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# Virus-Like Particle and Nanoparticle Vaccines

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## INTRODUCTION

Currently licensed vaccines can be divided into three broad categories: (1) genetically attenuated “live” pathogens that remain replication-competent but have greatly diminished ability to cause disease; (2) “killed” pathogens that have been chemically inactivated; and (3) isolated “subunit” fragments of pathogens. The first two categories are represented, respectively, by the classic oral (Sabin) and injectable (Salk) poliovirus vaccines. During their initial development, it was quickly appreciated that the live-attenuated immunogen confers durable immunity against wild-type poliovirus replication in vaccinated individuals (i. e., sterilizing immunity). In contrast, the inactivated immunogen generally provides more transient protection and primarily serves to prevent the spread of poliovirus to the central nervous system, as opposed to fully preventing viral replication in the gut. These patterns are recapitulated in the responses elicited by other vaccines. For example, live-attenuated immunogens, such as measles and smallpox vaccines, provide long-term sterilizing immunity, while killed pathogen vaccines induce more transient responses that require periodic boosting.<sup>1</sup> The differences can be at least partially attributed to the fact that live immunogens display a variety of pathogen-associated molecular patterns (PAMPs) that engage innate antimicrobial sensors, such as toll-like receptors (TLRs).<sup>2</sup> This contrasts with replication-incompetent immunogens, which lack these innate “danger” signals and therefore induce a diminished, delayed, and less durable immune response.

Subunit vaccine responses tend to follow the same pattern as killed pathogen vaccines. A familiar example is the tetanus toxoid vaccine, which requires periodic boosting to maintain protective levels of tetanus toxin-neutralizing

antibodies. In the current review, we focus on a new class of more immunogenic subunit vaccines in which the immunogen of interest is organized into a multivalent array that resembles the surface of geometrically rigid virions. The resulting immunogens, termed “virus-like particles” (VLPs), are typically composed of recombinant virion structural proteins that self-assemble into nanoparticles in the absence of viral genomic DNA or RNA. It is now well established that properly assembled VLPs can induce potent, diverse, and durable serum antibody responses that are comparable to responses against live or live-attenuated pathogens. The remarkable potency of rigidly multivalent subunit immunogens has been most comprehensively documented in studies of current commercial VLP-based vaccines against human papillomaviruses (HPVs).<sup>3,4</sup> An emerging view is that at least some types of VLP immunogens, including current HPV vaccines, fall into a special class of subunit vaccine that can show the same potency and durability as live-attenuated immunogens. Recent clinical trials have demonstrated that VLP immunogens can even be potent enough to overcome the self-tolerance of human B cells, allowing the induction of auto-antibody responses of therapeutic interest.<sup>5,6</sup>

Not all VLP antigens show the same high immunogenicity as HPV vaccines. For example, the commercial hepatitis B virus (HBV) vaccine, which is comprised of VLPs assembled from the HBV surface (S) envelope protein, induces relatively poor antibody titers that decline over time.<sup>7</sup> It may be that the S protein is arrayed in a relatively mobile and flexible way and thus fails to qualify as a geometrically rigid multivalent structure. Similar flexibility issues might explain why emerging VLP-based vaccines against Dengue virus appear not to have elicited stable high-titer neutralizing antibody responses in clinical trials.<sup>8</sup>

## HUMORAL RECOGNITION OF VIRUSES

Nearly all known animal viruses have virion diameters in the 10–1000 nanometer (nm) range. Smaller (<500 nm) virions can passively traffic through lymphatic system and can also be directly taken up by immune cells such as macrophages, dendritic cells, and B cells. Either pathway efficiently brings antigens to the lymph nodes, where adaptive immune responses can be induced. In the setting of the lymph node, successfully transported virions are taken up by specialized macrophages in the subcapsular sinus of the draining lymph node. Virions are then transferred to follicular dendritic cells (FDCs) where they are directly presented to B cells. Durable attachment to FDCs can be enhanced by decoration of the presented antigen with low affinity IgM antibodies or complement components.<sup>9</sup> When antigen-specific surface immunoglobulins (Igs) on the B cell (B cell receptors (BCRs)) recognize the antigen, these BCRs form microclusters to promote a signaling cascade that activates the B cell. The recognized antigen can then be endocytosed and processed for presentation on an MHC class II protein, which in turn recruits the help of antigen-specific CD4 + T cells.

## RIGIDLY MULTIVALENT IMMUNOGENS: THEORY AND PRACTICE

In 1993, Bachmann, Zinkernagel and colleagues showed that B cells recognize multivalent antigens with repetitive 50–100 angstrom (Å) spacing in a distinctive way that leads to potent induction of cell activation and survival signals.<sup>10</sup> Rigid epitope spacing of this type is commonly found on the surface of many types of virions but is rare among vertebrate self antigens. In fact, some of the few host protein complexes with 50–100 Å spacing are immune gene products, such as IgM antibodies, complement component C1q, and collectins like the mannose binding lectin. Repetitive spacing can thus be seen as a type of PAMP that B cells have evolved to specifically detect via engagement of cell surface BCRs. Strong B cell responses can be elicited by other types of PAMP signals through engagement of Toll-like receptors (TLRs) or by persistent interaction with FDCs through IgM or C1q. However, the Bachmann-Zinkernagel effect operates independently of conventional adjuvant signaling. Instead, epitope spacing and rigidity appear to be the primary variable in triggering this effect.<sup>11</sup> There is some indirect evidence that multivalent immunogens trigger a special type of signaling separate from adjuvant effects. This comes from the observation that it is extremely difficult or perhaps impossible to generate hybridomas from mice immunized with rigid virus-like particles under dosing schedules typically used for production of monoclonal antibodies.<sup>12,4,13</sup>

Using mouse model systems, Übelhart and colleagues have recently proposed a model in which soluble monovalent antigens trigger signaling primarily through IgM-isotype BCRs, while multivalent antigens are able to trigger signaling through IgD-isotype BCRs found on fully mature B cells.<sup>14</sup> The hinge region of each surface Ig isotype is critical for determining sensitivity to rigidly multivalent antigens. A current hypothesis is that internalization of multivalent antigens via IgD may lead to enhanced presentation of the antigen to CD4+ T cells.<sup>15</sup>

Initially, it was unclear whether the Bachmann-Zinkernagel effect would be applicable to humans. The extraordinary success of the current rigid multivalent VLP vaccines against HPV has confirmed its relevance to human populations. Participants in early trials of HPV vaccines who received only one dose of the vaccine demonstrated high titer neutralizing antibody responses that plateaued for at least six years after vaccination (Fig. 4.1).<sup>16,17</sup> Booster doses of the HPV vaccine cause only incremental increases in antibody titers. This type of response stands in dramatic contrast to more conventional immunogens, such as traditional tetanus toxoid vaccines, where booster doses trigger major, albeit temporary, rises in neutralizing antibody titers (Fig. 4.2).<sup>18</sup> A possible interpretation is that a given B cell's initial contact with the VLP immunogen triggers a differentiation pathway—presumably mediated by IgD BCRs—that predisposes the cell to expand and differentiate into a set of

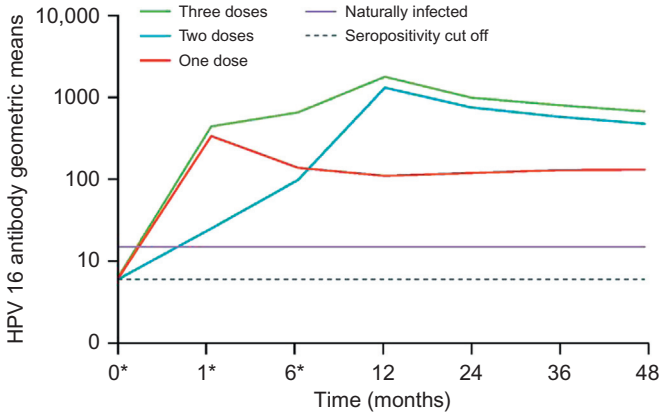


FIGURE 4.1 Serological responses to an HPV VLP vaccine.

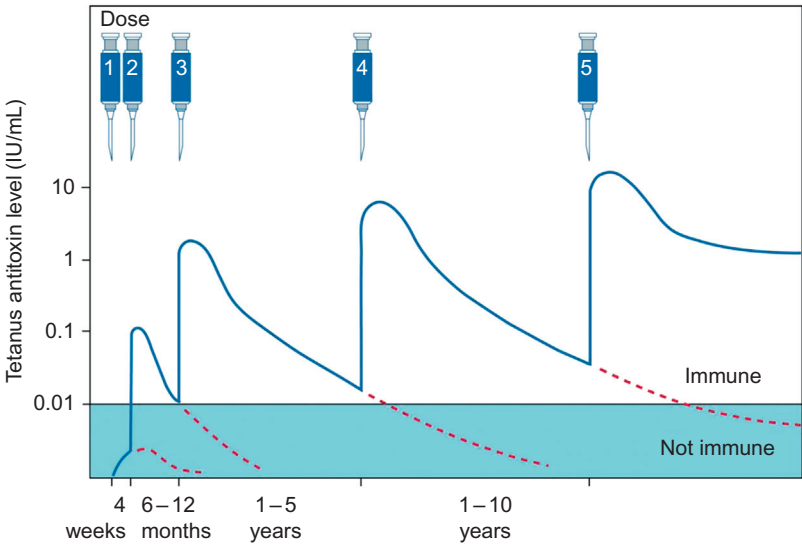


FIGURE 4.2 Typical serological response to tetanus toxoid vaccine.

long-lived plasma cells that reside in the bone marrow without necessarily passing through a durable memory B cell phenotype.

### NATURALLY ICOSAHEDRAL VIRIONS

The success of current HPV vaccines demonstrates that recombinant particles assembled from naturally rigid icosahedral virion proteins can provide

durable antibody-based sterilizing immunity to pathogenic viruses. A surprising feature of HPV vaccines is that they induce antibody responses capable of cross-protecting women against infection with HPV serotypes that aren't present in the vaccine preparation.<sup>19</sup> A newer version of the HPV vaccine contains VLPs that represent nine different HPV serotypes, and the presence of multiple antigens in the single vaccine has little impact on the induction of responses to all nine types.

The outcome of HPV VLP vaccines suggests a promising path for developing VLP vaccines for other diverse families of viruses with naturally rigid icosahedral virions. The knobby nonenveloped icosahedral surface of polyomavirus virions is one example. Polyomaviruses are so similar to papillomavirus virions that the two viral families were originally classified into a single viral family, the *Papovaviridae*, based on their appearance in electron micrographs. VLPs based on two pathogenic human polyomaviruses known as BKV and JCV are currently under investigation as possible vaccine immunogens.<sup>20–22</sup> In a “compassionate use” case study, an elderly immunodeficient patient given an experimental JCV VLP vaccine demonstrated a remarkably high JCV-neutralizing serological titer of approximately 25 million. The result is consistent with the idea that polyomavirus VLP immunogens may be at least as potent as current HPV VLP vaccines.

A number of other important human pathogens have naturally rigid icosahedral surfaces. One example is Chikungunya virus, a member of the *Alphavirus* family. VLPs assembled from the icosahedrally rigid envelope glycoprotein of Chikungunya virus have elicited high-titer neutralizing antibody responses in both animal models and in human trials.<sup>23,24</sup> Noroviruses represent another promising target for VLP vaccine development. Results from clinical trials of VLP-based vaccines against norovirus are currently under consideration by the US Food and Drug Administration. The recent success of a VLP-based vaccine against canine parvovirus suggests that comparable VLP-based vaccines against human parvovirus B19 should be feasible.

Human rhinoviruses are naturally icosahedral non-enveloped viruses and thus may represent another facile target for VLP vaccine development. Rhinoviruses collectively cause 70% of mild upper respiratory infections in humans. Although there are at least 100 known rhinovirus serotypes, it may eventually be possible to construct a panel of recombinant rhinovirus VLPs for inclusion of a multi-VLP vaccine analogous to the nine-serotype HPV VLP vaccine. Based on the assumption that a multi-VLP rhinovirus vaccine immunogen would, like HPV VLP vaccines, elicit at least some degree of cross-neutralization of rhinovirus serotypes not contained in the vaccine, this approach could theoretically offer broad protection against the common cold. The project could serve to pave the way for the development of VLP-based vaccines against viruses in the Picornaviridae family, including emerging pathogens such as EV68, which has been associated with flaccid paralysis.

As mentioned above, VLPs based on flaviviruses, such as dengue virus, have so far elicited poor immunogenicity despite the potentially icosahedral display of the surface pre-membrane (prM) and envelope (E) proteins. Structural studies of dengue virions and VLPs indicate that E can exist in a range of highly distinct conformations. A major conformational shift is triggered by proteolysis of the PrM by the cellular protease furin. Structure-guided design and engineering of more complete proteolysis of recombinant dengue VLPs might result in more rigid display of a uniformly “mature” herringbone configuration of E proteins, thereby more effectively triggering the Bachmann-Zinkernagel effect. The initial work that revealed the Bachmann-Zinkernagel effect relied on use of a vesicular stomatitis virus (VSV) model system. Like other members of the viral family *Rhabdoviridae*, the surface envelope glycoprotein (G) of VSV is not icosahedrally ordered. VSV G is, however, arrayed at very high density on the bullet-shaped surface of the virion. This suggests that strict geometric rigidity is not necessarily required to trigger the Bachmann-Zinkernagel effect and that other forms of extremely high-density polyvalency can suffice.

## SYNTHETIC STRUCTURE-BASED NANOPARTICLE PLATFORMS

Advances in structural biology and nanotechnology have opened new opportunities for the design of synthetic vaccine immunogens on the basis of atomic-level information. Designs mimic a number of biological macromolecules including cargo proteins, multimeric enzymes, subviral particles, viral capsids, and other self-assembling proteins. Typically, vaccine antigens are displayed on these scaffolding molecules to form defined nanostructures such as nanoparticles, nanotubes and nanosheets. Vaccine antigens are typically conjugated to these scaffolds by genetic fusion either (i.e., single polypeptide construct), through adaptor molecules (e.g., streptavidin-biotin interactions) or through covalent chemical crosslinking. Each approach has advantages and challenges, and the best approach may vary depending on the target immunogen. Genetic fusion is not always feasible as many fusion partners disrupt the assembly of the underlying multimeric scaffold protein. On the other hand, genetic fusion systems allow precise control over antigen orientation and occupancy (i.e., surface density of the antigen) and can be generated with simple production pipelines. Genetic fusion systems have recently been used to create self-assembling vaccine immunogens for the influenza virus<sup>25,26</sup>, HIV-1,<sup>27</sup> respiratory syncytial virus (RSV),<sup>28</sup> and Epstein-Barr virus (EBV).<sup>29</sup> In the influenza nanoparticle studies, the viral hemagglutinin (HA) envelope protein or truncated HA stem protein was able to trimerize at the three fold symmetric axes of a self-assembled nanoparticle based on a bacterial ferritin protein. Many other types of viral envelope proteins exist as homotrimers on the surface of natural virions. Examples of viruses with trimeric envelope proteins include HIV-1, RSV, human metapneumovirus, coronaviruses (e.g., Middle Eastern

respiratory syndrome virus) and Ebola viruses. Thus, the suitability of the ferritin platform for rigid multivalent display of trimeric antigens opens opportunities to create complex nanoparticulate vaccine immunogens. It is also possible to use other symmetric axes of ferritin or encapsulin, which is another bacterial protein that has recently been successfully engineered as a nanoparticle vaccine platform.<sup>29</sup>

## **Viral Capsid Scaffolds**

Viral capsids were the first and now the most widely used nanoparticle scaffolds for displaying antigens of interest in a rigid multivalent array. In one of its earliest uses in 1987, VLPs assembled from the core protein of hepatitis B virus (HBcAg) were able to display an exogenous foot-and-mouth disease virus (FMDV) epitope.<sup>30</sup> The  $\sim 27$  nm HBcAg particles were able to elicit FMDV-specific immune responses in animals at a substantially higher magnitude compared to responses elicited by a nonnanoparticulate  $\beta$ -galactosidase scaffold or free peptide epitope alone. Since then, as structural biology and biochemical techniques have continued to mature, other viral capsids and proteins have been utilized to display various epitopes and protein antigens.<sup>6</sup> One advantage of using viral capsids, such as HBcAg or bacteriophage-derived capsid proteins, is that the VLP can be produced in high-yield bacterial expression systems that are easily scalable for vaccine manufacturing. Typically, the extension of genes or insertion of exogenous target antigen is limited in these platforms by either size, shape, orientation of the antigen, or a combination of these factors. Thus, it is often difficult to display larger antigens, such as an entire viral envelope protein. For example, the HBcAg is a versatile platform to present some relatively large proteins but proper nanoparticle assembly is disrupted if the N- and C-termini of the antigen of interest are too distant from one another. This problem has been partially resolved by introducing enzymatic cleavage sites near the HBcAg-insertion site, thereby releasing one terminus of the insert and relaxing the core protein scaffold to allow particle assembly.<sup>31</sup>

In prokaryotic expression systems it is possible to incorporate unnatural amino acids into nanoscaffolds, making them suitable for chemical conjugation. Multiple VLP systems have used this technology to conjugate exogenous antigens on to the surface of particles. The method is especially powerful if the antigen of interest requires a specific tertiary or quaternary structure, disulfide bond formation or glycosylation or other posttranslational modifications to elicit the desired immune response. Conjugation of the antigen to an acceptor scaffold allows separate expression of the scaffold and the antigen in heterologous expression and purification systems. The efficiency of chemical conjugation varies for different systems as the distribution, occupancy and orientation of the antigen on the scaffold surface can be difficult to control. These factors can be partly manipulated by conjugation conditions (i.e., concentration of antigen and scaffold) or genetic design (restricting the number of reactive residues



on both scaffold and antigen). Recently, an alternative conjugation technique has been described on the P22 bacteriophage capsid system, in which an adaptor protein called Dec (decoration protein) is used to link the target antigen to the particle scaffold.<sup>32</sup> The Dec protein assembles into trimers so that it can, in principle, be attached to trimeric proteins such as influenza HA, HIV-1 envelope, Ebola virus glycoprotein, RSV fusion protein or coronavirus spike proteins. Another recent study reports use of the *Streptococcus pyogenes* fibronectin binding protein-based SpyTag/SpyCatcher system for predictable covalent linkage of antigens.<sup>33</sup> One of the earliest but now well-established systems made use of chemical conjugation of biotin to an HPV VLP scaffold surface, thereby allowing tetrameric streptavidin to serve as a “bridge” to display a biotinylated protein antigen.<sup>11</sup>

### Self-Assembling Proteinaceous Nanoparticles

In addition to virion structural proteins, there are many groups of proteins that naturally form higher-order multimers that are geometrically rigid and suitable for vaccine antigen display. Some of these multivalent nonviral proteins are chaperones or enzyme complexes that require a multimeric state for specific biological functions. Ferritin is perhaps the best-characterized example of a nonviral protein nanoparticle that has been engineered for multiple applications, including semiconductors, in vivo contrast agents and diagnostic biosensors. It has also been used as a carrier protein to deliver therapeutic agents and vaccine antigens. Early attempts primarily focused on attaching simple peptide epitopes to the surface of self-assembled ferritin particles using tractable prokaryotic production systems. As outlined above, the ferritin platform has shown robust humoral immunogenicity in animal models and seems likely to move forward toward to clinical trials.

Another successful example in this category is based on a bacterial enzyme, lumazine synthase. Analogous to ferritin, a single subunit of this protein self-assembles into an icosahedral particle ( $T = 1$ ), with 60 identical building blocks, thus having potential use for multivalent display of monomeric antigens.<sup>27</sup> Although ferritin and lumazine synthase nanoparticles are relatively small in diameter ( $\sim 12\text{--}20$  nm) compared to typical virions or viral capsids, some recombinant antigen-decorated nanoparticles in this size range have now been shown to recapitulate the Bachmann-Zinkernagel effect. Furthermore, it has been demonstrated that certain mutations in lumazine synthase can lead to assembly of larger nanoparticles.<sup>34</sup>

In a study of EBV gp350-nanoparticle vaccine candidates, the receptor-binding domain of EBV gp350 was linked either to ferritin or to another slightly larger (24 nm) diameter scaffold assembled from a bacterial encapsulin protein subunit. Both types of antigen-nanoparticles elicited similar magnitudes of humoral response to the EBV antigen, suggesting that nanoparticle size did not significantly affect immunogenicity in this context.<sup>29</sup>

The encapsulin platform has also been engineered to encapsidate proteins in the lumen of the nanoparticle by attaching a specific signal sequence on the encapsidated proteins.<sup>35</sup> This accentuates the potential for creating nanoparticulate vaccine immunogens that deliver encapsidated adjuvant molecules, such as co-stimulatory molecules or TLR agonists. These proteinaceous nanoparticles can be made in mammalian cells by simply introducing a secretion signal sequence. The secreted subunit protein (and fused antigen) can spontaneously self-assemble into nanoparticles in the milieu of the ER lumen and be purified as nanoparticles from culture supernatant by straightforward chromatography techniques. An advantage of this approach is that the fused antigen of interest can fold properly and acquire a natural pattern of post-translational modifications such as glycosylation, which is known to be critical for proper folding of many viral envelope proteins. Many additional classes of nanoparticles remain to be explored as vaccine antigen display scaffolds. Some examples of unexplored areas in this discipline include bacterial microcompartment complexes as scaffolds<sup>36</sup> and vertebrate immune system components that recognize multi-valent antigens. The latter may include complement component C1q, IgM, mannose binding protein (MBP) or related highly oligomeric lectins. These vertebrate-derived scaffolds might have the advantage of persisting on FDCs or other immune cells that have specific receptors for C1q, IgM, or MBP.

A next generation of nanoparticles could include features such as conditional assembly/disassembly or higher order and regulated coassembly. Prototype systems for conditional assembly have been explored with an engineered ferritin with a redesigned monomer-monomer interface carrying metal-binding site that “smoothens” the surface and renders assembly dependent on the presence of the metal ions.<sup>37</sup> At present, there are no examples of using proteinaceous nanoparticles to accommodate multiple inserts, which might allow display of hetero-oligomeric antigens of interest. It is possible that this type of display could be achieved using scaffolds that are comprised of more than one type of building block. Such platforms might include a two-subunit insect ferritin<sup>38</sup> or a three-subunit F420-reducing hydrogenase nanoparticle.<sup>39</sup> Alternatively, it might be possible to obtain the same complexity with “split” designs by breaking down a single subunit protein into two or more smaller parts. In sum, the field of vaccine design utilizing the synthetic proteinaceous self-assembled nanoparticle platforms is already well past its infancy and a few of the aforementioned candidates will soon be entering human clinical trials. It is likely that this new class of immunogens will possess superior immunogenicity and will provide for rapid, flexible and scalable manufacturing of subunit vaccines in the near future.

## **De Novo Design of Synthetic Self-Assembling Nanoparticles**

Modern computational approaches are increasingly poised to solve challenging structural biology questions that have previously been impervious to

experimental investigation. The first attempts to utilize synthetic building blocks to make a self-assembling molecule as a scaffold to display exogenous antigens started with very simple structures—two helices linked with a loop—to recreate two-, three- and fivefold symmetry axes of canonical icosahedral particles.<sup>40</sup> As protein modeling has become increasingly accurate and reliable, larger and more complex subunit building blocks are being designed. Such protein building blocks can now be designed in silico to self-assemble into a quaternary complexes, such as nanoparticles.<sup>41,42</sup> These computational approaches might offer a path to implementing functions such as conditional assembly/disassembly or may offer improved immunogenicity for more challenging antigens, such as the structurally plastic HIV-1 Env protein.

## CONCLUSION

VLPs and self-assembling proteinaceous nanoparticles are powerful platforms to present antigens to the humoral immune system. These platforms represent a geometrically rigid repetitive array of antigens that, in contrast to soluble monomeric protein subunits, are capable of robustly triggering PAMP-mediated immune stimulation of B cells. In addition to the highly successful current VLP-based vaccines against HPVs, many other VLP/nanoparticle-based vaccine candidates have recently entered preclinical and clinical trials. The potential applications of this new class of vaccine platforms are very broad and seem poised to serve as an important next generation of vaccines.

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