively characterized. All of these features facilitate the engineered presentation of specific peptide and protein antigens on the bacteriophage surface in a highly immunogenic format.

RNA phages and their VLPs have been utilized in two major modes for immunogenic display of epitopes, chemical conjugation and genetic fusion. Each has its advantages. Bacteriophage VLPs present a repetitive array of reactive groups that may be utilized with commercially available reagents to cross-link an immunogen to the particle surface. The advantages of this approach are firstly, both peptide and non-peptide immunogens can be presented in dense repetitive arrays, secondly, full length polypeptide antigens can be conjugated to the particle in their native conformations, thus avoiding the folding and assembly problems that sometimes accompany genetic fusion to viral structural proteins, and thirdly, a wide range of molecular sizes is compatible with chemical conjugation. Larger targets increase the likelihood that immunization will induce a broad range of antibodies that recognize both linear and conformational epitopes on the target molecule, although a potential disadvantage is that critical epitopes cannot be specifically targeted. Cytos, a Swiss biotechnology company has used the chemical conjugation approach to develop a number of Qß bacteriophage VLP-based vaccines. Several of these vaccines have entered human clinical trials and have demonstrated favorable safety and immunogenicity profiles [16,17••,18].

Genetic insertion of a foreign sequence into viral structural protein enables its display on the VLP surface. Although this technique is limited to protein targets, successful incorporation of an epitope into a VLP guarantees that the antigen will be displayed in the same structural environment and at high density on the particle surface. This technique also has advantages from a manufacturing standpoint; chimeric particles can be purified using the same well-established methods used to purify unmodified parental VLPs. Nevertheless, it is a common observation that peptide insertions into viral capsid proteins often result in protein folding failures that interfere with VLP assembly. To solve this problem, we have engineered a version of a bacteriophage coat protein that is much more stable thermodynamically and

dramatically more tolerant of foreign insertions [19]. Taking advantage of the close physical proximity of the N-terminus of one subunit to the C-terminus of the other, we duplicated the coat coding sequence, and fused the two copies in a single reading frame. This single chain dimer coat protein is highly tolerant of foreign peptide insertions at several sites on the surface of the VLP [20–23]. When expressed from a plasmid, recombinant coat protein readily self-assembles into a non-infectious VLP. These engineered VLPs consist of 90 coat protein dimers, can be expressed from plasmids in *Escherichia coli* as about half of total soluble protein and are rapidly purified in a single chromatographic step [24]. Like others have shown using similar VLP-based systems [25], we can engineer vaccines by displaying specific, previously identified epitope on the surface of VLPs. We have developed a broadly protective Human Papillomavirus vaccine using this approach [15]. However, an additional advantage of this system is that we can create very large libraries of bacteriophage VLPs displaying random peptide sequences. We can then use these libraries for vaccine discovery using a methodology that is described in more detail below.

Eliciting targeted responses against critical epitopes

The conventional view is that vaccines should elicit broad polyclonal responses against pathogens rather than targeting individual neutralizing epitopes. In part, this view was due to the poor immunogenicity of peptide-based vaccines. Until the development of platform-based technologies it was nearly impossible to elicit peptide-specific responses of significant magnitude and longevity to have prophylactic effects. In addition, most pathogens have developed strategies to evade immunity by presenting epitopes to the immune system that can readily undergo antigen variation, while hiding highly conserved sites that are essential for protein function. Until recently, it was unclear whether it was even possible to target these conserved domains. However, a number of groups have used sensitive techniques to isolate rare human monoclonal antibodies that target conserved epitopes and have broadly neutralizing activity. The existence of these broadly neutralizing monoclonal antibodies against HIV, Influenza, Respiratory Syncytial Virus (RSV), and other pathogens [26,27] and detailed structural information about their epitopes indicate that it may be possible to identify immunogens capable of eliciting similar antibody responses. Unfortunately, there has been little success in translating the knowledge of these epitopes into useful vaccines, primarily because these epitopes are poorly immunogenic in their native context. Most promising have been attempts to design protein scaffolds that accurately mimic the structure of critical viral epitopes (including epitopes from HIV, influenza, and RSV) but also allow them to be exposed in a format that is more conducive to activation of antibody responses. To date, most of the epitope scaffold immunogens designed by these methods have induced structure-specific antibodies but have failed to induce neutralizing antibodies [28,29]. In a breakthrough, however, Correia and colleagues recently used a computational design method to successfully engineer an immunogen that elicits potent neutralizing antibodies against RSV [30••].

A VLP platform for vaccine discovery

As an alternative to this rational design approach for targeting specific epitopes, we have developed a more empirical approach to vaccine discovery. Using a system based on bacteriophage MS2 VLPs, we have developed a vaccine discovery technology that integrates epitope identification and immunogenic presentation of epitopes on a single platform. This technology is inspired by the method called phage display, but differs from it in important respects. The basic idea is to create vast libraries of random peptide sequences that are displayed on a bacteriophage structural protein. Traditional filamentous phage display typically displays peptides on the pIII protein of M13 phage. Screening of M13 phage libraries using mAbs and polyclonal sera has facilitated the identification of numerous specific epitopes and mimotopes [31,32•]. However, filamentous phage are typically poor immunogens because they do not readily present foreign peptides at the high densities required for potent immunogenicity [33]. As a consequence, the use of M13-identified epitopes as vaccines requires that the peptides be produced synthetically and then linked to a more immunogenic carrier protein. The display of peptides in a new structural context unrelated to the filamentous display frequently lose the ability to induce antibodies with activities mimicking those of the selecting antibody [34]. Therefore, the ability of our VLP technology to identify epitopes on the same structural platform to be used later in their presentation as a vaccine has critical advantages for vaccine discovery.

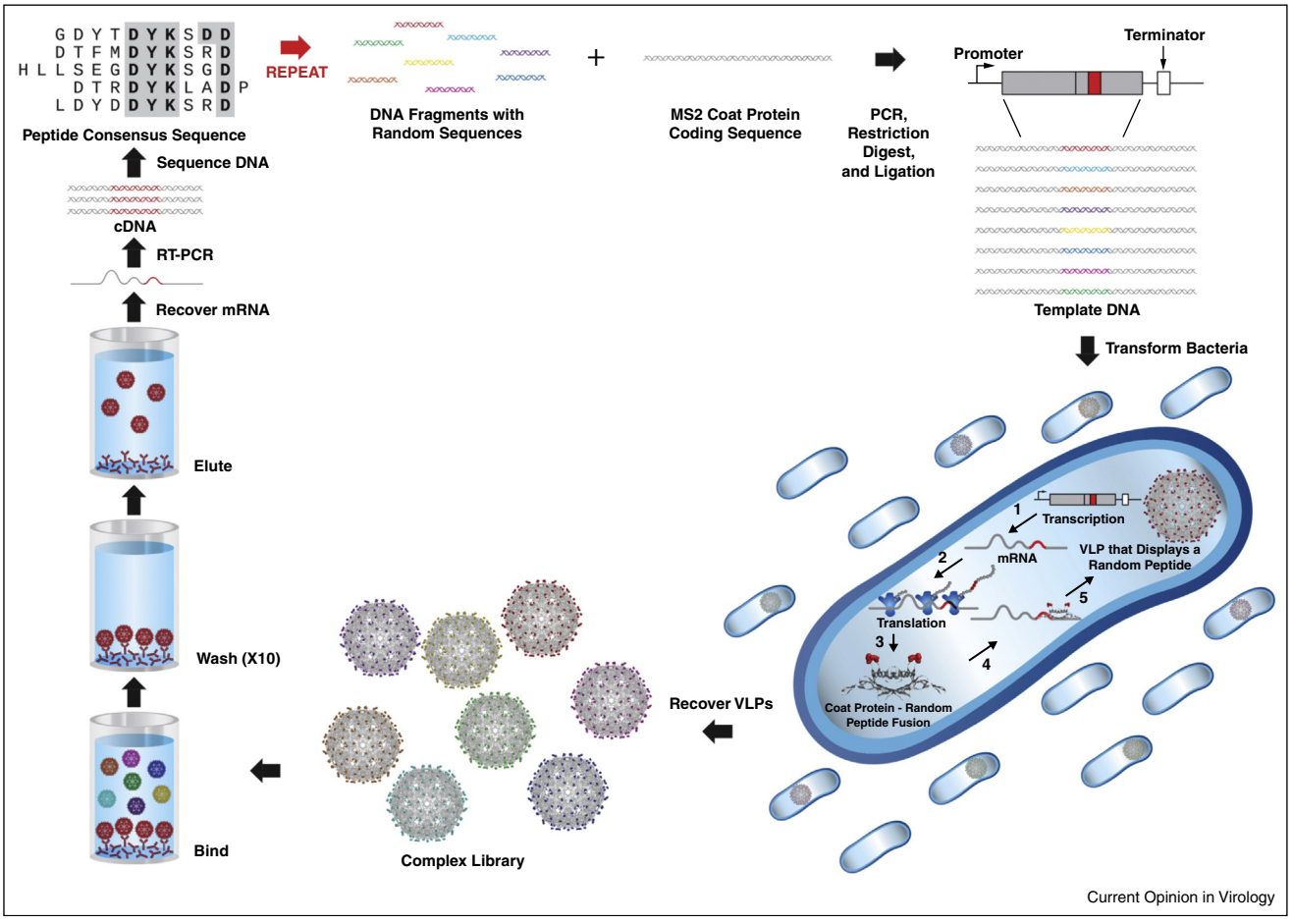
The use of the MS2 VLPs for affinity selection applications depends on three key features: firstly, a surface-exposed site in coat protein that tolerates insertions without disruption of coat protein folding or VLP assembly, secondly, the encapsidation of nucleic acid that encodes the coat protein and any guest peptide it displays, allowing recovery of the genetic material post-selection and thirdly, the ability to create very large diverse libraries of VLPs. To satisfy the first criterion, we engineered a single-chain dimer version of coat protein described above. The second requirement is satisfied by the ability of recombinant VLPs to encapsidate the mRNA that encodes coat protein and any guest peptide it carries [20,22]. Packaging of coat-specific RNA establishes the genotype/phenotype linkage that makes affinity selection possible. The third requirement, library construction, has been facilitated by creation of a plasmid vector that has allowed us to construct highly complex libraries of VLPs displaying random peptides. Using this system, we have produced libraries with complexities in

excess of 10^{10} individual members with the random sequence of $[NNS]_{6–15}$ (i.e. individual plasmid libraries displaying either random 6-amino acid, through 15-amino acid peptides). The nucleotide sequence NNS [N = any base, S = G or C] allows us to encode all 20 amino acids and minimizes the likelihood of stop codons. The generation of these complex libraries at the level of plasmid DNA allows us to produce VLPs displaying the random peptide sequence in a surface-exposed loop of the coat protein (the AB-loop) or at the exposed N-terminus, which can then be subjected to affinity-selection, typically using a monoclonal antibody (mAb) [21••]. Selected sequences can be recovered by RT-PCR, used to regenerate a library of selectant plasmids, and then this process can be repeated iteratively (Figure 2). Additionally, we can monitor the status of selection in each round by amplicon-mediated deep sequencing techniques, allowing a comprehensive view of the peptides obtained through the selection process.

Using antibodies to guide vaccine development

The ultimate goal of this system is to take advantage of the growing number of broadly neutralizing antibodies and to affinity select VLPs that can, in turn, elicit potent antibody responses with activities that mimic the selecting mAb (Figure 2). We have previously validated the use of the MS2 affinity selection system against model antigens [21••]. More recently, using mAbs against Nipah Virus (NiV, unpublished data), the malaria blood stage antigen Rh5 [35•], and the *Staphylococcus aureus* quorum sensing peptide AIP4 [36], we have used this affinity selection technique to identify VLPs that elicit antibodies with neutralizing activity.

Figure 2



VLP affinity selection. Large (10^{10}–10^{11} individual members) libraries of VLPs are constructed at the plasmid level by Kunkel mutagenesis. These libraries are used to transform *E. coli* in order to generate complex libraries of VLPs displaying random peptides sequences. Upon affinity selection using a monoclonal antibody or polyclonal sera, RNA is isolated and amplified from VLPs by RT-PCR and then subcloned into the MS2 coat protein expression vector. After each round the progress of selection can be monitored by Ion Torrent deep sequencing or by sequencing individual clones. This process can be repeated iteratively with increasing stringency to find the highest affinity binders.Figure courtesy of Carlee Ashley, Sandia National Laboratory.

Using a neutralizing mAb against the NiV, we performed affinity selections using a mixture of VLP libraries displaying random peptides. Sequencing of selected VLP inserts identified a peptide that extensively matched a sequence within the NiV G-protein itself. This shows the utility of our system to identify and map linear epitopes. Serum from mice immunized with one of the VLP selectants strongly neutralized cellular entry of a luciferase-producing NiV-G pseudotyped vesicular stomatitis virus. For the blood-stage malaria antigen Rh5, we performed selections using an anti-Rh5 mAb (5A8) with high activity in *Plasmodium falciparum* growth inhibition assays (GIA). The selected VLPs elicited antibodies that bound to recombinant RH5 in ELISA and recognized native RH5 in rhoptries (by immunofluorescence staining). Most importantly, this sera potently blocked *P. falciparum* invasion of erythrocytes in an *in vitro* GIA assay [35•].

We also used this affinity selection system to identify a vaccine that could elicit inhibitory antibodies against auto-inducing peptide 4 (AIP4), an important *S. aureus* virulence factor. AIP4 is a short secreted cyclized peptide produced that is a key signal in activating expression of a host of *S. aureus* virulence factors. Using a selecting mAb (AP4-24H11) that blocks the activity of AIP4 [37], we identified eight VLPs that bound specifically to the antibody and were competed off with bioactive cyclic AIP4, but not linear AIP4 peptide. These VLPs displayed peptides that had no primary sequence homology to the AIP4 peptide, suggesting that they were structural mimics of the AIP4 epitope. Mice immunized with two of the VLPs (VLP-2 and VLP-4) had decreased ulcer size upon intradermal challenge with an AIP4-expressing *S. aureus* strain as well as decreased levels of virulence factors and decreased virulence associated cytokine production [36].

Structural considerations — identification of linear epitopes and mimotopes

Filamentous phage display has often been used to identify linear epitopes, but it has been possible in some cases to utilize affinity-selection to isolate mimotopes, molecular mimics of epitopes [38]. Sometimes it has been possible to mimic the structures of complex, conformational epitopes, and occasionally the peptides identified using the process are even able to elicit antibodies that react with the original epitope in its native environment [39–41]. However, in many cases these antibodies react well with the immunizing synthetic peptide, but do not recognize the epitope in its native environment. Because the MS2 VLP platform allows both affinity selection and immunization to be carried out on a single structural framework, we think that it is likely that this system will increase the frequency with which we can identify mimotopes able to induce a desired antibody response. Furthermore we display our peptide libraries in either a constrained, cyclical manner in the AB-loop or in an unconstrained, linear fashion at the N-terminus of the coat protein. This greatly increases the conformational diversity of our library display system, which may lead to more opportunities to identify immunogenic mimotopes.

Our experience performing selections using the mAb against AIP4 serves as a case in point. These selections resulted in the identification of eight VLPs displaying peptide sequences that were, for the most part, unrelated to one another. In addition, none of the selected peptides had any sequence similarity to the AIP4 peptide, suggesting that the selectants were mimotopes of AIP4. While two of the selectants elicited antibody responses that blocked AIP4-mediated pathology, the other selectants did not neutralize AIP4 activity. These selectants likely represent peptides that can bind to paratopes on the selecting mAb, but do not represent true immunologic mimics of the AIP4 epitope.

We suspect that even linear epitopes can exist in a conformationally optimized context. For, example, after two rounds of selection with the anti-RH5 antibody we obtained a virtually homogeneous population of selectants, each of which displayed the 8-mer sequence, SAIKKPVT [35•]. This peptide contains a 4-amino acid identity to a sequence near the Rh5 N-terminus (AIKK), suggesting this site represents the epitope recognized by this mAb. The fact that affinity-selection so clearly favored one sequence over the other members of the sequence family (some of which were identified after sequencing round one selectants), suggests that amino acid residues outside the AIKK identity may serve to most effectively present the core epitope to the antibody in the constrained context of the coat protein AB-loop.

Summary

The MS2 VLP display system has a number of advantages for vaccine development. The VLP has a simple chemical composition, is stable, and is easy to produce and purify in large amounts. It allows affinity selection of peptides in a highly immunogenic format, thus integrating the epitope discovery and vaccine functions into a single particle. Its amenability to construction of complex random-sequence and antigen-fragment libraries facilitates its application to affinity-selection on both mono- and polyclonal antibodies. By targeting specific epitopes, it can elicit specific high-titer responses against vulnerable conserved domains of pathogens, and can also avoid complications associated with non-neutralizing and infection-enhancing antibody responses. A major limitation of the VLP system is that currently we can only display short peptides on VLPs. This may make it difficult to identify mimics of more complex conformation epitopes or very large epitopes. Nevertheless, we have presented evidence that this approach can open a path to vaccines based on linear epitopes as well as mimotopes of protein

conformational epitopes. The empirical approaches described here have the potential to substantially accelerate the development of new vaccines against diverse targets.

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