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Review

Potentiating Virus-like Particles for Mucosal Vaccination Using Material Science Approaches

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Abstract: Virus-like particles (VLPs) exhibit such unique colloidal and structural properties that make them ideal candidates for various bio-nanotechnology applications, among which mucosal vaccination is particularly promising. However, since mucosal surfaces present harsh environments to VLPs, stabilization of VLP capsids or alternative delivery strategies are necessary. Addressing these challenges requires interdisciplinary research, and the intersection of material science and immunology is presented in this review. Approaches such as crosslinking capsid coat proteins, incorporating VLPs in polymer matrices and hydrogels, or forming crystalline nano-/micro-structures show potential for developing muco-stable VLP vaccines or for delivering these vaccines in a sustainable manner. This review explores recent material science approaches that leverage VLPs as nanotools for various applications and with the potential for translation to mucosal vaccination.

Keywords: virus-like particles (VLPs); mucosal vaccination; bio-nanotechnology; stability enhancement; material science; antigen delivery

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1. Introduction

Mucosal surfaces of the respiratory, gastrointestinal, and urogenital tracts consist of specialized epithelial and immune cells, as well as cellular secretions that make a gel layer (mucus) over the epithelium (Figure 1A). Mucus is the first line of defense against foreign objects, including viruses, bacteria, and fungi (pathogens), that come into contact with mucosal surfaces [1]. Mucus physically impedes pathogens from approaching the epithelium. Pathogens are then removed via mechanisms such as mucociliary clearance in the respiratory tract or peristalsis in the gastrointestinal tract, which push mucus to the outside of the body. Mucus is also a reservoir for enzymes and secretory immunoglobulin A (sIgA), which kill, aggregate, and neutralize pathogens [2,3].

Below mucus, the epithelial cells create a tightly packed cellular barrier that further prevents pathogen entry into the tissue. Within epithelium, microfold (M) cells take up the pathogens and deliver them to antigen-presenting cells (APCs) in mucosa-associated lymphoid tissues (MALTs) for presentation to and further processing by immune cells. Innate immune cells, such as macrophages and dendritic cells, and adaptive immune cells, like T cells and B cells, are concentrated in MALTs. These cells move between various MALTs and mucosal inductive sites, and coordinate and execute immune responses against pathogens [2,3].

Despite the protective barrier functions and defense mechanisms of mucosal surfaces, they remain vulnerable to pathogen invasion from constant exposure to the external environment, vulnerabilities in mucus (e.g., large structural pores and non-adhesivity to near-neutral surface structures of viruses allowing their diffusion) or susceptibility to pathogens that have developed mechanisms of mucus invasion (e.g., bacteria producing mucolytic enzymes).

To enhance mucosal defenses, vaccination with the aim of stimulating the production of pathogen-specific sIgA antibodies is necessary. Since the more traditional injection

methods (such as intramuscular or subcutaneous injections) have generally resulted in limited mucosal protection, direct delivery of antigens to mucosal surfaces or a combination of systemic and mucosal vaccinations are considered the most potent strategies [4,5].

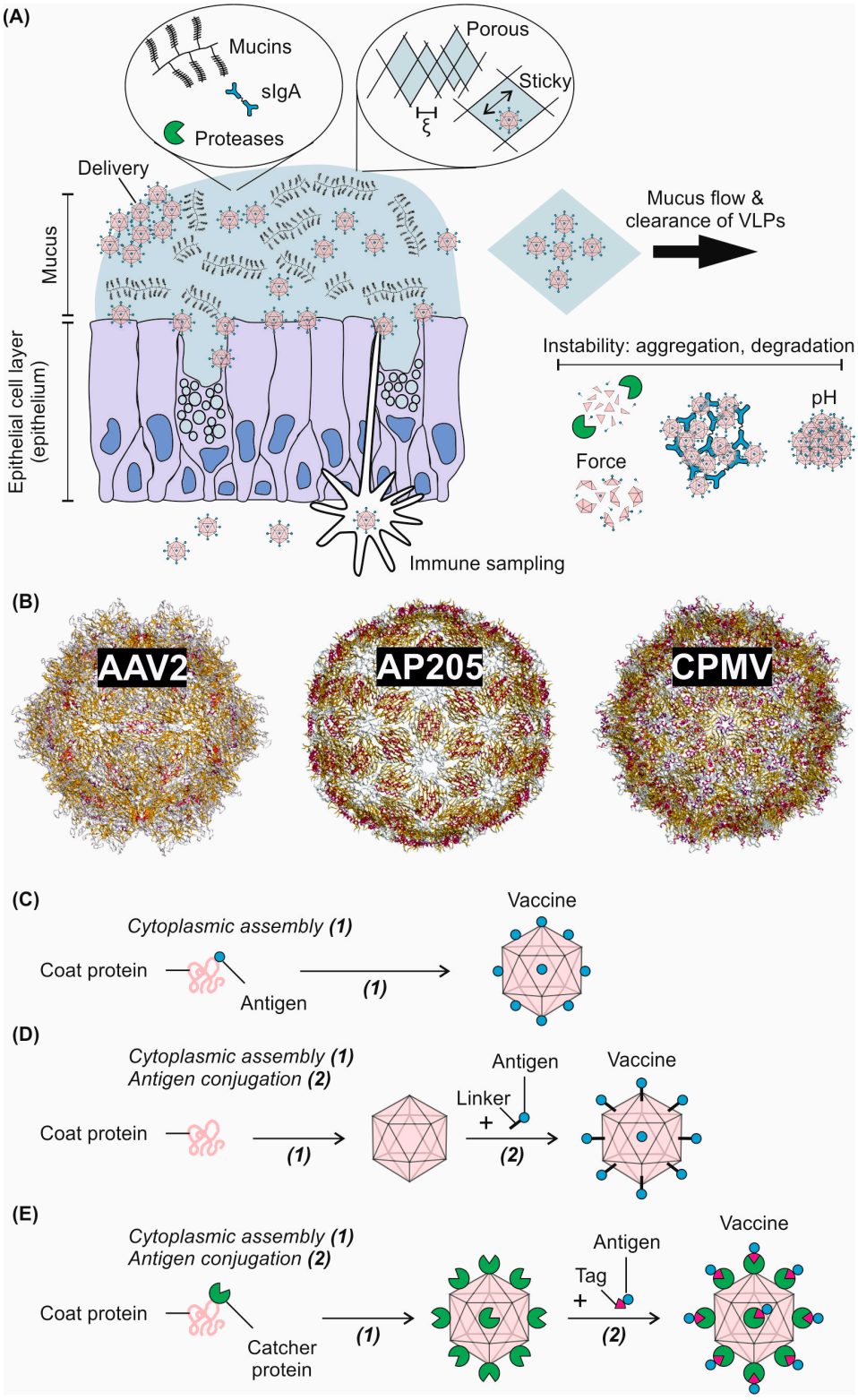

Figure 1. (**A**) Stability of virus-like particle (VLP) vaccines on mucosal surfaces is a critical factor for eliciting robust immune responses. Key determinants of antigen stability include interactions between

VLPs and mucin glycoproteins leading to subsequent clearance in flowing mucus, destabilization by high concentration of proteases, aggregation due to variations in pH or secretory immunoglobulin A (sIgA), and mechanical agitation. ξ denotes mucus porosity. (**B**) Atomic resolution structure of several VLPs including adeno-associated virus type 2 (AAV2) (PDB DOI: https://doi.org/10.2210/pdb5IPI/pdb), the VLP constructed from the coat protein of Acinetobacter phage (AP205 VLP) (PDB DOI: https://doi.org/10.2210/pdb5LQP/pdb), and cowpea mosaic virus (CPMV) (PDB DOI: https://doi.org/10.2210/pdb5A33/pdb). (**C**) Genetic fusion approach for VLP vaccine production: Antigens are genetically fused to coat proteins, enabling a one-step cytoplasmic assembly process (1) to generate the VLP vaccine. (**D**) Chemical conjugation approach for VLP vaccine production: Antigens are chemically attached to preassembled capsids. The process involves (1) cytoplasmic assembly of the capsid and (2) attachment of antigens to coat proteins via molecular linkers, resulting in the final VLP vaccine. (**E**) Tag/Catcher conjugation approach for VLP vaccine production: Coat proteins fused with Catcher proteins are assembled into capsids (1) during cytoplasmic assembly. In a subsequent step (2), antigens labeled with specific Tag peptides are conjugated to the Catcher proteins on the capsid surface, forming the final VLP vaccine. Alternatively, Tag peptide can be conjugated to the capsid, and Catcher protein to the antigen. Panel (**A**) is adapted from Ali et al.;

When mucosal surfaces are immunized against a pathogen through the delivery of its antigenic compound(s) (e.g., receptor binding domain of SARS-CoV-2 or O-antigen of *Salmonella Typhimurium*), the antigen molecules similarly experience mucus as a physicochemical barrier, although, by the definition of their intended function, these molecules must efficiently navigate mucus and be delivered to the immune cells across the epithelium. The difficulty arises partly from the poor stability of antigens in mucosal environments, caused by factors such as a wide range of pH levels, high concentrations of degrading enzymes, neutralizing sIgA, as well as adhesive interactions with mucin glycoproteins. These factors can lead to antigen degradation in mucus and elimination from the mucosal surfaces via physiological clearance mechanisms such as gut peristalsis, nasal and tracheal mucociliary clearance, and cough.

To address this limitation, antigen delivery using nanoparticle carriers has been investigated. The main idea is that nanoparticles can protect antigens from physical elimination by efficient navigation through mucus (e.g., by muco-penetrating nanoparticles) or by forming a depot on mucosal surfaces through adhesive interactions with mucus (muco-adhesive nano- and microparticles), from enzymatic degradation and sIgA aggregation by forming a protective shell around the antigens, and further enhancing immune stimulation by acting as adjuvants (substances that enhance the immune response to an antigen) [6].

Of particular interest among nanoparticle carriers are non-enveloped virus-like particles (VLPs). VLPs are proteinaceous nanostructures which are derived from mammalian virus, bacteriophage, and plant virus capsid proteins. Structurally, VLP capsids are composed of coat protein units that self-assemble into organized geometries, often with icosahedral symmetry, but sometimes with other symmetries or filamentous forms [7]. The coat protein units can be identical or composed of different subunits, which highlights the structural diversity of VLPs [8]. Unlike actual mammalian viruses, VLPs lack genetic material that could lead to VLP replication in mammalian cells and are therefore safe and noninfectious to humans and animals. Some common VLPs, including adeno-associated virus type 2 (AAV2), the VLP made up of Acinetobacter phage coat protein AP205, and cowpea mosaic virus (CPMV), are shown as examples in Figure 1B.

For vaccination purposes, mammalian virus-derived VLPs can be used directly as vaccines, as these inherently carry their native virus antigens and are highly immunogenic. Examples include human papillomavirus HPV VLPs (Cervarix®, Gardasil®, and Gardasil9®) which have been in clinical use since 2006/2007, and hepatitis B VLPs (Engerix®, Sci-B-Vac™, and RTS®), with Engerix® approved in 1989. The VLPs derived from plant virus or bacteriophage capsids are generally less immunogenic. In their cases, antigens can be displayed on the surface of their capsids through various methods. These include genetic

fusion, where coat proteins are genetically modified to present antigens directly on the VLP surface (Figure 1C), chemical conjugation to link antigens to pre-assembled VLPs using a molecular linker (Figure 1D), and the tag/catcher system, which covalently binds antigens to the VLP surface using specific peptide–protein pairs (Figure 1E) [9]. To this end, several VLP vaccines are in use or are in advanced stages of development, and a list can be found in recent reviews [10]. VLPs present antigens in an ordered and multimeric form, which leads to avidity interactions with APCs and immune cells, further enhancing immune activation.

Because VLPs mimic native mammalian viruses (e.g., adenovirus, rhinovirus, poliovirus, Norwalk virus, rotavirus, human papillomavirus) in colloidal properties, including size, ranging from 20 to 200 nm, and patchy surface charge and hydrophobic group distributions on capsid surfaces, they are expected to present muco-penetrability [11–13]. Moreover, given the overall similarity in structural properties, including features such as pathogen associated molecular patterns (PAMPs) [14,15], VLPs are expected to engage the immune system, either directly in the case of mammalian virus-derived VLPs, or through adjuvanticity in the case of plant virus and bacteriophage VLPs and presentation of foreign antigens. This is, however, dependent upon surviving the harsh mucosal environment during diffusive transport through mucus.

The mucin glycoproteins in mucus bundle and from a network with structural pore size (correlation length in polymer physics [16,17]) ranging ξ = 50–500 nm (Figure 1A) [18]. The viscosity of fluid within these pores, which is mainly composed of water, electrolytes, and possibly short mucin glycoprotein fragments, is assumed to be $\eta = 10^{-2}-10^{-1}$ Pa s [19]. The diffusion constant $D = (k_B\ T)/3\pi\eta d$ of a VLP of diameter d = 30 nm inside the pores is D = 0.2–2 $\mu m^2/s$, where k_B is the Boltzmann constant and T the temperature (37 °C). The time required for this VLP to navigate the mucus thickness and reach the apical surface of the epithelium can be estimated from $t \cong L^2/D$. The thickness L of mucus layer varies in different mucosal tissues. Assuming delivery to the respiratory tract, where the thickness of mucus layer is in the range 2–5 µm [20], one obtains a period from a few tens of seconds to minutes depending on the mucus thickness and the effective viscosity. Indeed, physical interference from the mucin network attenuates the diffusion constant through (a) adhesive interactions, and (b) convolution of the diffusion pathway (steric hindrance), which further prolong the duration of diffusive displacement in mucus [11–13,21]. Interestingly, a single amino acid mutation in the AAV6 capsid, K531E, significantly reduced its ability to displace in human cystic fibrosis sputum, resulting in diffusion rates which were comparable to the more mucoadhesive AAV1 serotype [21].

Within this period of time, VLPs encounter several stability challenges as schematically shown in Figure 1A. Varying pH levels and sIgA can cause VLPs to aggregate and be rapidly cleared from mucosal surfaces. Additionally, the abundance of proteases and the constant mechanical agitation from mucociliary clearance, peristalsis, and cellular interactions can compromise the stability of VLPs. Moreover, adhesive interactions with mucin glycoproteins can contribute to VLP destabilization (by denaturation of capsid coat proteins due to hydrophobic interactions) or aggregation (by a mechanism similar to bridging flocculation) [22].

To overcome these challenges, material science can offer innovative solutions to enhance the stability and efficacy of VLPs for mucosal vaccination. Approaches such as surface modification with macromolecules [23], encapsulation in hydrogels, polymeric nanoparticles and metal–organic frameworks [24,25], alternative delivery methods such as microneedle patches [26–30] and implants [29,31,32] have shown great potential in general biomedical applications. Building on these advancements, ongoing research is increasingly focused on integrating material science and immunology to develop even more resilient VLP formulations [10]. Recent reviews explored the synergies of material science and immunology for the production of next-generation vaccines [33–35]. In continuation of this research, this review focuses on mucosal applications of VLPs, particularly where material science approaches can lead to significant breakthroughs.

It is noted that while this review focuses on non-enveloped VLPs, their enveloped counterparts also hold significant promise in immunization strategies. However, few intrinsic attributes of non-enveloped VLPs make them potentially more practical for mucosal vaccination. Non-enveloped VLPs have symmetric and rigid structures which allow for high-resolution X-ray crystallography and cryo-electron microscopy, and therefore a controlled conjugation of antigens via the three strategies mentioned earlier (Figure 1C,E). In contrast, enveloped VLPs are less characterized structurally due to their pleomorphic nature and the complexity of their lipid bilayers. The structural simplicity of non-enveloped VLPs also translates into easier and higher-yield production and purification processes, as they can be efficiently expressed in systems like bacteria without requiring additional lipid removal. Meanwhile, the production and purification of enveloped VLPs are more challenging, requiring eukaryotic expression systems and complex purification to remove lipids and contaminants. Nevertheless, the presence of lipid bilayer gives these VLPs greater structural flexibility, allowing for the incorporation of diverse and large antigens. Furthermore, non-enveloped VLPs exhibit better biochemical stability (which is important in mucosal environments), while enveloped VLPs are more sensitive, possibly due to their lipid composition. For an overview on the current state of vaccines produced using both non-enveloped and enveloped VLPs, the reader is referred to a recent review [36].

2. Mucosal Vaccination Using Virus-like Particles

There are several examples of successful mucosal administration of mammalian virus VLPs, intranasally [37–40] as well as orally [41–45], in which the VLP vaccines induced both local mucosal immunity and systemic immune responses. Simultaneously, there has been growing interest in exploring the potential of plant virus and bacteriophage VLPs for antigen delivery to mucosal surfaces [46,47]. The following sections present recent advances in this area, highlighting the considerable potential of this technology.

2.1. Vaccination Against Viruses (Seasonal Influenza A & SARS-CoV-2)

Seasonal influenza A virus poses a significant global health challenge, causing millions of severe illnesses and deaths annually. While vaccination is the most effective prevention method against influenza, vaccines must be updated yearly to meet the frequent genetic shifts in the main circulating virus serovars. Vaccination strategies that are faster in production than the current embryonated egg strategy are therefore needed. The development of VLP-based influenza vaccines can potentially answer these needs. In this direction, bacteriophage P22 capsid was used to display the hemagglutinin (HA) head domain of the PR8 strain of influenza A (Figure 2A), employing SpyTag–SpyCatcher dipeptide bonding for the conjugation (Figure 1E) [48].

Intratracheal vaccination of mice with the VLP vaccine (P22-HA_{head}) led to minimal weight loss after challenge with a 50% lethal dose of the virus, while PBS-treated mice and mice vaccinated with SpyCatcher-HA_{head} or a mixture of P22 and SpyCatcher-HA_{head} (i.e., without covalent conjugation of HA_{head} to P22 capsid) experienced 10–15% weight loss [46]. Furthermore, vaccination with P22-HA_{head} vaccine led to a 100% survival rate, compared to 60% and 50% in the cases of SpyCatcher-HA_{head} (SC-HA_{head}) and PBS treatments, respectively (Figure 2B) [48]. The VLP vaccine further induced a robust anti-HA IgG antibody response, which remained elevated up to 14 days post-challenge [48]. The mixture of P22 and SC-HA_{head} (without conjugation) also led to 100% animal survival as well as antibody generation [48], which is potentially due to the adjuvanticity of the P22 capsid. The response was, however, delayed and, at lower levels, compared to the P22-HA_{head} vaccine [48].

These findings underscore the potential of VLPs to generate effective mucosal vaccines that can be rapidly adapted in order to address emerging and mutating pathogens such as influenza.

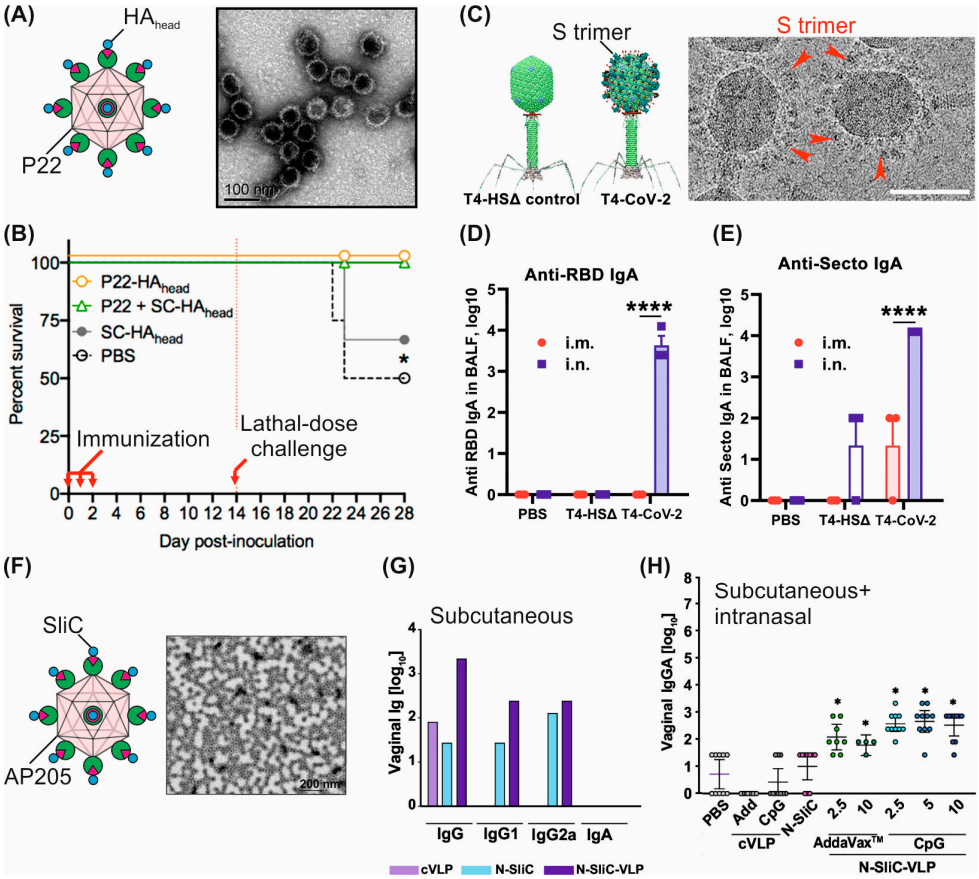

Figure 2. Examples of recent virus-like particle (VLP)-based mucosal vaccines against viral and bacterial infections. (**A**) Schematic and transmission electron microscopy (TEM) image of P22-HA$_{head}$ VLP vaccine. Scale bar 100 nm. (**B**) Representative example of immunity induced by P22-HA$_{head}$ vaccination, showing the survival of immunized mice following exposure to a lethal virus challenge. (**C**) Schematic and cryo-TEM image of T4-CoV-2 VLP vaccine and T4-HSΔ (T4 vector control lacking outer capsid proteins Hoc and Soc, or any SARS-CoV-2 antigens). The red arrowheads indicate the S-trimer displayed on capsid surface. Scale bar 100 nm. (**D**,**E**) Selected examples of mucosal immunological response, showing anti-receptor binding domain (anti-RBD) IgA (**D**) and anti-spike ectodomain trimer (anti-Secto) IgA (**E**) titers in bronchoalveolar lavage fluid. Data presented as mean ± SEM, pooled from three independent experiments (n = 12 for T4-CoV-2, n = 10 for T4-HSD, and n = 5 for PBS). Titers between the intramuscular (i.m.) and intranasal (i.n.) routes were compared using two-way ANOVA (**** $p < 0.0001$). (**F**) Schematic and TEM image of SliC-AP205 VLP vaccine. Scale bar 200 nm. (**G**,**H**) Selected examples of immunological responses showing total IgG, IgG1, IgG2a, and IgA titers in vaginal lavage after subcutaneous immunization of mice with SpyCatcher-conjugated VLP (cVLP) alone, SliC with the N-terminal SpyTag (N-SliC) alone, or N-SliC-VLP vaccine (**G**) and IgA titers in vaginal lavages after subcutaneous and intranasal immunizations of mice with N-SliC-VLP vaccine with AddaVax adjuvant, N-SliC-VLP vaccine with CpG adjuvant, N-SliC, cVLP with AddaVax adjuvant, cVLP with CpG adjuvant, or PBS (**H**). Graphs show geometric mean titers with error bars representing 95% confidence intervals. Statistical significance among groups was determined using the Kruskal-Wallis test with Dunn's multiple comparisons. * $p < 0.05$. Panels (**A**) and (**B**) are adapted with permission from Sharma et al., Copyright © 2020, American Chemical Society. Panels (**C**), (**D**) and (**E**) are adapted from Zhu, Ananthaswamy et al. and Zhu, Jain et al., respectively, under the terms of Creative Commons Attribution Noncommercial License 4.0 (CC BY-NC) and Creative Com-mons Attribution 4.0 International license. Panels (**F**), (**G**) and (**H**) are adapted from Martinez et al. under the terms of Creative Commons Attribution 4.0 International license.

Despite significant efforts, intramuscular COVID-19 vaccines, including mRNA and inactivated viral vaccines, did not prevent virus shedding and transmission from infected individuals [5]. This has been attributed to failure in inducing robust mucosal immunity in the respiratory mucosae, particularly the generation of vaccine-induced sIgA [5]. To induce mucosal sIgA, mucosal vaccination or a combination of systemic and mucosal vaccinations (e.g., prime and boost) are the potent strategies. In this direction, using bacteriophage T4 (a tadpole-like bacteriophage consisting of an icosahedral capsid and a tail structure), a study developed an anti-SARS-CoV-2 vaccine for mucosal administration (Figure 2C) [49,50]. The vaccine incorporated SARS-CoV-2 spike protein ectodomain trimers (*S*-trimers), which were covalently conjugated to the capsid surface through SpyTag–SpyCatcher dipeptide bonding (Figure 1E). Additionally, a peptide of the putative external domain of E protein was fused to the capsid surface genetically (Figure 1C). Furthermore the capsid encapsidated SARS-CoV-2 nucleocapsid proteins [50].

Using transgenic and non-transgenic mice, the researchers assessed the ability of the vaccine to induce immune responses, including antibody generation, T-cell responses, and virus-neutralizing activities [50]. In particular, anti-receptor binding domain (RBD) and anti-spike ectodomain trimer (Secto) antibody titers were determined in bronchoalveolar lavage fluid (Figure 2D,E, respectively) [50]. It was found that intranasal (i.n.) administration elicited high titers of mucosal sIgA antibodies, in contrast to intramuscular (i.m.) immunization, which failed to produce sIgA [50]. Intranasal immunization further induced strong systemic immunity and provided complete protection and apparent sterilizing immunity against SARS-CoV-2 challenge [50].

These findings highlight the potential of VLPs to stimulate the production of antigen-specific sIgA in mucosal vaccination.

2.2. Vaccination Against Bacteria (Enterotoxigenic Escherichia coli & Neisseria Gonorrhoeae)

In pig farms, enterotoxigenic *Escherichia coli* (*E. coli*) colonization, which is facilitated by *E. coli* fimbriae adherence to specific intestinal receptors and enterotoxin release, is a major contributor to poor growth in piglets [51]. Current prophylactic treatments rely on passive immunity through sow vaccination; however, this strategy is insufficient for preventing post-weaning infection [51]. To address this issue and to reduce antibiotics consumption in weaner pigs, a recent study investigated a VLP-based vaccine targeting FaeG, the major structural subunit of F4 fimbriae of *E. coli* [52]. In the study, FaeG was conjugated to the capsid of AP205 VLP using the SpyTag–SpyCatcher system (Figure 1E) and the immunogenicity of the resulting vaccine, FaeG.cVLP, was evaluated in mice, piglets, and sows [52].

Intranasal immunization of mice induced fecal and serum IgA and serum IgG antibody responses [52]. However, neither intramuscular nor heterologous intramuscular/intranasal immunization of newly weaned piglets from vaccinated sows elicited a significant immune response, potentially due to the presence of maternally derived antibodies that neutralized and cleared the VLP vaccines displaying FaeG [52]. Additionally, intramuscular immunization in piglets from non-vaccinated sows did not result in a robust antibody response [52]. Therefore, while the VLP-based vaccine showed promise in eliciting immune responses, especially intestinal IgA following intranasal vaccination in mice, further investigations and optimizations are needed to enhance its effectiveness in post-weaning piglets.

These findings further highlight the critical differences in immune responses between laboratory animals like mice and farm animals like pigs and underscore the challenges of translating findings from controlled laboratory settings to practical applications in farming contexts.

Neisseria gonorrhoeae is responsible for a sexually transmitted infection (gonorrhea) that presents a major public health concern [53]. Particularly due to the emergence of multidrug- and antibiotic-resistant *Neisseria gonorrhoeae* strains, there is an urgent need for an effective vaccine [53]. A recent study developed a VLP-based vaccine targeting a conserved surface lipoprotein of *Neisseria gonorrhoeae* (SliC) that contributes to bacterial defense against human

lysozyme [54]. The SliC antigen was conjugated to AP205 VLP via SpyTag–SpyCatcher dipeptide bonding (Figure 2F), and two adjuvants, namely AddaVax and CpG, were additionally tested in combination with the vaccine to enhance the immune response.

Two independent mouse immunization study groups were formed. In the first group, mice were immunized subcutaneously, and in the second one, mice were immunized subcutaneously followed by an intranasal boost. Subcutaneous immunization induced systemic IgG and IgA responses and vaginal IgG; however, no vaginal IgA could be detected (Figure 2G). The combination of subcutaneous and intranasal immunization produced systemic IgG and IgA responses, and additionally elicited IgA in vaginal lavages (Figure 2H). The use of CpG as an adjuvant in the combination routine showed the most significant increase in total serum IgG, IgG3 titers, and bactericidal antibodies.

These findings suggest that VLP-based vaccines are promising for developing effective immunization against *Neisseria gonorrhoeae*.

2.3. Vaccination Against Parasites (Toxoplasma Gondii)

Toxoplasma gondii is another significant global health concern that particularly affects immunocompromised individuals and pregnant women [55]. Despite its widespread prevalence, an effective vaccine for toxoplasmosis remains unavailable. A recent study evaluated the effectiveness of a heterologous immunization strategy using recombinant baculovirus and VLP, both expressing Toxoplasma gondii Rhoptry Protein 18 (rBV-ROP18 and ROP18-VLPs, respectively) as candidates against toxoplasmosis [56]. Mice were immunized through a regimen of prime and first boost with rBV-ROP18, and second boost with ROP18-VLP, via the oral, intranasal, or intramuscular routes and subsequently challenged with a lethal dose of Toxoplasma gondii to compare the immune responses and protection levels elicited by the different administration routes.

The findings revealed that the vaccine induced significant Toxoplasma gondii-specific antibody responses across all routes, with intranasal and intramuscular immunizations producing stronger systemic IgG responses, and oral immunization yielding the highest mucosal IgA levels in the intestines and intranasal immunization in the brain. Robust CD8+ T cell and germinal center B-cell responses were observed across all routes, with the intranasal immunization showing the highest CD8+ T cell proliferation. The vaccines further reduced pro-inflammatory cytokine production in the brain and led to 100% survival of the immunized mice, with the intranasal route providing the greatest reduction in brain cysts. These findings suggested that heterologous immunization using recombinant baculovirus and VLP vaccines, particularly delivery through the oral route, is an effective strategy against toxoplasmosis.

These reviewed examples show the potential of VLPs in diverse mucosal immunizations. It is expected that the effectiveness of these nanostructures can be further enhanced once unfavorable mucosal factors are recognized and strategies to overcome them are considered in the design process. Below, several material science-based approaches that could combat mucosal challenges are reviewed.

3. Material Science-Based Approaches That Can Potentiate Mucosal Vaccination Using Virus-like Particles

The limited availability of clinical VLP vaccines for mucosal administration is partly due to the challenges associated with delivering antigens directly to mucosal surfaces, despite the proven potential of these vaccines to induce immune responses in systemic administration (e.g., HPV VLP and hepatitis B VLP vaccines) as well as in mucosal administration (as given by the examples in the previous section). However, systemic administration does not typically generate mucosal immunity, therefore, antigen delivery via the mucosal routes is critical for achieving localized protection against mucosal pathogens [4,5]. These challenges partly stem from inactivation and destabilization of VLP vaccines by mucosal proteases and sIgA, varying pH levels, mechanical stresses, as well as rapid elimination through mucus clearance and feces excretions (Figure 1A).

To address these obstacles and realize the full potential of VLP vaccines for mucosal applications, innovative approaches are necessary. While the following material science-based innovations have not yet been fully explored in the context of mucosal applications, they hold significant potential for enhancing the stability, delivery, and immunogenicity of VLP vaccines in the respiratory, gastrointestinal, and urogenital mucosal environments.

3.1. Stabilization by Crosslinking Capsid Coat Proteins Using Bifunctional Polymers

Traditionally, polymers have been attached "from one end" to proteins to extend their circulation time in vivo and to reduce their nonspecific adsorption [57]. The same approach has been adapted for VLPs [58]; for example, polyethylene glycol (PEG) conjugation (PEGylation) was used to protect adenovirus vectors from neutralizing antibodies, leading to prolonged transgene expression and reduced immune activation against the vector [59–62]. Similarly, the use of poly-[*N*-(2-hydroxypropyl)methacrylamide] (pHPMA) to coat adenovirus particles has been shown to effectively shield the virus from neutralizing antibodies and enable retargeting, resulting in improved infection specificity and resistance to immune neutralization [63].

Polymers with functional groups at both ends (bifunctional polymers) can crosslink reactive amino acids on the capsid interior and/or exterior surfaces if the interatomic distances between the reactive moieties are about the most probable end-to-end distance of the polymer [16,17,64] (Figure 3A). The interatomic distances can be estimated from the atomic structure of capsids, obtained from the Protein Data bank [65,66], and analyzed through software such as PyMOL [67]. The end-to-end distance of a polymer molecule is $\langle R \rangle = bN^{\nu}$, where b is about the size of one monomer, N is the degree of polymerization, and the Flory exponent $\nu = 1/2$ or $3/5$, for polymers in theta or good solvents (e.g., PEG in an aqueous solution) [16,17,64].

A proof of concept was shown with poly(2-oxazoline), crosslinking the coat proteins of bacteriophage Qβ VLP. It was demonstrated that, after crosslinking Qβ coat proteins, the thermal stability, and the structural integrity of the capsid were significantly increased and, for example, the capsid became stable at elevated temperatures up to 100 °C [23].

In a recent investigation, PEG_x molecules (where x = 5 to 25 is the number of PEG monomers) with a functional Sulfo-*N*-hydroxysulfosuccinimide (Sulfo-NHS) moiety on each end were used to crosslink surface lysine residues of AP205 VLP [68]. Crosslinking was shown to enhance the stability of VLPs against varying pH and salt conditions. In particular, the PEG-crosslinked VLP became stable in phosphate buffered saline (PBS) in a pH range 4.0–7.0, unlike the native VLP, which had a narrower pH range of stability, 6.0–7.0 [68]. The crosslinked VLPs further showed a longer half-life in pig gastric fluids compared with the simply PEGylated or native VLPs (Figure 3B) [68]. In particular, while the native VLP destabilized under 5 min of exposure, the crosslinked VLPs showed stability in between 5 and 30 min [68]. In addition to biochemical stability, the capsid's mechanical behavior also varied where the capsid stiffness increased by a factor of two after crosslinking [68]. Of particular relevance to mucosal applications, crosslinking did not impair the ability of VLPs to traverse the mucus barrier, as demonstrated in 3D human nasal epithelial tissues (Figure 3C) [68]. Specifically, on tissue with motile cilia, a noticeable portion of VLPs were transported to the tissue edge via mucociliary clearance, while the remaining portion translocated mucus and accumulated on top of the epithelial cell layer (Figure 3C) [68]. When ciliary beating was halted via the addition of trans-cinnamaldehyde, the VLPs were distributed more uniformly over the epithelium (Figure 3C) [68]. Moreover, in vivo studies of subcutaneous vaccination in mice indicated that crosslinking did not inhibit anti-AP205 serum IgG immune responses (the model antigen in this vaccination study was the mildly immunogenic capsid coat protein AP205) (Figure 3D) [68]. Meanwhile, the adjuvanticity of AP205 coat proteins led to anti-PEG serum IgG responses (Figure 3E) [68].

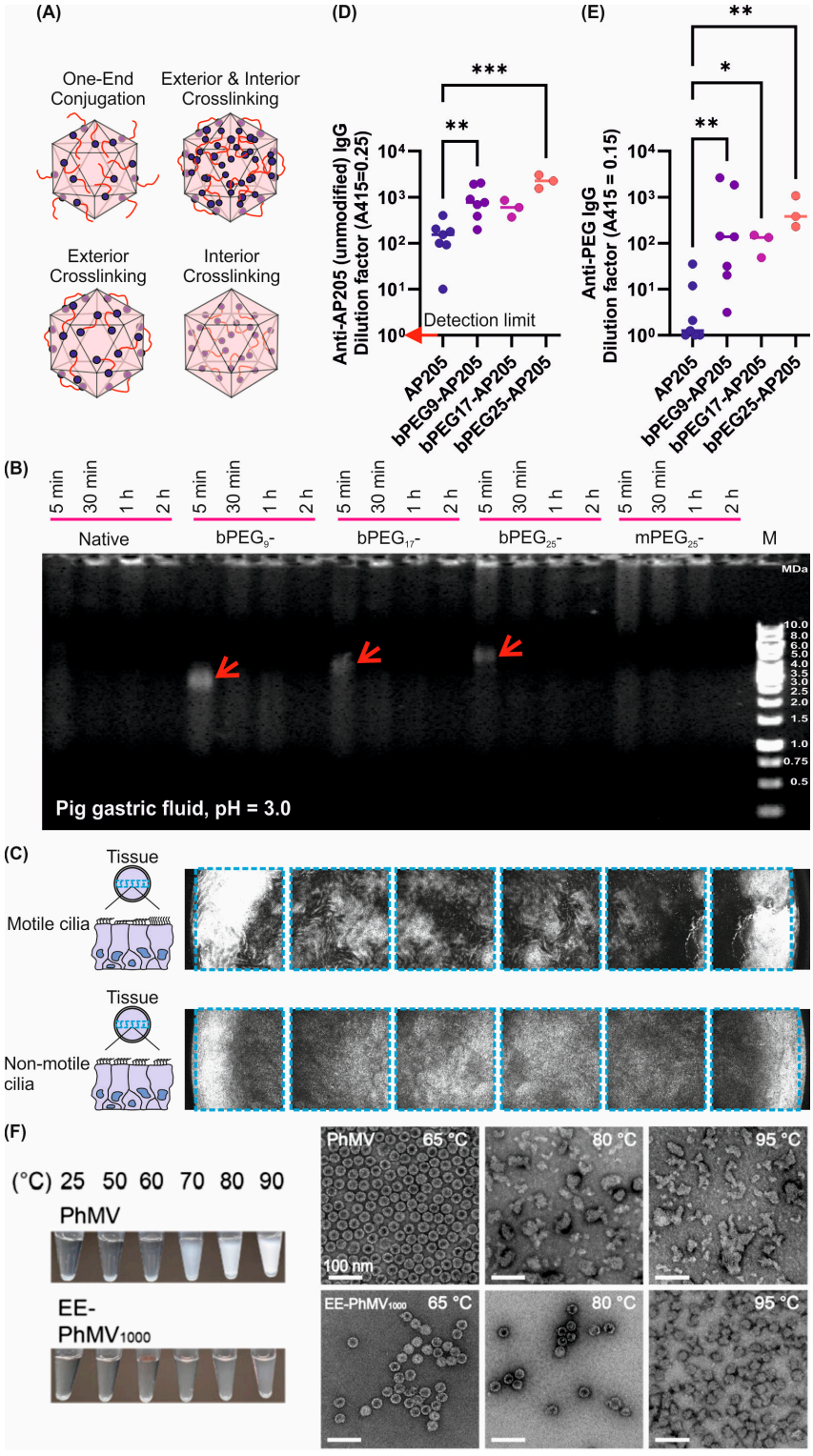

Figure 3. (**A**) Schematic representation of one-end conjugation of polymers to virus-like particle (VLP) capsids, or conjugation from both ends (crosslinking) using bifunctional polymers. Crosslinking can be on the exterior or interior capsid surfaces or both. (**B**) A cut section of agarose gel electrophoresis of native AP205 VLP, and PEGx-crosslinked AP205 VLPs (bPEG$_x$-, where x is the number of monomers) and simply PEGylated AP205 VLP (mPEG$_{25}$−) in pig gastric fluid at pH 3.0. Incubation times are indicated. Arrows at 5 min indicate stable capsids in gastric fluid. The complete gel can be found in

Ref. [68]. (**C**) Confocal microscopy images of the distribution of VLPs on human nasal epithelial tissue with motile cilia (top) and on human nasal epithelial tissue with non-motile cilia (bottom). Cyan boxes show areas of confocal microscopy along the diameter of the tissue culture. (**D**,**E**) Generation of serum IgG antibodies upon subcutaneous immunization of mice with native AP205 VLP and PEG-crosslinked AP205 VLPs, against AP205 coat protein (**D**) and against PEG (**E**). Statistical significance was determined by ordinary one-way ANOVA with Dunnett's multiple comparisons test, with a single pooled variance on log-normalized data (* $p = 0.0332$; ** $p = 0.0021$; *** $p = 0.0002$). (**F**) Samples of physalis mosaic virus (PhMV) and crosslinked PhMV (EE-PhMV) at various temperatures, together with TEM images showing thermally induced morphological changes to PhMV and stability of EE-PhMV at temperature range 25–90 °C. Scale bar 100 nm. Panel (**B**) to (**E**) is adapted from Ali et al.; Copyright © 2024 The Authors. Published by American Chemical Society. This publication is licensed under CC-BY 4.0. Panel (**F**) is reprinted with permission from Wu et al. Copyright 2024 American Chemical Society.

A similar strategy for enhancing the stability of physalis mosaic virus (PhMV) was postulated [69]. PEG_{15} with maleimide end functionalities was used to crosslink capsid coat proteins containing free cysteine residues on the interior surface of the capsid, and the resulting VLPs were named endoskeleton-enhanced (EE) VLPs [69]. The methodology was shown to significantly improve the chemical, thermal, and mechanical stability of the VLPs. For instance, the crosslinked VLPs could withstand temperatures nearing 90 °C for 15 min without significantly losing colloidal stability and structural integrity (Figure 3F) [69]. This methodology also demonstrated a 6-fold increase in the rupture distance and a 1.9-fold increase in the rupture force of individual coat proteins from the capsid for the crosslinked VLP as compared to the native VLP, indicating enhanced mechanical stability [69]. The study further assessed stability under other stress conditions including different pH levels, surfactants, and organic solvents, finding that the crosslinked VLP maintained its integrity much better than the native VLP capsid [69].

Polymers conjugated to a capsid surface, in both cases of one-end conjunction and two-end conjunction (crosslinking), provide a protective shield that minimizes capsid aggregation and enhances stability [70]. This polymer coating, through steric hindrance, effectively prevents protease attacks and degradation by obstructing proteases' access to the capsid and interferes with sIgA neutralization by reducing antibody binding [17,70]. However, due to the limited number of reactive amino acids on a capsid surface, the density of the polymers on the surface is relatively diluted.

Particularly, the reviewed studies suggest that enhancing the structural stability of VLPs via surface crosslinking of capsid coat proteins can lead to more stable nanocarriers, without loss of mucus penetrability or immunogenicity. This strategy clearly shows potential for VLP application in mucosal immunization, although the immunogenicity of crosslinking polymer molecules, or generation of polymer-specific antibodies, could be a limiting factor [71–74].

3.2. Protection Against Enzymes and sIgA and Achieving Sustained Release by Incorporation in Polymer Matrices

Overcoming the challenges associated with protease degradation and sIgA aggregation are important for mucosal vaccination using VLPs. Polymeric nano- and microparticles composed of biocompatible materials, such as poly(lactic-co-glycolic acid) (PLGA) and chitosan, can incorporate VLPs and provide a protective barrier against proteases and sIgA in mucosal environments. Moreover, the VLPs are released gradually from the matrix, leading to a sustained antigen delivery to the mucosal immune system. Several strategies have been used to incorporate VLPs in polymer matrices, including spray-dried polymeric nano- and microparticles as well as hydrogels (Figure 4A,B).

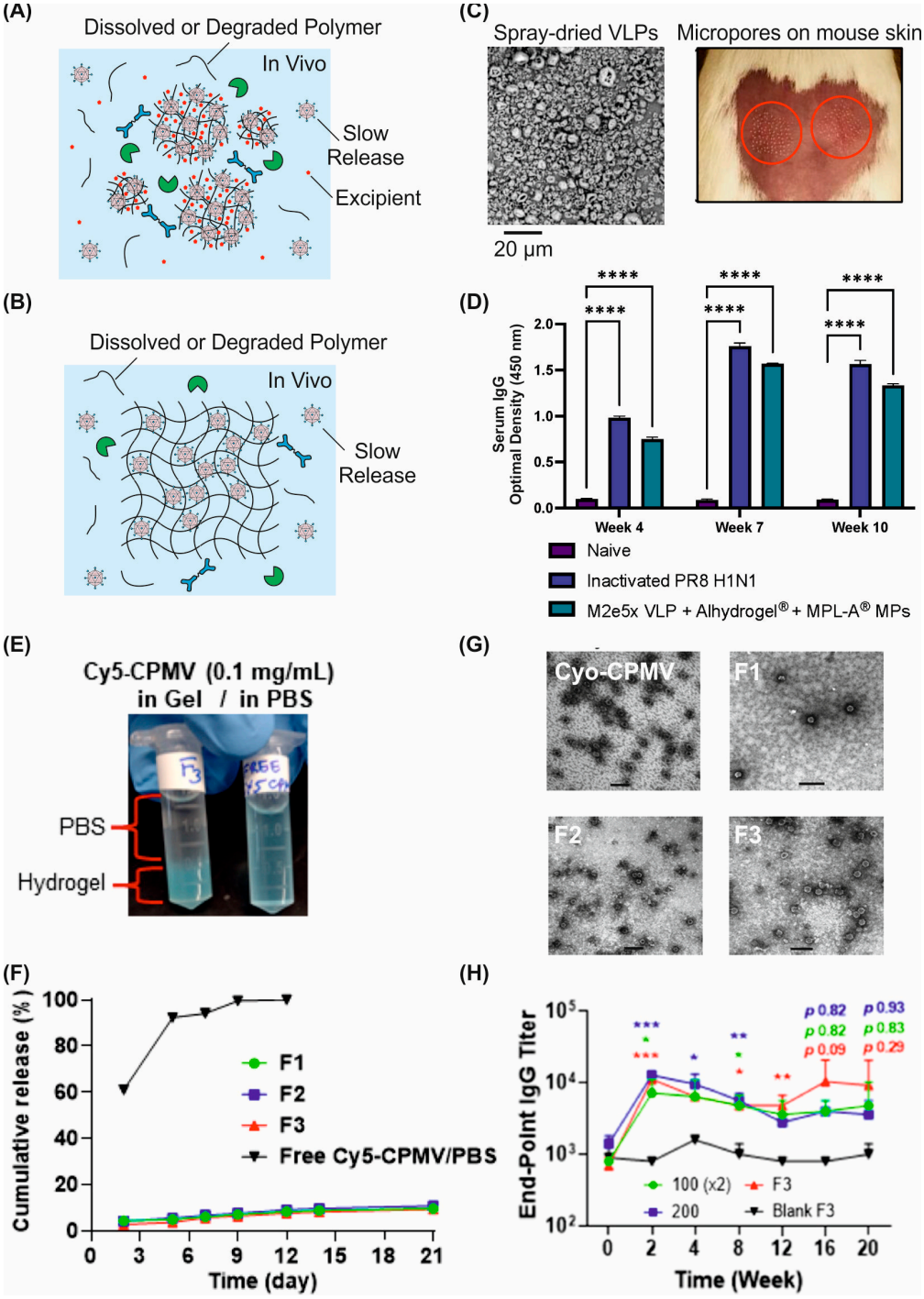

Figure 4. (**A**,**B**) Schematics of virus-like particle (VLP) vaccines incorporated in polymer matrices for protection against proteases and secretory immunoglobulin A (sIgA): spray-dried microparticles (**A**) and hydrogels (**B**). (**C**) Scanning electron microscopy (SEM) image of spray-dried microparticles containing M2e5x VLPs, and micropores on mouse skin created by ablative laser. (**D**) Representative immune response showing serum IgG levels generated after immunization with inactivated PR8 H1N1 and M2e5x VLP + Alhydrogel® + MPL-A® microparticles. Data are expressed as mean ± standard deviation (SD). For multiple comparisons, one-way ANOVA was performed with Tukey's post hoc test. A p-value < 0.05 was considered statistically significant (**** $p < 0.0001$). (**E**,**F**) Experimental

settings used for in vitro release analysis of CPMV from hydrogel (**E**), and data showing the release of hydrogel-incorporated Cy5-CPMV (F1, F2, and F3 samples) versus free Cy5-CPMV/PBS at 37 °C (**F**). Hydrogel formulations contained 4.5 mg/ml CPMV dispersed in low molecular weight (MW), medium MW, and high MW chitosan, and named F1, F2, and F3, respectively. (**G**) TEM images of Cy5-CPMV released from hydrogels in vitro, showing the integrity and stability of VLPs within the hydrogel matrix. (**H**) Example of immunological response in mice vaccinated with hydrogel-incorporated CPMV Covid-19 vaccine. Blank F3: negative control, F3: 200 μg of CPMV vaccine in hydrogel, 200:200 μg of CPMV vaccine in PBS, and 100 (×2): prime/boot immunization with 100 μg of CPMV vaccine in PBS. Data were statistically analyzed using one-way ANOVA with Tukey's multiple comparison test or two-way ANOVA with pairwise comparisons followed by Holm-Šidák correction. Asterisks in the figures indicate significant differences between groups (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Panels (**C**) and (**D**) are adapted from Gomes et al. under an open access Creative Common CC BY license. Panels (**E**) to (**H**) are adapted with permission from Nkanga et al., Copyright © 2022 American Chemical Society.

The influenza A virus challenge to global health, and vaccination being the most effective prevention method were mentioned earlier. The ectodomain matrix-2 protein (M2e) of influenza A is highly conserved across influenza A strains and is therefore a potential antigen for a universal vaccine; however, in soluble form it has shown poor immunogenicity [75]. To enhance its immunogenicity, a strategy utilized five tandem repeats of M2e antigen, M2e5x, and genetically presented this construct to the surface of a VLP assembled from influenza matrix-1 protein (M1) [76,77]. The resulting M2e5x VLP vaccine was then incorporated in bovine serum albumin (BSA) polymeric matrix using spray drying [76]. The one-step incorporation procedure involved aerosolization of a VLP–polymer solution to droplets, followed by water evaporation by heating and subsequent cooling, resulting in spray-dried particulates [76]. The VLP-loaded microparticles had an average size of about 4.8 μm and a negative surface charge (Figure 4 C) [76]. Other microparticles, containing the adjuvants Alhydrogel® and monophosphoryl lipid A (MPL-A®), were similarly formulated [76].

Mice were immunized by applying a suspension of spray-dried microparticles onto laser-ablated micropores on their skin (Figure 4C) [76]. The immunization produced high levels of M2e-specific serum IgG antibodies, comparable with inactivated PR8 H1N1 virus, and persisting over 10 weeks post-immunization (Figure 4D) [76]. The immunized mice further showed heightened CD4+ and CD8+ T-cell responses in lymph nodes and spleens compared to naive mice [76].

In another investigation, spray-drying was used to incorporate VLPs composed of respiratory syncytial virus (RSV) fusion (F) protein and influenza virus M1 protein (RSV-F VLPs) into a biodegradable, cellulose-based polymer matrix with an incorporation efficiency of about 85% [78]. The resulting microparticles were about 2.5 μm in diameter and had a positive surface charge [78]. These VLPs could also be loaded in a poly(lactic-co-glycolic acid) (PLGA) matrix using a double emulsion solvent evaporation technique, forming 0.5-μm particles with a negative surface charge with an incorporation efficiency of about 73% [79]. Incorporated VLPs were stable for at least 1 year when stored at 4 °C [79].

This technology was investigated against RSV infection [78]. RSV is a major cause of severe respiratory illness, leading to significant global morbidity and mortality, particularly in children, the elderly, and immunocompromised individuals, with millions of infections and hospitalizations each year. During infection, the fusion protein (F) of RSV plays the key role in viral entry into host cells. Mice were immunized with vaccine microparticles via transdermal administration using a microneedle patch. The immunization was followed by a live virus challenge [78]. Immunized mice, especially when the adjuvant MPL-A® was incorporated in the formulation, showed high IgG titers and maintained stable body weight. Additionally, vaccination significantly reduced lung viral loads and lung histopathology revealed reduced inflammation.

Hydrogels can also provide a platform for protection and slow release of VLP vaccines. In fact, various studies have explored the use of chitosan-based nano- and microparticles

(muco-adhesive hydrogels) as delivery systems for mucosal vaccination. For instance, chitosan microparticles were successfully utilized to deliver Porcine Circovirus Type 2 (PCV2) VLPs orally, which showed immune responses comparable to injectable formulations [80]. Similarly, a thermal-sensitive hydrogel based on *N*-[(2-hydroxy-3-trimethylammonium) propyl] chitosan chloride was investigated for intranasal delivery of an Ebola virus glycoprotein antigen and provided enhanced humoral and cellular immune responses through prolonged antigen residence time in the nasal cavity [81]. Chitosan nanoparticles have also been used for the delivery of a live Newcastle Disease Virus (NDV) vaccine via oral or intranasal routes, and demonstrated improved mucosal immunity compared to conventional methods [82]. Additionally, quaternized chitosan nanoparticles were utilized for the combined delivery of attenuated live Newcastle Disease Virus (NDV) and Infectious Bronchitis Virus (IBV) vaccines via the intranasal route, which led to effective immune responses in chickens [83].

These investigations highlighted the potential of muco-adhesive hydrogels in mucosal vaccination. In a recent advancement, a study used an injectable slow-release hydrogel formulation using chitosan and β-glycerophosphate to incorporate CPMV VLPs [84] (Figure 4E). The formulation gelled at 37 °C and slowly released cyanine 5-loaded CPMV in in vitro (Figure 4F) as well as in vivo experiments. Hydrogels containing CPMV VLPs displaying an epitope of SARS-CoV-2 spike protein were then developed [84]. In vitro, it was found that the hydrogel effectively protected the VLP vaccines from rapid degradation and allowed a sustained release over several months, while preserving the immunogenicity of the incorporated vaccine particles (Figure 4G). In mouse models, a single subcutaneous injection of the hydrogel elicited a sustained antibody response for up to 20 weeks (Figure 4H).

These studies suggest that, by protecting VLPs from proteases and sIgA, while allowing their sustained release, spray-dried or hydrogel particles can enhance immune responses. Importantly, muco-adhesive hydrogels, which can also form from spray-dried microparticles coming in contact with mucosal surfaces, require careful consideration of several critical properties. These include rheological characteristics, such as storage modulus (G′) and loss modulus (G′′), muco-adhesion which is influenced by the surface charges of polymer constituents, and wetting properties on mucus. Pore size plays a crucial role in ensuring controlled VLP release, and the choice of physical or chemical crosslinking methods impacts gel stability and degradation rates. Other aspects such as thermal- and pH-sensitivity could be considered to ensure responsiveness in physiological conditions. The design could vary depending on the route of administration: gels tailored for rectal delivery, encapsulation in capsules for oral delivery, or nano- and microparticles optimized for nasal delivery. Collectively, these parameters underscore the versatility and adaptability of hydrogel-based systems for mucosal vaccine delivery.

3.3. Sustained Delivery of Antigens from Crystalline VLP Structures

In addition to incorporating polymer matrices, other platforms have been developed for the slow release of VLP vaccines at the delivery site. The cucumber mosaic virus $CuMV_{TT}$ incorporates a tetanus toxin (TT)-derived peptide and a universal T-helper cell epitope [83]. During synthesis, $CuMV_{TT}$ VLP additionally packages *E. coli*-derived ssRNA that stimulates toll-like receptors TLR7/8 [85]. In a recent investigation, microcrystalline tyrosine (MCT), an adjuvant used in allergy immunotherapy, was shown to become decorated with $CuMV_{TT}$ VLPs upon mixing (Figure 5A) [85]. The resulting microcrystalline particles were injected intratumorally into mouse models of melanoma tumors. Inside the tumor the particles formed persistent depots and prolonged the lifetime of $CuMV_{TT}$ VLP in the tumoral environment. To show this effect, the authors formulated fluorescently labeled $CuMV_{TT}$ with and without MCT and conducted intratumoral injections in mice bearing B16F10 melanoma. Tumors were harvested at one- and five-days post-treatment, and the percentage of CD11b+ cells positive for fluorescently labeled $CuMV_{TT}$ was evaluated using flow cytometry. The results showed that labeled VLPs were detectable at similar

levels in both groups one day after the injection; however, by day five, the percentage of CD11b+ cells positive for $CuMV_{TT}$ was significantly higher in the group injected with MCT compared to the $CuMV_{TT}$-alone group (Figure 5B). With $CuMV_{TT}$ + MCT injection, it was further found that polyfunctional CD8+ and CD4+ T cells were induced, and tumor growth was inhibited both locally and systemically. Increased IFN-γ and TNF-α producing CD8+ T cells were also observed. Additionally, the particles increased granulocytic cell populations and decreased monocytic cells, correlating with tumor weight reduction. Finally, a significant abscopal effect was observed, where treatment of one tumor induced antitumor responses in untreated tumors [85].

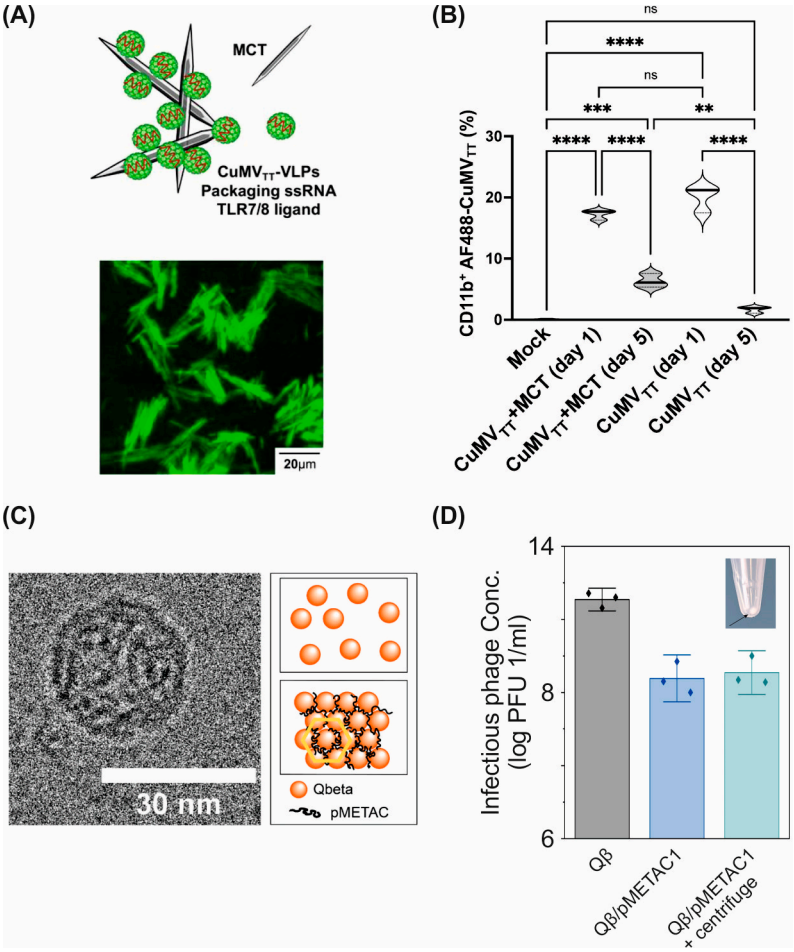

Figure 5. (**A**) Schematic and optical image of cucumber mosaic virus containing a tetanus toxin-derived peptide, $CuMV_{TT}$, decorating microcrystalline tyrosine (MCT). Scale bar 20 μm. (**B**) Representative immunological response, showing the percentage of CD11b+ AF488-labeled $CuMV_{TT}$ cells in tumor after 1 or 5 days of intratumoral injection. Comparisons involving more than two groups were performed using one-way analysis of variance (ANOVA), while comparisons between two groups were conducted using the non-parametric Student's *t*-test. Statistical significance is indicated as follows: **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; ns = not significant. (**C**) Cryo-TEM image of native bacteriophage Qβ, and schematic of Qβ in a hexagonal arrangement induced by polycation pMETAC1 interactions. Scale bar 30 nm. (**D**) Infectivity of native Qβ, Qβ/pMETAC1 suprastructure, and Qβ/pMETAC1 suprastructure + centrifuge (i.e., nanostructures separated from the liquid phase as macroscopic aggregates using centrifugation (inset) and resuspended in fresh medium). Panels (**A**,**B**) are reproduced from "In situ delivery of nanoparticles formulated with micron-sized crystals protects from murine melanoma", Mohsen et al., 10, e004643 [85], 2022 with permission from BMJ Publishing Group Ltd., London, UK; Panels (**C**,**D**) are reproduced from Tran et al. [86] under the terms of the Creative Commons CC BY license.

In another development, cationic polymer poly[2-(methacryloyloxy)ethyl]trimethylammonium chloride (pMETAC) was used to derive the formation of colloidal crystalline arrays of bacteriophage Qβ (Figure 5C) [86]. The VLP packing process was controlled by the solution pH and pMETAC chain length. Colloidal crystals with 2D hexagonal arrangements formed at pH > 7.0, and reversibly disassembled at lower pH values [86]. The polymer chain length was shown to significantly affect the structure formation, with shorter chains (3.3 kDa) leading to highly ordered structures compared to the longer chains (16.5 kDa) [86]. Specific adsorption of pMETAC onto Qβ surfaces was found to be crucial for structure formation, and charge inversion was observed at high polymer concentrations [86]. The study explored the stability of colloidal crystals under different temperatures and ionic strengths, and demonstrated stability up to 60 °C and sensitivity to changes in ionic strength [86]. Specifically, the crystalline VLP assemblies could be separated from the solution by centrifugation and up-concentrated. The infectivity of Qβ in the concentrate was then tested and, while it showed some reduction, it maintained activity (Figure 5D) [86].

These studies show that VLP assemblies, in packed crystalline forms, can sustain long-term release of the VLPs and potentially contribute to the efficacy of their function.

4. Discussion

VLPs have emerged as a promising platform for prophylactic treatments due to their colloidal and structural similarities to actual mammalian viruses, and their ability to present a wide variety of antigens. However, despite their significant potential, various stability and delivery challenges hinder their application in mucosal vaccination. In particular, the hostile conditions of the mucosal surfaces require innovative solutions to enhance VLP stability and efficacy on these surfaces.

4.1. Stability Enhancements Through Material Science Approaches

A major issue in developing VLP-based mucosal vaccines is overcoming destabilization exerted by the mucosal environments (Figure 1A). Crosslinking capsid proteins has shown great promise in improving VLP stability under these conditions. Particularly, crosslinking using bifunctional polymers, such as poly(2-oxazoline) and PEG derivatives, have demonstrated substantial improvements in the chemical, thermal, and mechanical stability of VLPs. This approach ensures that VLPs can withstand harsh environmental conditions and remain effective over a broader pH range and in exposure to enzymes, making VLPs more viable for mucosal immunization.

The efficiency of these systems in inducing mucosal immunity needs further validation through preclinical and clinical studies. However, care must be taken regarding the immunogenicity of these polymers. For instance, anti-PEG antibodies have been shown to reduce the efficiency of PEGylated therapeutic and prophylactic drug molecules [71,87]. This underscores the importance of considering polymer immunogenicity in future applications [88,89].

4.2. Delivery Strategies Through Material Science Innovations

Despite advances in stabilizing VLPs, challenges still remain in generating sufficient levels of antigen-specific sIgA. Incorporation in polymer matrices and hydrogels, in addition to protection against enzymes and sIgA, results in sustained release of VLP vaccines that can potentially enhance the generation of antigen-specific sIgA. Other approaches, such as forming colloidal crystalline structures, can also lead to a sustained VLP release. The ability to fine-tune the crystalline structure based on environmental factors such as pH and ionic strength offers a versatile platform for future designs.

4.3. Benefits Beyond Mucosal Vaccinations: Material Science Solutions for Vaccine Stability

Material science innovations in vaccine formulation can also address challenges that extend beyond mucosal vaccination, offering benefits to all vaccination strategies. For

example, freeze-drying AP205 VLP vaccines with stabilizing agents such as trehalose, sucrose, and TWEEN® 20 enabled stable storage at 18–37 °C for up to six months without loss of immunogenicity, as shown in murine studies [90]. Similarly, CPMV VLPs formulated with Pluronic F127 retained structural and functional integrity during storage at elevated temperatures and demonstrated efficacy in tumor models [91]. These advances further underscore the potential of material science to enhance thermostability and broaden vaccine accessibility, complementing both mucosal and systemic immunization strategies.

4.4. Regulatory Challenges

Regulatory challenges are an important consideration in the development of VLP-based vaccines. Issues such as random RNA packaging and unintended protein associations can complicate the production of highly pure VLP vaccines. This, in turn, makes the licensing process more complex. This aspect was not covered in the present work, and readers are encouraged to refer to references [92,93] for further information.

5. Conclusions

VLP-based mucosal vaccines offer a compelling strategy for controlling infectious diseases that enter the body through mucosal surfaces. The ability of VLPs to carry multiple antigens could be exploited further to design vaccines that target several mucosal pathogens simultaneously. Material science approaches offer promising solutions to the stability and delivery challenges for mucosal vaccination. Further research is clearly required to validate currents innovations in biomimetic mucosal tissues (human organ-on-a-chip models of mucosal epithelium), and then to translate them into preclinical and clinically viable solutions. By addressing the gaps in understanding antigen stability relations to mucosal immune responses and optimizing material science techniques for VLP stabilization and delivery, the field is prepared to make significant breakthroughs in the prevention of infections.

6. Future Directions

Moving forward, research efforts should concentrate on addressing the following critical areas to fully realize the potential of VLPs for mucosal vaccination:

6.1. Refining Mucosal Models for Preclinical Testing

While significant progress has been made in stabilizing VLPs for mucosal applications, the complexity of mucosal surfaces in vivo continues to pose a challenge. In vitro models generally fail to replicate the full spectrum of physiological conditions found in mucosal tissues, such as mucus composition and thickness, pH variations, enzymatic and sIgA activity, and mechanical forces like mucociliary clearance. Future work should prioritize developing more sophisticated in vitro models that accurately mimic these features, including the incorporation of dynamic mechanical environments and viable mucus layers. These models will help bridge the gap between in vitro studies and in vivo applications, improving the predictive value of preclinical research.

Additionally, investigating the colloidal state of VLPs in mucus could be achieved using synthetic mucus or mucus collected from human organ-on-a-chip models, which offer a more realistic simulation of mucosal environments. Moreover, the stability of VLPs in the gastrointestinal tract could be evaluated using artificial gastric fluid and other simulated digestive media to better understand their behavior under the harsh conditions of the digestive system [94].

6.2. Enhancing VLP Stability in Mucosal Environments

Improving the stability of VLPs in mucosal tissues is critical for their success as vaccine platforms. While surface crosslinking and incorporation in biocompatible polymers have shown promise, further exploration is required to optimize these approaches. A more comprehensive understanding of how different polymers interact with mucosal

components such as mucins and sIgA is necessary. Moreover, innovative polymer designs that enhance VLP stability without compromising immunogenicity or inducing adverse immune responses should be prioritized. In particular, the immunogenicity of commonly used polymers should be carefully examined, and alternative materials explored [95–97].

6.3. Optimizing Antigen Delivery and Immune Response

Ensuring that VLPs efficiently deliver antigens to mucosal immune cells remains a key area for improvement. Advanced delivery strategies such as incorporation in biodegradable hydrogels, and crystalline VLP structures can enhance durability of antigen presentation and uptake. However, the immunogenicity of these delivery systems needs further examination. Future research should explore how these methods can induce robust antigen-specific sIgA, and whether incorporation of adjuvants or multiple antigens can further enhance the breadth and durability of immune responses. Furthermore, the importance of designing vaccines which give precise control over spatial and temporal immune responses have been emphasized [98]. Building on these principles, future work should focus on the development of VLP formulations that not only protect against mucosal degradation but also allow for controlled VLP release and timing of immune responses to maximize efficacy.

6.4. Investigating New Biomaterials for VLP Delivery

The exploration of new biocompatible and biodegradable materials for delivering VLPs presents an opportunity for innovation. While current research has mostly focused on chitosan and its derivatives due to muco-adhesive properties, there is considerable potential to discover new polymers that can enhance VLP stability as well as immunogenicity through adjuvanticity. Future work should also investigate how these materials interact with the unique mechanical and biochemical environments of mucosal tissues.

6.5. Interdisciplinary Synergy in Advancing Mucosal Vaccines

Other scientific domains have provided insights into the complexity of mucosal surfaces, and the function of mucus in particular. For example, polymer physics has uncovered certain structural and functional behaviors of mucus, as demonstrated by models such as the gel-on-brush model, which explains the stabilization of respiratory mucus through osmotic and mechanical forces [99]. Similarly, in the gastrointestinal system, mucus compression and flow have been shown to depend on polymer interactions, as described using Flory–Huggins theory, which highlights the impact of dietary and therapeutic polymers on mucus structure and functionality [100]. Research in this direction has further provided insights into pathophysiology of muco-obstructive diseases [101].

This review highlighted how mucosal immunology can benefit from interdisciplinary insights, with contributions from material science, biophysics, polymer science, and bioengineering (e.g., by the development of physiologically relevant organ-on-a-chip mucosal tissues for in vitro screening). With continued interdisciplinary research at the intersection of immunology and these fields, VLP technology has the potential to revolutionize mucosal vaccination strategies.

The future of VLP-based mucosal vaccines is promising, but key challenges remain. By refining preclinical models, enhancing VLP stability, optimizing antigen delivery, addressing mucosal challenges in emerging diseases, and exploring new biomaterials, the field can advance toward developing effective and accessible vaccines for a wide range of mucosal infections.

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