REVIEW

Nanoparticle and virus-like particle vaccine approaches against SARS-CoV-2

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The global spread of coronavirus disease 2019 caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection has provoked an urgent need for prophylactic measures. Several innovative vaccine platforms have been introduced and billions of vaccine doses have been administered worldwide. To enable the creation of safer and more effective vaccines, additional platforms are under development. These include the use of nanoparticle (NP) and virus-like particle (VLP) technology. NP vaccines utilize self-assembling scaffold structures designed to load the entire spike protein or receptor-binding domain of SARS-CoV-2 in a trimeric configuration. In contrast, VLP vaccines are genetically modified recombinant viruses that are considered safe, as they are generally replication-defective. Furthermore, VLPs have indigenous immunogenic potential due to their microbial origin. Importantly, NP and VLP vaccines have shown stronger immunogenicity with greater protection by mimicking the physicochemical characteristics of SARS-CoV-2. The study of NP- and VLP-based coronavirus vaccines will help ensure the development of rapid-response technology against SARS-CoV-2 variants and future coronavirus pandemics.

Keywords: nanoparticle, virus-like particle, SARS-CoV-2, vaccine, COVID-19

Introduction

Coronavirus, a zoonotic RNA virus, can infect mammalian and avian hosts (Woo *et al.*, 2012). Only four "non-severe" strains (i.e., 229E, OC43, NL63, and HKU1) are known to circulate in humans and usually cause mild airway manifes-

tations (Garbino *et al.*, 2006). However, the impact of coronavirus on public health increased with the emergence of severe acute respiratory syndrome (SARS) in 2002 and Middle East respiratory syndrome (MERS) in 2012, which caused 774 and 858 deaths, respectively (Paules *et al.*, 2020). Unlike "non-severe" human coronaviruses, SARS coronavirus (SARS-CoV) and MERS coronavirus (MERS-CoV) utilize distinct host receptors and employ evasion mechanisms to escape host immune responses, which eventually contribute to serious infections in humans (Lee *et al.*, 2019a, 2019b; Molaei *et al.*, 2021). The unprecedented impact of coronavirus on public health has created the need for efficient prophylactic measures.

Vaccines are the most effective and economical way to control infectious diseases. However, they may not be the best option for rapid control of newly emerging pathogens because of the significant time needed to evaluate their safety and effectiveness for human application. Vaccine candidates against SARS-CoV were developed based on inactivated viruses, virus-like particles (VLPs), and DNA vaccine platforms but were not used in human subjects before the outbreak ended in 2003 (Taylor, 2006). While the MERS outbreak also led to vaccine development when the virus spread globally with a rapidly increasing incidence, the candidate vaccine remains under clinical study (Alharbi *et al.*, 2019; Bosaeed *et al.*, 2022). However, when SARS-CoV-2 was identified as a causative agent of coronavirus disease 2019 (COVID-19) and its gene sequences were reported in January 2020, research groups immediately began vaccine development. It seems obvious that the experience gained in earlier coronavirus outbreaks accelerated vaccine development. The COVID-19 pandemic has resulted in far more infections compared to previous outbreaks, highlighting the need for safe and effective vaccines. The speed of transmission also enabled the acceptance of innovative new vaccine platforms; namely, mRNA and adenoviral DNA vector vaccines, in addition to traditional inactivated virus vaccines.

The timeline for clinical studies of new COVID-19 vaccines was exceptionally short to meet the urgent demand. While the first COVID-19 vaccine was licensed in August 2021, vaccines were used in advance under the World Health Organization pre-qualification and emergency use authorization. While it is early yet to conclusively discuss adverse effects related to the new vaccine platforms, safety issues remain of interest as new SARS-CoV-2 variants sustain prolonged spread of infection (Kim *et al.*, 2021a). Risk-benefit assessment has also grown in importance as vaccination programs are extended

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to younger age groups. Therefore, safer vaccines using diverse platforms must also be investigated.

Nanoparticle and Virus-Like Particle Vaccines

Nanoparticle (NP) and VLP vaccines are terminologically interchangeable because they express the antigen of interest on virus-sized particles. Many NPs are derived from naturally oligomer-forming molecules, whereas VLPs utilize viral capsid proteins or replication-defective virus particles. More details about both nanoscale vaccine platforms against SARS-CoV-2 are covered later in this review (Table 1).

NP and VLP platforms can be classified by type (Fig. 1A). NPs are subdivided into two groups according to the simultaneous or separate expression of antigen and scaffold proteins. NPs can express scaffolds with antigen domains within a single peptide, enabling antigen display on the outer surface through self-assembly (Lim *et al.*, 2021). However, many NP approaches, especially for recent COVID-19 vaccine development, have separately expressed scaffold and antigen domains, which are later combined using linking systems. One advantage to the separate expression is the *Escherichia coli*-based production of the NP scaffold, while the antigenic portions can be produced in eukaryotic cell lines. *E. coli*-based expression enables the cheaper and faster production of scaffold proteins compared to eukaryotic expression systems. Another advantage is the flexible use of the NP platform for different antigen combinations based on common linking systems (Brune *et al.*, 2016; Zhang *et al.*, 2020; Boyoglu-Barnum *et al.*, 2021; Cohen *et al.*, 2021a). The use of a single antigen with linking system allows it to be easily shared by different types of NP scaffolds. Also, multiple antigens in a common linking system can be conjugated within a single particle to generate mosaic NP vaccines.

VLPs are intrinsically immunogenic substances, as they usually carry genetic materials and pathogen-associated molecular patterns (Yan *et al.*, 2005; Zepeda-Cervantes *et al.*, 2020; Mohsen *et al.*, 2022). The essential gene is removed to ensure safety, rendering VLPs replication-defective. For example, non-structural gene knockout influenza particles were produced by reverse genetics to yield replication-defective VLPs

Table 1. Nanoparticle (NP) and virus-like particle (VLP) vaccine approaches against SARS-CoV-2

Platform	Scaffold	Antigen(s)	Antigen production*	Animal model(s)	Linking system	Adjuvant(s)	Reference
NP	I50-50	RBD	Expi293F	Mouse	I53-50A/I53-50B	AddaVax	Walls *et al.* (2020)
		RBD	HEK293F	Mouse	SpyTag/SpyCatcher	SAS	Kang *et al.* (2021)
		Whole S	HEK293F	Mouse, rabbit, NHP	I53-50A/I53-50B	polyI:C, MPLA	Brouwer *et al.* (2021)
	Ferritin	RBD	293i	NHP	None	Aluminum	Li *et al.* (2021)
		RBD	Expi293	Mouse	None	Quil-A, MPLA	Powell *et al.* (2021)
		RBD	Expi293	NHP	None	ALFQ	King *et al.* (2021)
		RBD	HEK293	Ferret	None	AddaVax	Kim *et al.* (2021b)
		RBD	HEK293F	Mouse	SpyTag/SpyCatcher	SAS	Kang *et al.* (2021)
		RBD, HR	CHO-S	Mouse, NHP	SpyTag/SpyCatcher	SAS	Ma *et al.* (2020)
		RBD, S2G	ExpiCHO	Mouse	SpyTag/SpyCatcher	AddaVax, Adju-Phos	He *et al.* (2021)
		Whole S	Expi293	Hamster	None	ALFQ	Wuertz *et al.* (2021)
	mi3	RBD	Expi293	Mouse	SpyTag/SpyCatcher	Quil-A, MPLA	Guo *et al.* (2021a)
		RBD	Expi293F	Mouse	SpyTag/SpyCatcher	AddaVax	Halfmann *et al.* (2021)
		RBD	Expi293F	Mouse	SpyTag/SpyCatcher003	AddaVax	Tan *et al.* (2021)
		RBD	Expi293F	Mouse	SpyTag003/SpyCatcher003	AddaVax	Cohen *et al.* (2021a)
		RBD	HEK293F	Mouse	SpyTag/SpyCatcher	SAS	Kang *et al.* (2021)
VLP	AP205	RBD	Expi293F	Mouse, NHP	SpyTag/SpyCatcher	Aluminum, CpG-ODN	Guo *et al.* (2021b)
		RBM	*E. coli* C2566	Mouse	None	None	Liu *et al.* (2021a)
		S1 subunit	ExpiSf9	Mouse	SpyTag/SpyCatcher	Addavax	van Oosten *et al.* (2021)
	Ad5	Whole S	HEK293	Mouse	None	None	Wu *et al.* (2020)
		Whole S	HEK293	Mouse, NHP	None	None	Feng *et al.* (2020)
	ChAd	Whole S	293	Mouse	None	None	Hassan *et al.* (2020)
	CuMV	RBM	*E. coli* C2566	Mouse	None	None	Mohsen *et al.* (2022)
	F88-4 phage	S epitope	K91kan *E. coli*	Mouse	None	None	Staquicini *et al.* (2021)
	MLV	Whole S	293GP	Not tested	None	None	Roy *et al.* (2021)
	NDV	Whole S	BSRT7	Mouse, hamster	None	Addavax	Sun *et al.* (2020)
		Whole S	HEK293T	Mouse	None	SAS	Yang *et al.* (2021)
	RABV	S1 subunit	BEAS-2B, Vero	Mouse	None	MPLA, Addavax	Kurup *et al.* (2020)
	VSV	RBD	HEK293T	Mouse	None	None	Hennrich *et al.* (2021)
		Whole S	BHK-21, Vero E6	Mouse, hamster	None	None	Yahalom-Ronen *et al.* (2020)
		Whole S	BSRT7/5, Vero CCL81	Mouse	None	None	Case *et al.* (2020)

*Scaffold expression for NP vaccines that utilize linking system in an *E. coli* expression system. In these cases, antigen production system indicates expression cell line used for antigenic domain. Ad5, human type 5 adenovirus; ALFQ, army liposomal formulation QS21; ChAd, chimpanzee adenovirus; CuMV, cucumber mosaic virus; HR, heptad repeat; MPLA, monophospholipid A liposome; MLV, Moloney murine leukemia virus; NDV, Newcastle disease virus; RABV, rabies virus; RBD, receptor-binding domain; RBM, receptor-binding motif; SAS, Sigma adjuvant system; S2G, glycine-capped spike protein; VLP, virus-like particle; VSV, vesicular stomatitis virus.
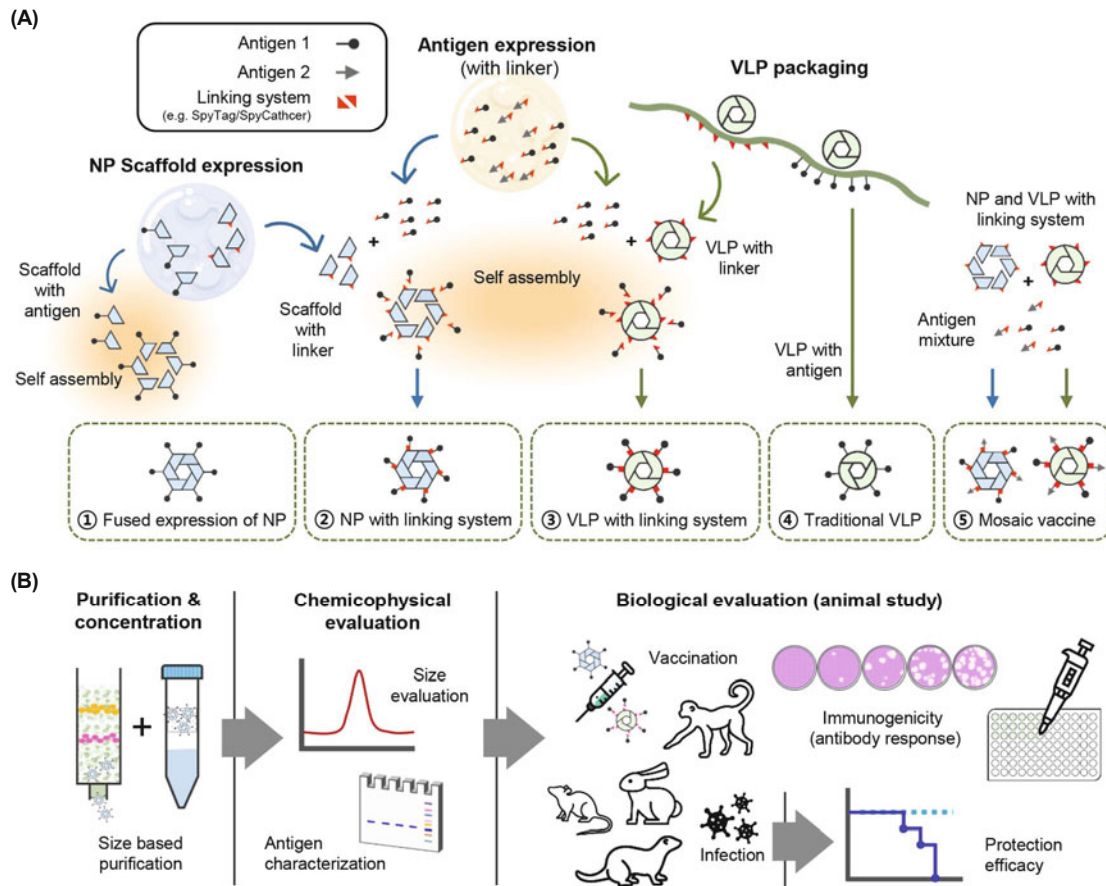
Fig. 1. **Production of nanoparticle (NP) and virus-like particle (VLP) vaccines against SARS-CoV-2.** (A) Classification of NP and VLP approaches. NP scaffold is structural protein that construct nanoscale protein structure. (1) Some NP vaccine approaches express antigen fused with scaffold protein which then form NPs that displays antigen on the outer wall of the particle. (2) Linker protein can be alternatively expressed on the NP surface to conjugate separately expressed antigen. (3) Likewise, VLP produced from packaging cell lines can express linker on surface for antigen display. (4) Direct display of antigen on VLP is traditional for generating VLP with foreign antigen. (5) A linking system can be used to generate mosaic vaccine by conjugating mixed antigens. Mosaic vaccine can induce immune response against multiple epitopes and can provide broader-spectrum protection. (B) NP and VLP are purified based on size and concentrated. Isolated vaccine candidates are chemicophysically evaluated before testing for biological safety and efficacy in animal models.

that protected mice from lethal challenge (Watanabe *et al.*, 2002). Based on their immunogenicity and safety advantages, VLPs have been used for vaccine development longer than NPs (Shirbaghaee and Bolhassani, 2016). VLP technology has also fused foreign antigens to the VLP coat or membrane proteins (Frietze *et al.*, 2016). VLPs also introduced a linking system and created efficient scaffold systems for foreign antigens. These continuous improvements broaden the scope of the application of VLP platforms for vaccine use. Viral capsid protein cloning and expression is another option for generating VLPs without incorporating genetic material (van Oosten *et al.*, 2021). Genetic material-free VLP vaccines are closer to the concept of NP vaccines and can also be used for drug delivery.

Antigen Construction and Linking System for COVID-19 Vaccines

Unlike whole-virus vaccines, subunit vaccines need to select antigens, including epitopes with higher immunogenic po-

tential. At present, the most popular SARS-CoV-2 antigen is the receptor-binding domain (RBD) located in the spike (S) protein, which comprises the S1 and S2 subunits (Ke *et al.*, 2020). Immunization with RBD can induce protective humoral immunity against viral pathogens, including SARS-CoV, MERS-CoV, and influenza virus (Jiang *et al.*, 2012; Zhou *et al.*, 2018). SARS-CoV-2 also has an RBD that plays a primary role in virus entry by interacting with angiotensin-converting enzyme 2 (ACE2). RBD-specific antibodies can efficiently block virus entry (Ju *et al.*, 2020; Seydoux *et al.*, 2020; Shang *et al.*, 2020). One study suggested that the RBD is more immunogenic than the entire S protein when equal amounts of each antigen are loaded and displayed on the same NP scaffold (Guo *et al.*, 2021a). An additional advantage of using RBD rather than the whole S protein is reduced antigen size, as the expression of smaller antigens can increase production yield (Salzer *et al.*, 2021). So far, most NP approaches against SARS-CoV-2 use RBD as an antigen, whereas VLP approaches more frequently use the whole S protein. The S2 subunit is more conserved than the S1 subunit and has a greater potential to induce cross-reactive antibody (Ma *et al.*, 2020;

Shah *et al.*, 2021). In one study, the heptad repeat antigen within the S2 subunit efficiently induced neutralizing antibodies against seasonal human coronaviruses, including 229E and OC43 (Ma *et al.*, 2020). However, another study reported that the S2 subunit expressing baculovirus-based VLP failed to induce neutralizing antibody responses, while whole S and S1 subunit expressing VLPs were successful (Chu *et al.*, 2021).

SpyTag/SpyCatcher is a paired linking system frequently used in SARS-CoV-2 NP vaccine development (Brune *et al.*, 2016). This "plug-and-display" system can link the SpyTag-antigen to the SpyCatcher-scaffold protein (or *vice versa*) by spontaneous isopeptide bond formation. Recently, another modification was introduced to SpyTag/SpyCatcher to achieve a more efficient antigen display and enhanced stability (Rahikainen *et al.*, 2021). Fc-tagged SARS-CoV-2 RBD has also been coupled with a protein A-expressing VLP scaffold (Geng *et al.*, 2021). The SnoopLigase-mediated linking system is another platform that can be used for antigen-vaccine conjugation (Andersson *et al.*, 2019). In an earlier study, both SpyTag/SpyCatcher and SnoopLigase systems were introduced into a single scaffold for twin antigen immunization (Brune *et al.*, 2017). That study expanded the application range of NPs to achieve stronger immunization by expressing additional antigens in a single vaccination.

There is a demand for the development of a novel linking system as people may possess linker-specific immune responses or develop such responses by repeated immunizations. Due to its bacterial origin, 98% of the tested human samples were positive for antibodies specific for SpyTag/SpyCatcher protein (Rahikainen *et al.*, 2021). It is not clear if linker-specific antibodies affect the immunogenicity of NP and VLP vaccines. These concerns may be overcome by chemical conjugation methods or by introducing genetic fusion so that antigen and scaffold proteins are expressed in a single system (Patel and Swartz, 2011; Charlton Hume and Lua, 2017; Lim *et al.*, 2021).

Production of NP and VLP Vaccines

Since NPs and VLPs use diverse cell lines for expression, the process itself can differ by platform. Protein subunits for NPs are purified using affinity tags and assembled into NPs, whereas VLPs are generally produced from packaging cell lines that are transiently transfected with viral gene-coding plasmids. An additional assembly step is required in the case of a linking system used for antigen display. NPs and VLPs separated by size-exclusion chromatography collected particles in the desired size ranges. Once NP and VLP particles are ready, many of their evaluation steps are common (Fig. 1B). NP and VLP size and morphology are evaluated by electron microscopy. Further biophysical techniques, such as dynamic light scattering and high-performance size-exclusion chromatography, are also used for size evaluation. It is also important to analyze the trimeric antigen formation and glycosylation patterns for COVID-19 vaccine development. Although the correlation between glycosylation and immunogenicity is not obvious, homogenous glycosylation is a parameters that requires assessment (van Oosten *et al.*, 2021). These biochemical analyses typically employ mass spectro-

metry techniques. In addition, protein stability evaluation is important in NP vaccine development as it affects the storage period and temperature requirements. Biolayer interferometry is useful for assessing antigenic characteristics by measuring the interaction between RBD or whole S antigen displayed on vaccine candidates and immobilized ACE2 (Forssén *et al.*, 2020).

Finally, vaccine candidates are tested for safety and immunogenicity. The mouse model is most frequently used to evaluate COVID-19 vaccine candidates. As laboratory mice (e.g., BALB/c, C57BL/6) lack ACE2 expression, K18-hACE2 transgenic mice are used instead (Winkler *et al.*, 2020). Syrian golden hamsters, ferrets, rabbits, and cynomolgus macaques have also been used for vaccine evaluation (Sia *et al.*, 2020; Brouwer *et al.*, 2021; Kim *et al.*, 2021b). Vaccine candidates are prepared with or without adjuvants and introduced to animals to monitor antibody responses. Enzyme-linked immunosorbent assay (ELISA) is a basic method used to evaluate antigen-specific antibody production. Neutralizing antibody production can be assessed by plaque reduction neutralization test; however, this test requires a biosafety level 3 facility due to the use of live virus. Thus, alternative methods for measuring the levels of neutralizing antibodies, such as competition ELISA have been developed (Wouters *et al.*, 2021). Levels of protection against SARS-CoV-2 infection are determined by the virus burden from nasal swabs and lungs, histopathology, weight loss, and survival rate.

NP-Based COVID-19 Vaccines

I53-50, an icosahedral protein scaffold generated by computational engineering, can display 120 antigens in a trimeric configuration (Bale *et al.*, 2016). Separately expressed trimeric antigens (containing I53-50A or I53dn5B) and scaffolds (I53-50B or I53dn5A) are assembled *in vitro*. This system was first used for respiratory syncytial virus (RSV), influenza, and human immunodeficiency virus (HIV) vaccine development (Brouwer *et al.*, 2019; Marcandalli *et al.*, 2019; Boyoglu-Barnum *et al.*, 2021). These I53-50-based vaccine candidates induced robust antibody responses in animal models. Notably, the NP scaffold itself acted as an immunogen and elicited specific antibody responses (Marcandalli *et al.*, 2019). However, NP scaffold-specific antibody responses were reduced when the scaffolds were masked by antigens. Pre-immunization with the NP scaffold did not affect the antibody response against subsequent immunization with antigen-containing NPs, suggesting that NP scaffold-specific antibodies do not adversely affect vaccine efficacy.

For COVID-19 vaccine development, self-assembling scaffold components (I53-50B) were expressed by *E. coli* and trimeric RBDs were purified from HEK293F supernatant (Walls *et al.*, 2020). Assembled RBD-I53-50 NPs elicited robust antibodies in immunized mice and nonhuman primates. Although this review does not discuss the role of adjuvants in NP vaccine development, adjuvant selection was not critical for the immunogenicity of the RBD-I53-50 NP vaccine (Arunachalam *et al.*, 2021). I53-30 is the closest COVID-19 NP vaccine for human use and is currently in clinical trials (NCT04742738, NCT05007951). Another study tested modified S proteins

instead of RBD in the I53-50 setting (Brouwer *et al.*, 2021). Immunization with S-I53-50 NP induced antibodies against multiple neutralizing targets and protected macaques from SARS-CoV-2 infection (Brouwer *et al.*, 2020, 2021).

Ferritin is a common natural protein cage that stores iron. It is found in animals, including humans and some bacteria. *Helicobacter pylori* ferritin forms a 24-mer structure containing eight threefold axes that can be used to display trimeric viral spikes, such as influenza hemagglutinin (Kanekiyo *et al.*, 2013). Ferritin has also been used for HIV (He *et al.*, 2016), Epstein-Barr virus (Kanekiyo *et al.*, 2015), RSV (Swanson *et al.*, 2020), and MERS-CoV (Kim *et al.*, 2018) vaccines. Several potential COVID-19 vaccines have also used ferritin as a scaffold system, mostly using RBD as an antigen (Ma *et al.*, 2020; He *et al.*, 2021; Kang *et al.*, 2021; Kim *et al.*, 2021b; King *et al.*, 2021; Li *et al.*, 2021; Powell *et al.*, 2021). Only one study used the entire S subunit (Wuertz *et al.*, 2021). While most of the COVID-19 vaccine studies used *H. pylori* ferritin, human and horse ferritin have also been evaluated (Qi *et al.*, 2018; Kalathiya *et al.*, 2021). Deletion of the 70 aa C-terminal in ferritin showed enhanced neutralizing antibody responses (Powell *et al.*, 2021).

mi3 NP, a mutant form of a rationally designed i301 nanocage, showed increased production yield, particle uniformity, and stability compared to the original structure (Bruun *et al.*, 2018). This self-assembling NP scaffold can display 20 trimeric antigens (60-mer). The platform is thermally stable and, therefore, useful for vaccine supply, where cold-chain storage is problematic (Liu *et al.*, 2021b; Rahikainen *et al.*, 2021). mi3 has been used to develop vaccines against influenza virus (Cohen *et al.*, 2021b), classical swine fever virus (Liu *et al.*, 2021b, 2021c), and malaria (Bruun *et al.*, 2018) in addition to vaccine candidates for SARS-CoV-2 (Cohen *et al.*, 2021a; Guo *et al.*, 2021a; Halfmann *et al.*, 2021; Kang *et al.*, 2021; Tan *et al.*, 2021). These RBD-based mi3 vaccines also induced neutralizing antibodies against SARS-CoV-2 variants and SARS-CoV (Halfmann *et al.*, 2021).

IMX313 contains the oligomerization domain of an avian C4-binding protein (Ogun *et al.*, 2008). During early development, antigen-conjugated IMX313 was designed to be expressed by the modified vaccinia virus Ankara (MVA). MVA-expressing *Mycobacterium tuberculosis* 85A-IMX313 showed an adjuvant effect and successfully induced CD4^{+} and CD8^{+} T cell responses in animal models (Spencer *et al.*, 2012). Similarly, the *Plasmodium falciparum* Pfs25-IMX313 complex was developed as a malaria vaccine (Li *et al.*, 2016). Psf25-IMX313 was delivered intramuscularly using a protein-in-adjuvant regimen, which demonstrated its potential for protein-based vaccine use (Li *et al.*, 2016; Brod *et al.*, 2018). DNA vaccine approaches have also been used to express HIV Tat-IMX313 (Tomusange *et al.*, 2016). IMX313-based *M. tuberculosis* and malaria vaccine candidates showed both safety and immunogenicity in human clinical trials (Minhinnick *et al.*, 2016; de Graaf *et al.*, 2021). Although IMX313 is a well-established NP system, it has not been used for COVID-19 vaccine development, possibly because NP systems that express antigens in trimeric configurations (i.e., I53-50, mi3, ferritin) are more suitable for displaying SARS-CoV-2 S protein or RBD.

VLP-Based COVID-19 Vaccines

VLP vaccines are sometimes indistinguishable from NP vaccines. When genetic material-free viral capsid structures are adopted for vaccine use, VLPs fit perfectly into the self-assembled and protein-based NP category. Due to this NP-like character, the protein coat of the AP205 bacteriophage (AP205 VLP) frequently utilizes the SpyTag/SpyCatcher linking system to display antigens (Brune *et al.*, 2016; Leneghan *et al.*, 2017; Cohen *et al.*, 2021b; Guo *et al.*, 2021b; van Oosten *et al.*, 2021). While one study comparing AP205 VLP and other NP vaccines, such as mi3 and IMX313, suggested that VLPs induced stronger antibody responses than NPs (Leneghan *et al.*, 2017), other studies showed comparable antibody responses (Bruun *et al.*, 2018; Cohen *et al.*, 2021b). More recently, in a COVID-19 vaccine study in animals, AP205 VLPs induced specific antibody responses (Guo *et al.*, 2021a; Liu *et al.*, 2021a; van Oosten *et al.*, 2021). Phage display is another way to use a bacteriophage for COVID-19 vaccine development. The dual display of the SARS-CoV-2 S protein epitope and the $\alpha_3\beta_1$ integrin ligand on the f88-4 bacteriophage efficiently induced antibody responses against the S protein (Staquicini *et al.*, 2021).

Many other plant virus- and eukaryotic virus-based pseudovirus platforms have been investigated for use in COVID-19 vaccines. Human type 5 adenovirus (Ad5) is the best-studied and is widely used as a replication-defective vector system. Ad5-based VLPs induced immunity against SARS-CoV-2 and protected mice and rhesus macaques from infection (Feng *et al.*, 2020; Wu *et al.*, 2020). Clinical studies of the Ad5 COVID-19 vaccine have demonstrated its safety and immunogenicity in human adults (Zhu *et al.*, 2020b; Wu *et al.*, 2021). Another study showed that chimpanzee adenovirus (ChAd) also induced robust antibody responses against SARS-CoV-2 (Hassan *et al.*, 2020), in which the intranasal administration of a ChAd-based vaccine induced both S protein-specific IgG and IgA antibodies. In another study, a gene sequence from tetanus toxin was introduced to cucumber mosaic virus (CuMV) to boost T cell responses (Zeltins *et al.*, 2017). CuMV VLP-expressing SARS-CoV-2 receptor-binding motif (RBM) induced an antibody response against RBD (Mohsen *et al.*, 2022). More VLP platforms show promise for COVID-19 vaccines, including those for Newcastle disease virus (Sun *et al.*, 2020; Yang *et al.*, 2021), rabies virus (RABV) (Kurup *et al.*, 2020), and vesicular stomatitis virus (VSV) (Case *et al.*, 2020; Yahalom-Ronen *et al.*, 2020; Hennrich *et al.*, 2021). RABV and VSV belong to the *Rhabdoviridae* family and have long been used for foreign antigen display. Glycoprotein was deleted to make replication-defective VSV, and RABV VLPs were tested in both live and inactivated settings. Inactivated RABV VLPs showed higher antibody responses than live VLPs (Kurup *et al.*, 2020). While most VLP approaches are not adjuvanted, this study also tested adjuvants and confirmed that adjuvants induced stronger antibody responses and changed the IgG2a/IgG1 ratio (Kurup *et al.*, 2020). In another study using NDV VLPs, adjuvant was effective when lower dose of antigen was used (Sun *et al.*, 2020).

While most NP studies used RBD as an antigen, those on VLPs more frequently used the whole S protein. Numerous attempts have also been made to enhance immunogenicity

by displaying the S protein. For example, when whole SARS-CoV-2 S or 19 amino acid-deleted S (DS) proteins were displayed on Moloney murine leukemia virus (MLV) VLPs, DS was more efficiently loaded on the VLP surface (Roy *et al.*, 2021). Although that study did not compare S and DS antigens for immunogenicity in animal studies, more compact antigen presentation will increase vaccine efficacy. Other studies substituted two residues (986 and 987), deleted the polybasic cleavage site, and optimized codons to enhance protein stability and increase VLP yield (Hassan *et al.*, 2020; Sun *et al.*, 2020; Wu *et al.*, 2020). As the whole S protein can induce antibodies that are cross-reactive with SARS-CoV (Wuertz *et al.*, 2021), current VLP approaches are somewhat more advantageous than NPs that mainly use RBD in terms

of broader protection against SARS-CoV-2 variants and newly emerging highly pathogenic coronaviruses.

Immunological Advantages of Nanoscale Vaccines

The protective efficacy of vaccines is determined by their capacity to induce potent antibody responses, as neutralizing antibody titers have been considered the mechanistic correlates of protection (Hogan and Pardi, 2021). This has been confirmed in COVID-19 vaccine studies (Yu *et al.*, 2020; Khoury *et al.*, 2021). However, the vaccine-induced activation of T cells is equally important. T follicular helper (Tfh)
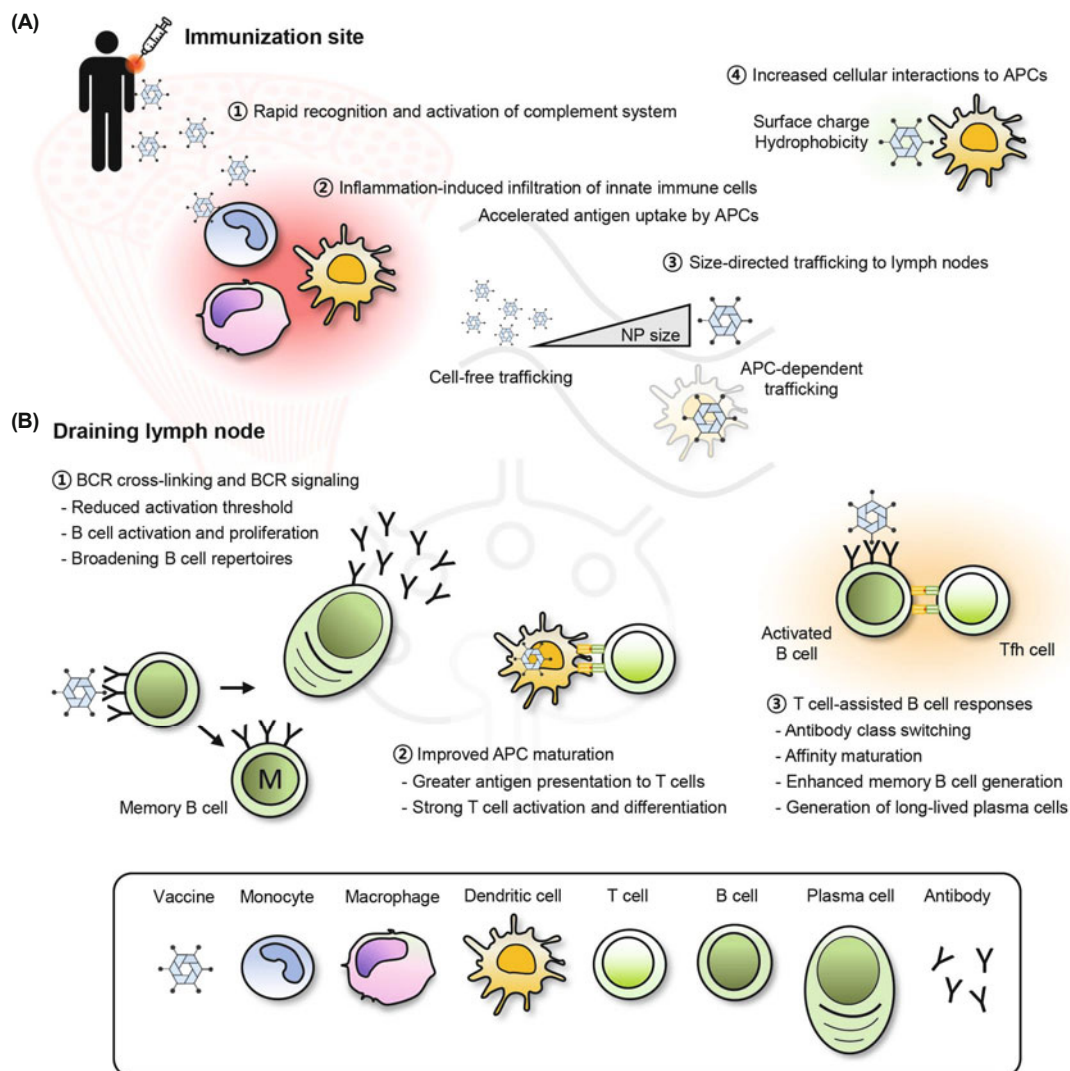

Fig. 2. Immunological advantages of nanoscale vaccines. Overview of immunogenic parameters of nanoparticle (NP) and virus-like particle (VLP) vaccines in inducing potent antibody, T cell, and B cell responses. (A) Accelerated engagement of nanoscale vaccines with the innate immune system at immunization site. Upon immunization, characteristic features of nanoscale vaccines, including high density of multimeric antigen, size, charge, and hydrophobicity, collectively influence their greater interactions with the innate immune system and trafficking to draining lymph nodes, leading to local inflammation, infiltration of innate immune cells, and uptake and processing of antigen by antigen-presenting cells (APCs). (B) Strong induction of humoral and cellular immunity in draining lymph nodes. Higher antigen density improves B cell receptor (BCR) cross-linking with multiple antigens, which lowers B cell activation threshold and thereby induces potent B cell activation. Also, enhanced antigen presentation by APCs induces strong T cell activation. Vaccine-induced T follicular helper (Tfh) cells provide help to cognate B cells for antibody class switching, affinity maturation, and generation of long-lived antibody-secreting plasma cells and memory B cells. DC, dendritic cells.

cells assist B cells in producing high-affinity antibodies as well as differentiation into memory B cells and long-lived antibody-secreting plasma cells (Crotty, 2019). As antibody titers decline and escape variants emerge, neutralizing antibodies often fail to maintain sterilizing immunity against SARS-CoV-2 infection (Noh et al., 2021). In this scenario, memory T cells are the ultimate agent of protection by eliminating infected cells, thereby limiting the spread of infectious agents. Indeed, the depletion of CD8 T cells impaired the protection of convalescent macaques against SARS-CoV-2 when antibody titers were suboptimal (McMahan et al., 2021). Accordingly, understanding the precise immune mechanisms elicited by vaccines is essential to determine their protective efficacy (Fig. 2). During a vaccine response, immunization triggers local inflammation and infiltration of innate immune cells, including antigen-presenting cells (APCs) such as monocytes, macrophages, and dendritic cells (DCs). APCs capture antigens at the site of immunization, migrate to draining lymph nodes, and present processed peptide antigens to cognate T cells that subsequently proliferate and differentiate into effector and memory T cells (Schenten and Bhattacharya, 2021). Antigens trafficked to draining lymph nodes are also directly recognized by B cells through their B cell receptors (BCRs) on the cell surface, triggering B cell activation, proliferation, and differentiation (Irvine and Read, 2020).

Traditional vaccine platforms, including inactivated and live attenuated viral vaccines, are highly effective in inducing antibody and T cell responses, but are not safe and may be potentially infectious, particularly in older individuals and adults with underlying diseases (Kim et al., 2017). Conversely, recombinant protein-based subunit vaccines are safe but often less immunogenic and suboptimal, requiring adjuvants to induce inflammation (Nguyen and Tolia, 2021). Adenovirus-based vaccines are another successful platform. Unfortunately, the prevalence of common adenovirus serotypes in humans along with immunization history could limit the selection of adenovirus serotypes to evade pre-existing immunity against the adenoviral vector itself in the vaccine development of newly emerging infectious diseases (Zhu et al., 2020a). Recent mRNA vaccines have shown high efficacy in protecting against SARS-CoV-2, with durable antibody and T cell responses (Polack et al., 2020; Baden et al., 2021; Hogan and Pardi, 2021). However, the nature of inflammatory factors and associated effector molecules responding to synthetic mRNA and lipid-NP carriers remain unresolved (Hogan and Pardi, 2021) and may lead to unexpected side effects such as myocarditis (Kim et al., 2021a). In addition, as type I interferon is a potent negative regulator of Tfh cell differentiation (Ray et al., 2014), the intrinsic property of synthetic mRNA in inducing a strong type I interferon response could skew CD4 T cell responses toward T helper type 1 (Th1) cell differentiation and away from desirable Tfh cell development (Guerrera et al., 2021).

NP vaccines have demonstrated enhanced vaccine responses and greater protection compared to traditional subunit vaccines. The high density of repetitive protein antigen is a major factor that contributes to the higher immunogenicity (Kelly et al., 2019; Brouwer et al., 2021). Multimeric protein antigens have long been associated with improved antibody responses for several reasons (Slifka and Amanna, 2019). Monomeric soluble antigens have limited interactions with the BCRs of the cognate B cells. In contrast, a high density of surface antigens leads to stronger interactions with multiple BCRs, which promote BCR cross-linking, activation of downstream signaling cascades, and potent B cell activation (Bachmann and Zinkernagel, 1997). Activated B cells can, in turn, interact with cognate Tfh cells and receive essential signals required for class switching, affinity maturation, and generation of long-lived antibody-secreting plasma cells and memory B cells (Crotty, 2019). Importantly, stronger interactions of antigens with multiple BCRs increase overall avidity to the cognate antigen and, therefore, lower the activation threshold of B cells (Bachmann and Zinkernagel, 1997). Accordingly, B cells with relatively weak avidity to antigens may also be recruited into the responses, broadening the repertoires of B cells responding to the antigen, as shown by one vaccine study that used multivalent HIV antigens (Abbott et al., 2018). Along with B cells, antigen multivalency with optimal size also facilitates an enhanced recognition of antigens by the innate immune system. Rapid recognition of NPs by the complement system induces local inflammation and antigen transfer to APCs. Multivalent antigens are also directly recognized by APCs, including DCs (Kelly et al., 2019). Collectively, accelerated antigen uptake and processing by APCs leads to greater antigen presentation to cognate T cells in the draining lymph nodes to promote T cell activation and differentiation.

Beyond antigen multivalency, other factors, including NP size, charge, and trafficking to draining lymph nodes, contribute to vaccine efficacy (Gause et al., 2017). For example, NP size controls lymph node trafficking, with sizes of 20–30 nm freely trafficking to draining lymph nodes, while APCs are strictly required for the transport of larger NPs from the injection site to lymph nodes (Reddy et al., 2006; Manolova et al., 2008). Although results have varied, studies have shown that size also influences the distribution of particles within draining lymph nodes, with 20-nm polystyrene beads preferentially localized in the B cell area to facilitate their interactions with B cells (Manolova et al., 2008). Such size-dependent changes in antigen trafficking to lymph nodes appear to be functionally important, as 40-nm polystyrene bead–conjugated OVA antigen induced the highest CD4 and CD8 T cell responses as well as potent antibody production compared to larger particle sizes in immunized mice, presumably due to the enhanced antigen presentation by DCs in the lymph nodes (Fifis et al., 2004). The biochemical properties of NP surfaces are important determinants. Positively charged surfaces preferentially interact with negatively charged membranes of innate immune cells (Fromen et al., 2015). Similarly, hydrophobic NPs lead to increased cellular interactions, resulting in enhanced immune responses (Moyano et al., 2012). The major advantage of NP vaccines is their ability to modulate these properties, including antigen multivalency, size, charge, and lymph node trafficking, to improve antibody and T cell responses and, ultimately, vaccine efficacy.

Conclusion

There is ongoing debate regarding the usefulness of the

COVID-19 vaccine in the post-pandemic era as it is difficult to predict the future of COVID-19 (Telenti *et al.*, 2021). Despite systematic studies suggesting the possibility of seasonal circulation of COVID-19 (Audi *et al.*, 2020; Li *et al.*, 2020), it is not clear whether virulence will remain sufficiently high to require vaccination programs. SARS-CoV-2 may disappear or become endemic within restricted areas, similar to previous SARS-CoV and MERS-CoV outbreaks (Al-Omari *et al.*, 2019). Nonetheless, a rapid vaccine production platform is critical to minimize the impact of unexpected respiratory virus pandemics.

The introduction of new types of vaccines against SARS-CoV-2 undoubtedly reduced morbidity and mortality rates among high-risk groups (McNamara *et al.*, 2021). However, young people, who have lower SARS-CoV-2 morbidity and mortality, need assurance of greater safety (Brandt *et al.*, 2021). The adjuvanted subunit vaccines are already in the vaccine market, and accumulating studies have evaluated their safety and efficacy. Protein-based NP vaccines are believed to be similarly safe and effective for human use. Cost-effectiveness will be an ongoing concern once multiple fast-responding vaccine platforms are prepared. Therefore, continuous efforts to reduce the costs for NP and VLP vaccine production will be the key to competitive pricing. Production of the NP vaccines in yeast may lower costs (Dalvie *et al.*, 2021). Also, for NP and VLP production, use of linking systems that adopt *E. coli* to express scaffold protein may be advantageous. In these systems, the folding process or post-translational modification is less strict (Kang *et al.*, 2021); however, *E. coli* lacks post-translational modification which may affect glycosylated antigen expression. Further, *E. coli* expression may pose a problem with protein folding. However, *E. coli*-based expression of influenza and MERS-CoV vaccine candidates successfully induced neutralizing antibody responses, suggesting that *E. coli* is an attractive expression system for NP vaccine production (Kim *et al.*, 2018; Lim *et al.*, 2021). Further developments employing the innovative chaperone technology will enhance *E. coli*-based soluble expression systems and provide an efficient method for NP vaccine production (Son *et al.*, 2021). Similarly, the plant-based generation of VLPs may be useful for high-yield vaccine production (Maharjan and Choe, 2021).

Efforts are ongoing to develop a universal influenza vaccine to control continuous seasonal outbreaks and occasional pandemics (McMillan *et al.*, 2021). Likewise, prophylactic vaccination with a universal vaccine may be beneficial in controlling seasonal and new coronavirus pandemic outbreaks. Although some RBD-specific antibody clones isolated from patients with COVID-19 show cross-reactivity to SARS-CoV or MERS-CoV, they have greater reactivity to SARS-CoV-2, suggesting that a single RBD is not sufficient to induce immunity against a broad spectrum of coronaviruses. To overcome this problem, one group recently developed mosaic NPs that display multiple RBDs from distinct clades, and this vaccine candidate elicited immunity against heterologous coronavirus strains (Cohen *et al.*, 2021a). Alternatively, a mixture of NPs that express different RBDs can induce broader immune responses.

NP and VLP vaccines also have inherent hurdles to overcome to make them more attractive for the COVID-19 vaccine platform. Safety issues that might arise from the pathogen-derived features of VLPs and the use of immune-stimulatory adjuvants are critical for vaccine development. Some adverse effects have been reported following AS04-adjuvanted human papillomavirus VLP vaccination (Harris *et al.*, 2014). As traditional aluminum adjuvants are less effective in weak immune responders (Miller *et al.*, 1999), newer adjuvants such as pattern-recognition receptor agonists are necessary despite their pro-inflammatory potential (Wu, 2016). Compared to monomeric subunit vaccines, NP and VLP approaches demand more thorough validation for antigen and scaffold protein expression because incorrect folding significantly disrupts particle formation. Molecular engineering can enhance antigen stability and solubility. For example, modification of the VLP capsid to remove the surface charge increased solubility and improved assembly (Lu *et al.*, 2015). Despite these concerns, NP and VLP technologies are flexible platforms with remarkable expandability. Further studies will identify promising options for vaccine development for the current COVID-19 pandemic and future coronavirus outbreaks.

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Conflict of Interest

The authors have no conflicts of interest to disclose.

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