Review: Development of SARS-CoV-2 immuno-enhanced COVID-19 vaccines with nano-platform

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ABSTRACT

Vaccination is the most effective way to prevent coronavirus disease 2019 (COVID-19). Vaccine development approaches consist of viral vector vaccines, DNA vaccine, RNA vaccine, live attenuated virus, and recombinant proteins, which elicit a specific immune response. The use of nanoparticles displaying antigen is one of the alternative approaches to conventional vaccines. This is due to the fact that nano-based vaccines are stable, able to target, form images, and offer an opportunity to enhance the immune responses. The diameters of ultrafine nanoparticles are in the range of 1–100 nm. The application of nanotechnology on vaccine design provides precise fabrication of nanomaterials with desirable properties and ability to eliminate undesirable features. To be successful, nanomaterials must be uptaken into the cell, especially into the target and able to modulate cellular functions at the subcellular levels. The advantages of nano-based vaccines are the ability to protect a cargo such as RNA, DNA, protein, or synthesis substance and have enhanced stability in a broad range of pH, ambient temperatures, and humidity for long-term storage. Moreover, nano-based vaccines can be engineered to overcome biological barriers such as nonspecific distribution in order to elicit functions in antigen presenting cells. In this review, we will summarize on the developing COVID-19 vaccine strategies and how the nanotechnology can enhance antigen presentation and strong immunogenicity using advanced technology in nanocarrier to deliver antigens. The discussion about their safe, effective, and affordable vaccines to immunize against COVID-19 will be highlighted.

KEYWORDS

COVID-19, SARS-CoV-2, vaccine, nanotechnology, antigen presenting cells (APCs)

1 Introduction

The seventh β -coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was firstly reported on Dec. 2019 at Wuhan, China. Previously, α CoV (HCoV-229E and HKU-NL63) and four types of β CoV (HCoV-OC43, HCoV-HKU1, SARS-CoV, and MERS-CoV) were discovered. The SARS-CoV-2 causes symptoms in the upper respiratory and gastrointestinal tracts. Phylogenetic analysis has revealed that SARS-CoV-2 has four amino acids (PRRA) insert at the edge of S1 and S2 which add the new functional furin cleavage site and improve the ability of the SARS-CoV-2 for binding to enzyme 2 (ACE2) receptor (Fig. 1). The disease from SARS-CoV-2 was named as “coronavirus disease 2019 (COVID-19)” by the World Health Organization (WHO) on 11 February 2020. SARS-CoV-2 has an ancestor sequence derived from bat-CoV-RaTG13. The virus in the bat contains the amino acids polyacrylic acid (PAA) by recombination of sequences that derived from RmYN02 in *Rhinolophus malayanus* bat. However, the receptor-binding domain (RBD) of SARS-CoV-2 is similar to beta-CoVs in Malayan pangolins

(*Manis javanica*). Major challenges in designing effective vaccines with minimal adverse effects are to fully understand the virus and how our body stimulates the immune responses. The candidate COVID-19 vaccines in clinical trial consist of the conventional development approach (inactivated virus, viral-based vaccine) and the modern development using nanotechnology platform (nucleic acid-based, protein-based vaccine). Both approaches elicit an immune response and prevent COVID-19. However, common limitations of the conventional vaccines include being administered mainly through parenteral routes and requiring multiple doses [1], cold-chain maintenance that needs to be kept at +2 to +8 °C to ensure vaccine potency [2], potential risk of virus-based vaccine localization in the central nervous system [3], and taking many years for safety and efficacy approval [4]. The accelerating vaccine development and deployment to combat SARS-CoV-2 by using modern strategies such as nanoparticle formulated vaccines and the evaluation are urgently needed [5–7]. Nanotechnology was first applied in medicine to increase the efficacy and bioavailability of drugs in 2005 [8]. It plays an important role on vaccine design and applications. It truly

Figure 1 SARS-CoV-2 structure and genome organization. Adapted with permission from Ref. [17], © Chan, J. F., et al. 2020 and Ref. [68], © Published by Elsevier B. V. 2020. An annotated SARS-CoV-2 map (GenBank NC045512.2) created by Snapgene. (a) TEM of SARS-CoV-2 virus particles isolated from a patient. Adapted with permission from Ref. [342], © Massachusetts Medical Society 2020. (b) Illustration of SARS-CoV-2 morphology. (c) Comparative genome organization of coronavirus-related strains. Display genes in the 5′–3′ direction.

shortens the usual timeline by using antigens delivery platforms with safer and higher effectiveness than the conventional vaccines, owing to the properties of nanoparticles are able to modify in size, shape, and surface to target the immune cells. In this review, we will begin with an introduction (Section 1), basic biology to understanding of new virus (Section 2), a summary of the vaccines that have been developed (Section 3), how nanoparticles show advantages in the vaccine development (Section 4), and the discussion of COVID-19 vaccines in Phase 3 clinical trial (Section 5).

2 Biology of SARS-CoV-2

2.1 Genomic organization of SARS-CoV-2

SARS-CoV-2 genome was deposited in the GenBank database

(NC045512.2; MN908947; MN975262.1). SARS-CoV-2 genome consists of 29,903 nucleotides. Phylogenetic analysis cluster SARS-CoV-2 in the same group of SARS-CoV and MERS-CoV with similarity score 79% and 50%, respectively [9]. The order of genes (5′ to 3′) is as follows: open reading frame 1ab (ORF1ab), spike (S), ORF3a, envelope (E), membrane (M), ORF6, ORF7a, ORF7b, ORF8, nucleocapsid (N), and ORF10 (Fig. 1). The nine-amino acid sequence “LLRKNKGK” in *nspl* was highly conserved between SARS-CoV (accession No. NC004718) and SARS-CoV-2 [10]. SARS-CoV-2 has completely changed four genes: spike (S), ORF3, ORF8, and ORF10. The crown-like appearance under microscope comes from Spike (S) proteins, which mediate virus invade into the host [11]. The insertion in O-linked glycan residues (PRRA: amino acids 681–684) at the S1–S2 junction

provides a new feature of furin cleavage site. Moreover, the receptor binding domain “DISTEIYQAGSTPCN” (amino acids 467–481) derived from pangolin beta-CoVs, allowing a high affinity binding to human angiotensin converting ACE2 receptors [12]. ORF3 of SARS-CoV-2 has mutation in translational process. ORF3b in SARS-CoV-2 has a premature stop codon, a completely novel short protein which is a more potent inhibitor of human IFN-1 and the length of its C-terminus affects the anti-IFN activity [13]. The clinical symptoms of severe COVID-19 patients were reported as the impaired Type I interferons (IFN-1) activity [14]. Type I interferons are soluble immune mediators secreted from viral infected cells and play three major roles. First, they limit the spread of infectious viruses. Second, they modulate innate immune response by promoting natural killer (NK) cell functions, antigen presenting cell (APC), pro-inflammatory, and cytokine production. Third, they activate the adaptive immune responses and immunological memory [15, 16]. Furthermore, the novel ORF8 of SARS-CoV-2 was predicted to form an alpha helix with six strands of beta-sheet(s) proteins which the annotation of this motif is unknown [17]. Previously annotated ORF8 of SAR-CoV has virus replicative fitness function in the human-to-human transmission [18]. Clinical data of COVID-19 patients infected with 382-nucleotide deletion in ORF8 had mild symptom, more effective T-cell responses, and lower concentrations of proinflammatory cytokines. It was hypothesized that the major deletion ORF8 in SARS-CoV-2 may lead to weakened of COVID-19 symptoms [19, 20]. ORF10 is the new ORF which is not found in SARS-CoV. ORF10 is a unique short protein or peptide of 38

residues length, which does not have any similar proteins in the National Center for Biotechnology Information (NCBI) database. No annotated function was found [21] and could not be detected by liquid chromatography–mass spectrometry/mass spectrometry (LC–MS/MS) in proteomics analysis [22, 23].

2.2 The SARS-CoV-2 life cycle

2.2.1 Infectious cycle in the host

SARS-CoV-2 infectious cycle begins once its Spike binds to the ACE2 receptors which are found in various tissues such as lungs, adipose tissue, and small intestine [24]. After receptor binding, the host serine protease TMPRSS2 cleaved viral S and the viral envelope fused to cell membrane along the endosomal pathway [25] (Fig. 2). The viral RNA was released into the host cell and translated into polyproteins, then viral polyproteins were cleaved into small products [26]. The viral replication proteins (16 non-structural proteins) were encoded from ORF1. Sub genomic messenger RNAs (mRNAs) were initiated at the 3' end of the viral genome and terminated at the transcriptional regulatory sequences (TRS). The nascent transcript transferred to the complementary leader TRS through the base-pairing interaction. The transcription started from the 5' end of the viral genome. RNA replication occurred on double membrane vesicles (DMVs) [27]. The replicated viral genomic RNA was assembling on ER–Golgi intermediate compartment (ERGIC) and formed mature virion inside Golgi vesicle. Then, they were released out through exocytosis pathway [28, 29].

Figure 2 The infectious cycle of SARS-CoV-2. Adapted with permission from “Coronavirus Replication Cycle”, © BioRender.com 2020, and Ref. [440], © Elsevier B. V. 2008. SARS-CoV-2 infectious cycle begins once its spike binds to the ACE2 receptors which are found in various tissues such as lungs, adipose tissue, and small intestine. After receptor binding, the host serine protease TMPRSS2 cleaves viral S and the viral envelope fused to cell membrane along the endosomal pathway. The viral RNA was released into the host cell and translated into polyproteins, then viral polyproteins were cleaved into small products. The viral replication proteins (16 non-structural proteins) were encoded from ORF1. Sub genomic mRNAs were initiated at the 3' end of the viral genome and terminated at the TRS. The nascent transcript transferred to the complementary leader TRS through the base-pairing interaction. The transcription started from the 5' end of the viral genome. RNA replication occurred on DMVs. The replicated viral genomic RNA was assembling on ERGIC membranes and formed mature virion inside Golgi vesicle. Then, they were released out through exocytosis pathway.

2.2.2 Neuropilin 1 (NRP1) receptor promotes SARS-CoV-2 infection to the cells

SARS-CoV-2 spike protein has an extra loop from multiple arginine (RRAR) [30] which is required for the efficient cleavage by furin (protease in the host cell) at the R685/S686 site into S1 and S2 polypeptides (Fig. 1). The S1 polypeptide exposes the CendR motif (RXXR) binding to the b1 domain of Neuropilin 1 receptor [31, 32]. Neuropilin-1 (NRP-1) is a transmembrane glycoprotein which has a role in a multifunctional receptor for many key biological processes including vascular endothelial growth factor (VEGF) signaling pathways [33, 34], coreceptors for class III Semaphorins as an essential axon guidance in primary olfactory neurons [35], and immune function [36]. Interestingly, SARS-CoV-2 uses NRP-1 as a coreceptor for promoting virus entry. S1 polypeptide is bound to both NRP-1 and ACE2 receptors then S2 polypeptide, which is anchored in the virus membrane, and is cleaved by cathepsins and TMPRSS2. As a result, S2 fusion peptide fuses viral envelope with the cell membrane [37]. NRP-1 and its homologue NRP-2 expression are found to be elevated in the bronchoalveolar lavage fluid (BALF) in severe COVID-19 cases. Nevertheless, NRP-1 expression alone causes low infectivity of SARS-CoV-2. The study shows the highest infection level of SARS-CoV-2 requires furin, NRP-1 receptor, ACE2 receptor, and TMPRSS2 [38]. The mutation in furin cleavage site (RXXR), monoclonal antibodies are raised against the NRP1 b1b2 ectodomain, a selective NRP-1 antagonist molecule (EG00229), furin inhibitors, and serine protease inhibitor could suppress the efficiency of viral infection [31, 39, 40]. Thus, the nanomedicine strategies to inhibit the furin and block NRP1 b2 ectodomain are promising approaches to develop antiviral drugs [30, 41, 42].

2.2.3 Viability outside the host

COVID-19 spread from human to human as shown in the first evidence came from a familial cluster who did not visit Wuhan [43]. SARS-CoV-2 is a respiratory virus that is able to spread through multiple modes including (1) contact transmission (direct contact by touching persons, e.g., handshake, indirect contact by touching contaminated objects, so called fomite transmission), (2) droplet transmission (droplet size $> 5 \mu\text{m}$; spread within 2 m distance), and (3) airborne transmission (droplet size $\leq 5 \mu\text{m}$; spread greater distance than 2 m) produced by coughing and sneezing from the infected patients [44–46]. Smaller aerosols are easily inhaled deposited in the lower respiratory tract (LRT) and promoting a more severe infection [47, 48]. The virus has vitality in the air for 3 h, causing potential airborne transmission [49], and more persistence up to one week varied on viral loads onto the object, the properties of surface, humidity, and temperature [50–52]. Outside the host cells, The virus can decay by far-ultraviolet light (UVC) light (222 nm) at $2 \text{ mJ}/\text{cm}^2$ within 25 min [53], heating at 56°C for 30 min [54], biocidal agents within 1 min such as (78%–95%) ethanol, (0.23%–7.5%) povidone iodine, (0.21%–0.5%) sodium hypochlorite [55, 56]. In order to stop spreading of SARS-CoV-2, social distancing, wearing mask, and keeping hands clean are the simplest ways to protect individual and community from COVID-19 pandemic during the vaccine development.

2.3 Clinical symptoms and immune response

The common symptoms are fever above 37.5°C , dry cough, and fatigue [57, 58]. The extreme cases are pneumonia, malfunction in the kidney, and respiratory distress syndrome [59]. Among the patients, losing the sense of smell (olfactory dysfunction; anosmia), distortion of the sense of taste (dysgeusia), or losing the sense of taste (ageusia) are reported [60, 61]. The cause of sensorineural

olfactory loss may be from the direct damage of the SARS-CoV-2 on the olfactory and gustatory receptors [62]. However, the COVID-19 patients will recover from the sensorineural dysfunctions within 1–3 weeks [63, 64]. A timeline of the typical clinical symptoms is shown in Fig. 3. The average incubation time after viral infection is 4–5 days before symptom onset [65, 66]. The nucleic acid test (NAT) has the highest sensitivity in the early phase of illness within 7 days. Viral RNA could be detected from various clinical specimens such as nasopharyngeal and oropharyngeal swabs from upper respiratory tract (URT), sputum, endotracheal aspirate, bronchoalveolar lavage from LRT, stool, urine, and blood [67]. A positive case of COVID-19 is confirmed from respiratory samples by using reverse transcription polymerase chain reaction (RT-PCR) test [57, 68]. However, the sensitivity of serological tests based on antibodies overtook that of NAT since day 8 after the onset [69]. The first line of defense could be detected immunoglobulin M (IgM) antibody after 3 to 6 days and peaked at 9 days and then declined in three weeks. While immunoglobulin G (IgG) antibody, a long-term immunity and immunological memory, is present in 5 days, and markedly increased in the second week after the symptom onset. It continues to elevate for the following 2 weeks [70–72]. Moreover, the sera from recovery COVID-19 patients have successful humoral responses *in vitro* plaque assay [73].

2.3.1 Innate immunity of COVID-19

The innate immunity is a broad mechanism of the host cell to control the production of virus by interferons (IFN- β and IFN- α) [74, 75]. The viral RNA is recognized by Toll-like receptors (TLR3, TLR7, and TLR8), RIG-I-like receptors (RLRs) locate in an endosomal or cytosolic compartment. The RNA sensors trigger a signaling cascade of pro-inflammatory cytokines and induction of Type I and III IFNs (IFN- λ). However, SARS-CoV-2 was reported to suppress type I IFNs [13] but up regulation of type III IFNs in primary human intestinal epithelial cells [76]. In the severe case, type I and III IFNs were elevated in the lower respiratory tract. IFN- λ contributed to the pathogenesis induced by RNA viruses. Sustained production of IFN- λ disrupted the lung epithelial barrier leading to susceptibility to bacterial superinfections in the mouse model [77]. The implication of pathogenesis could be the IFNs responses from aging [78]. High level of inflammatory cytokines, and MCP-1 probably activated Th1 cell responses and resulted in the activation of specific immunity [57]. The clinical data suggest that the disease severity correlated with high level of cytokines (IL-6, IL-2R, and IL-10). Total T lymphocytes count decreased in a moderate case, and remarkably decreased in severe cases. [79]. Addition of Type I IFNs treatment resulting in a dramatic reduction in virus replications, confirms the disease is sensitive to Type I IFNs [80].

2.3.2 Adaptive immunity of COVID-19

Second line of defenses against viral infection is responsible by B cells and T cells. CD4 $^+$ and CD8 $^+$ which are subcategories of T cells were identified by their surfaces [81]. CD4 $^+$ was made to be specific with the anti-spike protein receptor binding domain IgG titers in two weeks [82, 83]. The neutralizing antibody (NABs), which considered to be a key immune product for preventing virus infection, was positively related to the spike-binding antibodies targeting S1, RBD, and S2 regions [10]. This suggests that surface glycoprotein is a powerful immunogen for developing vaccine.

2.4 Laboratory testing to detect COVID-19

SARS-CoV-2 could be detected by NAT, serological tests based on antibodies, Chest CT Scan, and Chest X-rays. Currently, NAT

Figure 3 A timeline of the typical clinical symptoms of SARS-CoV-2 infection. Adapted with permission from Ref. [16], © Elsevier B. V. 2020, Ref. [79], © American Society for Clinical Investigation. 2020, and Ref. [441], © Elsevier B. V. 2020. The average incubation time after viral infection is 4–5 days before symptom onset. The NAT has the highest sensitivity in the early phase of illness within 7 days. However, the sensitivity of serological tests based on antibodies overtook that of NAT since day 8 after the onset. SARS-CoV-2 replicated viral genome in cytoplasm. Viral RNA was detected by TL3, TL7, TL8, RIG-I, and MDA5 receptor. The first line of defense could be detected IgM antibody after 3 to 6 days and peaked at 9 days then declined in three weeks. While IgG antibody, which is long-term immunity and immunological memory, is present in 5 days, and markedly increased in the second week after the symptom onset. It continued to elevate for the following 2 weeks. Clinical data suggest that the cytokine storm is positively correlated with the disease severity.

tests for RNA viruses include RT-PCR [84,85], isothermal amplification (reverse transcription loop-mediated isothermal amplification (RT-LAMP)) [86], and Cas13-based SHERLOCK [87]. Reverse transcription polymerase chain reaction is the gold standard to screen symptomatic people due to its simplicity and easy methodology by detecting ORF1b and nucleocapsid (N) of viral genome. The result of cycle threshold (Ct) values above 37 is considered negative. The sensitivity of detecting viral by quantitative polymerase chain reaction can be increased (lower Ct-value) by combining specimens from nasopharyngeal (NP) and oropharyngeal (OP) [88]. However, the collection of the LRT samples requires a skilled operator and may lead to false-negative result. Another assay method for rapid screening COVID-19 is serological assays. Serological tests for specific IgM and IgG antibodies which were produced in COVID-19 patients after the second week of viral infection can be tested with chemiluminescence assay (CLIA), immunofluorescence assay (IFA) [89], and enzyme-linked immunosorbent assay (ELISA) [90]. The main advantage of serological assay is to estimate the immune status of personal and herd immunity [91,92]. However, the accuracy of rapid tests and the biomarker of viral infection need to be evaluated. Computed tomography (CT) images are characterized by ground-glass opacification (GGOs). CT has been used on a massive scale to identify suspected cases with a low rate missed diagnosis of COVID-19 in China [93,94].

3 Traditional approaches to develop COVID-19 vaccine

The goal of vaccines is to induce an immunity against an immunogen in vaccinated person so that once exposed to the natural virus, second line of defenses against viral infection will be responsive. Characteristics of an ideal vaccine should be (1) high efficacy in target populations; (2) few or no adverse reactions; (3) stimulating humoral immune responses; (4) safe for pregnant women; (5) induction of life-long immunity; (6) inexpensive manufacturing and ease of translation into developing countries; (7) reproducibility and manufacturing process; (8) stable during transportation and storage; (9) easy and inexpensive to deliver [95]. Most of COVID-19 vaccine uses surface glycoprotein (S) in SARS-CoV-2 as immunogen. The WHO has categorized COVID-19 vaccines into 8 platforms: live attenuated virus, inactivated virus, replicating viral vector, non-replicating viral vector, virus like particle (VLP), DNA vaccine, RNA vaccine, and protein subunit. There are 110 candidate vaccines in clinical evaluation (8 vaccines in Phase 4, 19 vaccines in Phase 3, 9 vaccines in Phase 2/3, 10 vaccines in Phase 2, 27 vaccines in Phase 1/2, and 37 vaccines in Phase 1) and 184 candidate vaccines in pre-clinical evaluation that are published on 13 August 2021. Comparative COVID-19 vaccine platforms with advantages, disadvantages, and added values with nanotechnology are shown in Table 1. Candidate vaccines for COVID-19 developed by using

Table 1 A comparative COVID-19 vaccine platform with advantages, disadvantages, and added value with nanotechnology

Vaccine platform	Immunogen	Advantage	Disadvantage	Added value with nanotechnology
Live attenuated vaccine	Whole virion	Create a strong and long-lasting immune response	Need extensive safety testing	N/A
Inactivated vaccine	Whole virion	Same virus particle structure as SARS-CoV-2 wild type, safer than LAV vaccine Can infect a wide variety of dividing or nondividing cells, elicit strong immune responses	Weak immune response compared to LAV vaccine, require multiple doses	N/A
HAdV vaccine	S or N protein	Cross-reactivity of type-specific antisera with human adenovirus was absent.	Anti-adenovirus pre-existing immunity in humans could reduce the immunogenicity. Low viral yields compared to HAdV during virus production in HEK 293 cells.	N/A
ChAd vaccine	S or N protein			N/A
mRNA vaccine (conventional)	S or N protein	Very safe, mRNA does not integrate itself into the host genome. Clinical applications do not involve cell culture or fermentation.	Short half-life, instability of mRNA, transient, and low level of expression of antigen-encoding mRNA require a suitable delivery vehicle.	<ul style="list-style-type: none"> - Protect mRNA from degradation improving circulation time in blood - Overcome biological barrier with size, charge, ligands on NPs - Enhance immune responses by encapsulating with adjuvants - Protect sa-RNA from degradation
Self-amplifying RNA	S or N protein	Require low doses of mRNA, give high-level transient expression proteins	A large negatively charged molecule and it requires a suitable delivery vehicle	<ul style="list-style-type: none"> - Overcome biological barrier - Enhance immune responses - Protect pDNA, no cold-chain needed
DNA vaccine	S or N protein	Stable at room temperature, ability to be administered through a non-invasive route	Potential for integration of the vector DNA into the host genomic DNA	<ul style="list-style-type: none"> - AuNP encapsulated pDNA and protein for skin vaccination - Dry-powder for nasal vaccination
Virus-like particles vaccine	Co-expression of M, E, N, and S protein	Morphological mimic to the authentic virus without containing infectious RNA	The structure of the resulting VLP depends on the host cell expression system.	<ul style="list-style-type: none"> - Targeting, imaging, and enhancing immune response - Biodegradable polymers enhance immunogenicity and targeted of protein-based vaccines.
Subunit vaccine	S or N protein	Very safe, mimic fragment of an antigen, non-infectious	Requires multidose, and adjuvants to elicit the immune responses	<ul style="list-style-type: none"> - Protect pDNA vaccine vector - Targeting, imaging, and enhancing immune response - Stable for oral vaccination
Phage vaccine	S or N protein	Phage display and ability to deliver pDNA vector, immunotherapies, administer through a non-invasive route	Production involves bacteria fermentation. Precipitated phage is required to remove bacterial toxin and debris.	

conventional platforms are shown in Table 2 and candidate vaccines for COVID-19 developed by using nano-based platforms are shown in Table 3.

3.1 Live attenuated virus (LAVs)

Live attenuated viruses are the serially propagated viruses in cultured cells by carefully screening the passage to minimize pathogenesis. Most live vaccines create a strong and long-lasting immune response. There are two strategies to develop LAV SARS-CoV-2 vaccines: codon deoptimized, deletion, or mutation.

3.1.1 Codon deoptimized

Codon usage biases are preferred codons that frequently used in highly expressed genes, commonly found in all eukaryotic and prokaryotic cells [96]. In contrast, codon deoptimization (CD) can dramatically decrease gene expression by a replacement of commonly used codons with nonpreferred codons. This approach was used to develop poliovirus (PV) vaccines [97], and influenza A virus vaccines [98]. Bioinformatic analysis of genome content and frequently codon usage in SARS-CoV-2 reveals that highly biased frequencies used codons are A- and U-ending, while codons C- and G-ending are nonpreferred [99, 100]. Genes S and N are the best targets for codon deoptimization as vaccines and antiviral drugs. Currently, Codagenix and Serum Institute of India uses codon deoptimized approach for live attenuated COVID-19 vaccines in pre-clinical evaluation.

3.1.2 Deletion or mutation

The defective virus in replication by deletion or mutation is one approach for developing vaccine [101]. The study in SARS-CoV by deleting 29-nucleotide in ORF8 genes resulted in decreasing virus replication up to 23-fold when compared to the wild type. These data suggested that truncated product of ORF8 involved in attenuated viruses. It was hypothesized that the natural deletion within ORF8 genes occurred in the early stage of virus transmitting from human to human [18]. The phenomenon of nucleotide deletion in ORF8 genes was also found in new coronavirus [19]. SARS-CoV-2 variants from Vero-E6 cultured were found 15–30 bp deletion at the S1/S2 edge [43]. One variant (Del-mut-1) has deleted 30 nucleotides (10 aa: NSPRRARSVA) within the S protein leading to virus attenuation in hamster. A live vaccine with the deletion mutation as an attenuated vaccine requires high safety environmental laboratory because it can be transmitted from human to human [102, 103].

3.2 Inactivated virus

Inactivated virus vaccines are made by large production of virulent viruses then purified and concentrated prior to inactivation. Inactivation can be performed using chemicals such as formaldehyde, β -propiolactone (β -PL), or physical methods such as irradiation, heat, or a combination of chemical and physical methods [104, 105]. SARS-CoV inactivated vaccine was produced by propagating SARS-CoV strain BJ01. Infected Vero E6 cells

Table 2 Development of candidate vaccine for COVID-19 by using conventional platforms

Candidate vaccine developers	Vaccine platform	Number of doses	Timing of doses	Route of administration	Clinical stage	Ref.
PiCoVacc Sinovac Biotech, China	Inactivated virus	2	0, 14 days	IM	Phase 1/2 NCT04352608 Phase 3 NCT04456595	[107]
Wuhan Institute of Biological Products and Sinopharm, China	Inactivated virus	2	0, 21 days	IM	Phase 1/2 ChiCTR2000031809 Phase 3 ChiCTR2000034780	[443]
BBIBP-CorV vaccine Beijing Institute of Biological Products, China	Inactivated virus	2	0, 28 days	IM	Phase 1/2 ChiCTR2000032459 Phase 3 ChiCTR2000034780	[444]
BBV152A, B, C Bharat Biotech, India	Inactivated virus	2	0, 28 days	IM	Phase 1/2 CTRI/2020/07/02630 0	[445]
Ad5-nCOV CanSino Biologics, China	Nonreplicating human adenoviral vector	1	0 day	IM	Phase 3 NCT04313127 Phase 2 NCT04341389	[129]
Ad26.COV2.S Johnson & Johnson, USA	Nonreplicating human adenoviral vector	1	0 day	IM	Phase 3 NCT04526990 Phase 1 NCT04436276	[131]
Gam-COVID-Vac Gamaleya Research Institute, Russia	Nonreplicating human adenoviral vector	2	0, 21 days	IM	Phase 2 NCT04437875 Phase 3 NCT04530396	[446]
AZD1222 University of Oxford, AstraZeneca, UK	Nonreplicating chimpanzee adenoviral vector	2	0, 28 days	IM	Phase 1 NCT04324606 Phase 2 NCT04324606	[155, 447]
INO-4800 Inovio Pharmaceuticals, USA	Naked pDNA vector with electroporation delivery	2	0, 28 days	IM	Phase 3 NCT04400838 Phase 1 NCT04336410	[332]
					Phase 2 NCT04336410	

were harvested and inactivated by adding β -propiolactone and purified. SARS-CoV inactivated vaccines stimulated significant antibody responses against RBD in the S1 regions of the spike (S) protein. Antisera were able to block receptor binding and inhibited virus entry in immunized animals [106].

3.2.1 SARS-CoV-2 inactivated vaccine (PiCoVacc)

PiCoVacc candidate was developed by Sinovac Biotech in Beijing. PiCoVacc was made by isolating positive COVID-19 samples from BALF. China strain 2 (CN2) was selected to be inactivated with β -propiolactone. PiCoVacc CN2 has two amino acids substitution, E-A23D (changed from Ala to Asp at Envelope residue 23) and nsp10-T49I (changed from Thr to Ile at nsp10 residue 49). PiCoVacc CN2 has the same sequence of spike glycoprotein as wild type strain to avoid alter NAb epitopes. The study in BALB/c mice showed that PiCoVacc induced immune response by developing RBD-specific IgG, NAb at week 1 after immunization without any inflammation or other adverse effects. A non-human primate study was vaccinated with two doses 3 or 6 μ g at days 0, 7, and 14 then challenged 1 week later with different isolated strains of SARS-CoV-2. PiCoVacc showed fully effective at 6 μ g against viral infection [107]. Currently, PiCoVacc candidate vaccines reach Phase III clinical trial (NCT04456595).

3.3 Viral vector platforms

Viral vector-based vaccines are the viruses that are genetically engineered to safely deliver gene encoding desired antigens. Viral vector has a supramolecule size in nanoscale between 15–400 nm. Their nucleoprotein is covered with a lipid bilayer called enveloped viruses, or not covered with a lipid bilayer called nonenveloped viruses. Viruses internalize into the cells by specific receptor binding, endosomal escape, and uncoated nucleic acids into the cellular. Viral vector-based vaccines are the viruses that are genetically engineered to safely deliver gene encoding desired antigens which are able to induce the immune systems of the host cell against the respective target pathogen [108]. Viral vector-based vaccines are categorized into two groups based on their ability to replicate. Replicating viral vector can form infectious progeny in the vaccine's cells while non-replicating viral vector fails to produce new infectious progeny particles when infecting the cell of the vaccine [109, 110].

3.3.1 Replication viral vector-based vaccines

3.3.1.1 Recombinant measles vaccine (rMV)

Measles vaccine is a negative-stranded RNA live-attenuated virus. Reverse genetics approach generates heterologous live

Table 3 Development of candidate vaccines for COVID-19 by using nano-based platforms

Candidate vaccine developers	Vaccine platform	Number of doses	Timing of doses	Route of administration	Clinical stage	Ref.
mRNA-1273 Moderna, NIAID, USA	mRNA/LNP	2	0, 28 days	IM	Phase 1 NCT04283461 Phase 2 NCT04405076 Phase 3 NCT04470427	[290]
BNT162 Pfizer, BioNTech, Germany	mRNA/LNP	2	0, 28 days	IM	Phase 1 NCT04380701 Phase 2 NCT04368728 Phase 3 NCT04368728	[297, 298]
CVnCoV CureVac, Germany	mRNA/LNP	2	0, 28 days	IM	Phase 1 NCT04449276 Phase 2 NCT04515147	CureVac Inc.
LNP-nCoVsaRNA Imperial College London, UK	sa-RNA/LNP	2	0, 28 days	IM	Phase 1/2 SRCTN17072692	[312]
HDT-301 University of Washington, USA	sa-RNA/LION	2	0, 28 days	IM	Pre-clinical	[316]
CoVLP Medicago Inc. Canada	Plant-derived VLP adjuvanted with GSK or Dynavax adjs	2	0, 21 days	IM	Phase 1 NCT04450004	Medicago Inc.
NVX-CoV2373 Novavax Australia	Protein Subunit with adjuvanted Matrix M	2	0, 21 days	IM	Phase 1 NCT04368988 Phase 2 NCT04533399 Phase 3 NCT04583995	[228]
COVAX-19 Vaxine Pty Ltd. Australia	Protein Subunit with adjuvanted Advax-SM	2	0, 21 days	IM	Phase 1 NCT04453852	Vaxine Pty Ltd.

recombinant MV by using a helper cell-based rescue system [111]. This approach was used to develop MERS-CoV vaccine [112], SARS-CoV vaccine [113, 114], malaria vaccine [115], and HIV-1 vaccine [116]. Currently, rMV expressing spike glycoprotein SARS-CoV-2 or nucleoprotein (N) of SARS-CoV-2 is in pre-clinical phase. It was developed by a DZIF scientist at the German Center for Infection Research.

3.3.1.2 Vesicular stomatitis virus (VSV)

Vesicular stomatitis virus is a negative-sense, non-segmented, and single-stranded RNA virus with the enveloped capsid. There are three developers using live attenuated VSV-based approach to develop COVID-19 vaccines.

(1) IAVI and Merck use the same basis of Ebola rVSV vaccine which was approved by the European Commission and U. S. Food and Drug Administration (FDA). The rVSV SAR-CoV-2 vaccine candidate reaches a pre-clinical phase. (2) Rose laboratory from Yale. VSV-based SARS-CoV-2 vaccine was developed using similar approach to severe acute respiratory syndrome [117]. Currently, SARS-CoV-2 vaccine by Yale reaches the pre-clinical phase. (3) University of Western and Schulich Medicine & Dentistry developed COVID-19 vaccine using VSV-based. Currently, VSV-based COVID-19 vaccine reaches pre-clinical phase.

3.3.1.3 Horsepox

Horsepox virus (HSPV) is an orthopoxvirus (OPV) causing poxviral disease in horses. The SARS-CoV-2 (TNX-1800) was developed by Tonix Pharmaceuticals in Canada. TNX-1800 was constructed by inserting a viral promoter and SARS-CoV-2 Spike (S) genes into sHPXV vector. TNX-1800 reached the pre-clinical phase and a vaccination by scarification in primate was initiated in the 4th quarter of 2020.

3.3.2 Non-replication viral vector-based vaccines

Non-replicating viruses have defective in the gene related to replication. In normal cells, non-replicating viral vector can express gene encoding an immunogen. To propagate, the non-replicating viral vector requires the missing replication gene product to complement from the extra cell lines. Non-replicating viral vector vaccine includes adenovirus serotype 5 (Ad5), adenoviruses isolated from chimpanzees (ChAd), modified vaccinia Ankara (MVA), and adeno-associated virus.

3.3.2.1 Adenovirus serotype 5

Adenoviruses are nonenveloped viruses having linear double-stranded DNA (dsDNA) within an icosahedral nucleocapsid of 70–90 nm in diameter [118]. Their genome size is approximately 36 kb encoding two sets of transcriptional units: early genes and late genes. Early genes take control of the host cell and carry out viral DNA replication. Late genes express structural proteins of the virus [119–121]. There are at least 85 serotypes of human adenovirus (HAdV). HAdV 1–51 are distinguished by hemagglutination and neutralization assays [122]. HAdV 52–85 are discovered by genome sequencing and bioinformatic analysis [123]. HAdV serotypes 1, 2, 5, and 6 cause a common cold [124]. Recombinant adenoviral vectors are mostly derived from Ad5 which replicating defective constructs lacking essential viral genes *E1* and *E3*. The resulting vectors have the cloning capacity of 7.5 kb and have the most ORF in *E4* deletion excluding ORF6 that is important for Ad5 replication in HEK 293 cells [125, 126]. Recombinant adenoviral vectors have many advantages for vaccination such as (1) adenoviruses can infect both dividing and nondividing cells; (2) high titers and easy to purify; (3) they can accommodate insertion of foreigner DNA with stability up to net genome size of 37 kb; (4) adenoviruses do not integrate their DNA into the host genome [127, 128].

3.3.2.2 Ad5-nCOV

CanSinoBIO was the first team that developed COVID-19 vaccine trial in human by using Ad5 vector platform [129]. To generate vaccine using Admax system, the spike gene (GenBank YP_009724390) with the tPA signal peptide was carried with shuttle vector, then cotransfected in 293 cells with Ad5 vectored defective in *E1/E3* genes. The shuttle plasmid recombined the engineered spike gene to Ad5 vectored between loxP sites brought about the generation of viral progeny [130]. Clinical trial Phase 1 (ChiCTR2000030906) reported Ad5-based vaccine inducing T-Cell and antibody at day 14 and specific humoral immunity at four-week post-vaccination. However, the adverse effect was found in the high dose such as pain at the injection site, fatigue and shortness of breath. Currently, Adenovirus type 5 vector candidate vaccines reach Phase III clinical trial (NCT04526990).

3.3.2.3 Ad26.COV2.S

Ad26.COV2.S is the replication-defective human adenoviral vector serotypes 26 which was inserted SARS-CoV-2 Spike (S) genes into the *E1*-position by AdVac[®] technology [131, 132]. The Ad26.COV2.S vaccines were developed by Johnson & Johnson in Leiden, Netherlands. The vaccines were designed in seven constructs expressing difference of spike glycoproteins. The best construct of vaccine encoding S.PP containing full-length Spike gene with wild-type SP, mutations in furin cleavage site, and 2P substitutions gave the highest neutralizing antibody. The study in animal model showed that single dose intramuscular (IM) injection of varied titers virus particle (VP) Ad26.S.PP elicit dose dependent T cells especially CD4⁺ and CD8⁺ responses. Ad26.S.PP induced a Th1-biased after 2 weeks with high titers of NABs in Wuhan-Hu-1 strain [131], but elicited lower NABs against the new variant strain in non-human primate model [133]. The vaccinee Syrian golden hamsters with single dose showed S-specific and RBD-specific antibody responses at week 4. The highest dose (10¹⁰ vp) of vaccinee animal reduces TCID₅₀ infectious virus titers, protects against clinical disease, and no mortality after challenge [134]. A study in non-human primates (*M. mulatta*) that were immunized with single shot 1 × 10¹¹ vp of Ad26.S.PP showed great results of protecting viral infection. The samples from bronchoalveolar lavage or nasal swabs confirmed negative results by RT-PCR [135]. The Ad26.S.PP is renamed to Ad26.COV2.S for validated in a clinical trial (NCT04436276). A comparative study with low-dose or high-dose of Ad26.COV2.S vaccine or placebo administered on day 1 and day 57 in healthy adults (18–55 years) showed that Ad26.COV2.S induced binding antibodies against RBD and full-length Spike protein at day 8 of post vaccination. NABs were detected at day 15, and continued to increase overtime on days 29, 57, and 71 of observation. The boost vaccination on day 57 increased binding antibodies and NABs on day 71 [136]. The Phase III clinical trials are still ongoing (NCT04505722, NCT04614948). The interim results of a Phase1-2a trial reported that the local and systemic reactions occurred after single vaccination with either low-dose or high-dose. The mild adverse effects such as exhausted, headache, and pain in the muscle. However, the adverse effects will relieve in 24 h. NABs were detected in 90% and 100% at days 29 and 57 after the first vaccinated, respectively. The boost dose on day 57 provided an increase magnitude of NABs at day 71. The boosting effect of Ad26.COV2.S after vaccination at 6 months and 1 year was still an evaluation [137]. The Ad26.COV2.S vaccine was found less effective against four variants of SARS-CoV-2 strain: D614G, B.1.1.7 (UK), B.1.351 (South Africa), and P.1 (Brazil) with the reduction of NABs titers by pseudovirus neutralization assay (PsVNA) assay [138].

3.3.2.4 Adenoviruses isolated from chimpanzees

Simian adenoviruses (SAdVs: Latin simia meaning “ape”) are the closest relatives to human adenovirus but differ in their adenovirus hexon [139]. Chimpanzees adenovirus (ChAd) was isolated from explanted of chimpanzees (*Pan troglodytes*) [140]. The novel simian adenoviruses were characterized and named Pan 5 (SAdV-22; GenBank AY530876), Pan 6 (SAdV-23; GenBank AY530877), Pan 7 (Chimpanzee adenovirus serotype C7 (AdC7) or SAdV-24; GenBank AY530878), and Pan 9 (Chimpanzee adenovirus serotype C68 (AdC68), or SAdV-25; GenBank AC_000011.1) [141–143]. AdC68 and AdC7 were found to benefit the clinical test because these two serotypes did not circulate in humans and lacked neutralizing B-cell epitopes. Cross-reactivity of type-specific antisera with human adenovirus was absent [144, 145]. To solve the issue of the anti-adenovirus pre-existing immunity in humans which reduced the immunogenicity of human Ad5-based vaccine [146, 147], the scientists from University of Pennsylvania were the first team who developed a replication-defective vector based on AdC68 by deletion of E1 sequences [144]. AdC68 vector was used to develop HIV-1 vaccine [148], and rabies vaccine [149]. AdC7 vector was used to develop Zaire Ebola vaccine [150].

3.3.2.5 Chimpanzee adenoviral isolate Y25 (ChAdY25/ChAdOx1)

Chimpanzee adenoviral strain Y25 was isolated from fecal specimens of young chimpanzees and was passed 6 times in HEp-2 cells before characterization [151]. The researchers from the University of Oxford sequenced the whole genome Y25 (GenBank JN254802) and classified Y25 to be in the same cluster as that of human adenovirus E (HAdV 4) based on the nucleotide sequence in adenovirus hexon and fiber protein. Y25 was generated replication-defective vector by constructing in a bacterial artificial chromosome, which was the same method that constructed AdC68 vector. Chimpanzee adenoviral Y25 vector has a large insert capacity (~ 8 kb). It was deleted in *E1/E3* genes and modified in *E4* genes containing ORF from Ad5 to increased virus titers. ChAdY25-E construct (ChAdOX1) was selected to use in clinical trials [152] including ZIKV vaccine (NCT04015648), Influenza vaccine (NCT01818362), Tuberculosis vaccine (NCT01829490), Malaria vaccine (NCT03203421), CHIKV vaccine (NCT03590392), HBV vaccine (NCT04297917), and MERS-CoV vaccine (NCT04170829).

3.3.2.6 AZD1222

AZD1222 vaccine was developed by a similar approach for ChAdOx1 MERS [153]. To generate vaccine, the codon optimised spike (S) genes (GenBank: YP_009724390.1) with a tissue plasminogen activator (tPA) were synthesized and cloned into shuttle plasmid and recombined to destination DNA BAC vector at E1 locus of ChAdOX1 by using Gateway[®] recombination technology [152]. The study in nonhuman primate (Rhesus macaques) after immunized with AZD1222. Spike-specific antibody was detected at two weeks after vaccinated. No pulmonary disease developed in vaccinated group after challenge with SARS-CoV-2 [154]. The study in Phase 2/3 trial (NCT04400838) enrolled 560 participants aiming of finding dose frequencies and an optimal dose of vaccine administered in healthy adult. The study showed that the participants who received two doses of ChAdOx1 nCoV19 vaccine by intramuscular injection with low-dose (2.2 × 10¹⁰ virus particles) of the prime vaccination and boost vaccination with the standard doses (5 × 10¹⁰ virus particles), which were given 28 days apart, had lower adverse reactions than a single dose vaccination, especially in older adults (age ≥ 56 years) [155]. Currently, AZD1222 candidate vaccines reach Phase III clinical trial.

4 Nanotechnology and vaccine developments for COVID-19

The key for the future development of COVID-19 vaccine should focus on the two main points. First, the antigens which are the important part for the recognition of the immune systems [156]. Antigens can be designed in many forms including nucleic acid (DNA or mRNA), proteins, subunits of proteins, or the fragments of viruses [157]. The good design of antigens can increase the vaccine efficacy (VE) and reduce the amounts of antigens. Moreover, the selected of conserved region of virus such as nucleocapsid [158–160] or using the bioinformatic tools to design appropriated antigens in spike proteins could prevent the variation of SARS-CoV-2 [161, 162]. Second, the carrier of antigens. The good design of carrier enhances the vaccine efficacy by improving the retention time in the blood vessel and tolerated to the degradation by enzyme before release antigens at the target sites [163, 164]. The carrier in the nano scale is more uptake into the cells with the less toxicity which depends on its own properties [165, 166]. The strategies to enhance vaccine efficacy by designing the nanocarriers such as lipid-based nanoparticles, biodegradable polymeric nanoparticles, dendrimers, self-assembled macromolecules, and inorganic nanoparticles will discuss.

4.1 Role of nanotechnology

Nanotechnology could help to combat COVID-19 by four practical applications including (1) sensors for a specific target RNA sequence of viral by modifying gold nanoparticles [167]; (2) nanotechnology-based disinfectant to prevent fomite transmission by using silver nanocluster/silica-based coating on the surface [168]; (3) nanotechnology-based encapsulated antiviral drugs adhering to the lung by using chitosan nanoparticles [169]; (4) nano-based formulation vaccines to deliver the antigens, which could be divided into two main groups: (1) organic nanocarriers such as lipid-based nanoparticles, biodegradable polymeric nanoparticles, self-assembled macromolecules, and dendrimers; and (2) inorganic nanoparticles such as gold nanoparticles and carbon nanotubes.

4.1.1 Types of promising nanocarriers for COVID-19 vaccine development

4.1.1.1 Lipid-based nanoparticles

Lipid-based nanoparticles primarily consisting of phospholipids can be classified according to their sizes and chemical compositions [170]. Most popular lipid-based nanoparticles that were used for vaccine delivery include liposome (phospholipid vesicles) [171], niosomes (nonionic surfactant vesicles) [172], and nanoemulsions (small lipid droplets dispersed within an aqueous phase) [173]. Lipid-based nanoparticles provide several advantages including biocompatible, less toxic, and most successful approval for clinical application, easily changing size, charge, and surface properties by modifying new ingredient formula [8, 174]. However, drawbacks of the lipid-based nanoparticles *in vivo* are the rapid clearance from blood and non-specific uptake into the Reticuloendothelial system (RES) [175]. This drawback could be solved by (1) adding the surface-attached Toll-like receptor ligand (PAM and CpG) targeting the dendritic cell (DC) [176]; (2) PEGylation of liposomes enhancing uptake into APCs in lymph nodes (LNs) resulting in improved efficiency of vaccines [177].

4.1.1.2 Biodegradable polymeric nanoparticles

The biodegradable polymeric nanoparticles are classified by their polymer composition in the core and their morphology for example poly(lactide-co-glycolide) (PLGA) (poly-D,L-lactide-co-

glycolide), polylactic acid (PLA), and chitosan [178]. PLGA nanoparticle is the popular material for controlling released immunogen protein because of their high safety profile in human and approval by FDA [179]. The antigens can be encapsulated inside the core [180], conjugated [181], or adsorbed on the surface of the nanoparticles. The degradation of polymer occurs by the dissolution of chain fragments in noncross-linked systems when the polymer absorbs water and exchanges charges with water [182].

4.1.1.3 Dendrimers

Dendrimers are nano-sized polymeric molecules, which have branches like tree architectures consisting of a central core molecule, branching units, and end-groups (surface group). Dendrimer sizes depend on their increasing generation branch structure [183]. The advantages of dendrimers are the ability to modify the cavities to incorporate hydrophilic or hydrophobic molecules, modify the end-groups to attach peptide for targeting purposes, and they were approved for clinical applications [184]. Polyamidoamine (PAMAM) dendrimers are promising as nanocarriers of vaccines because their positive charges can form complex via electrostatic interaction with negatively charged of plasmid DNA molecules [185, 186]. Recently, a novel amphiphilic peptide dendrimer (AmPDs) was developed for safe and effective RNA delivery. AmPDs is composed of hydrophobic two C18 alkyl chains with unsimilar composition in hydrophilic dendrons, which can self-assemble and entrap RNA molecules into nanoparticles. AmPDs-based nanocarriers form stable complex, increase cellular uptake without notable toxicity, and have better RNA releasing ability than the previous class of synthetic polymers [187].

4.1.1.4 Self-assembled macromolecules

Self-assembled macromolecules represent well-controlled biomaterial structures from molecular building blocks such as DNA origami [188], peptide [189], and protein subunit [190] to form high-order hierarchical structures. An example of self-assembled macromolecules in nature is M13 bacteriophage which can be engineered as various functional nanostructures such as sensors [191], battery components [192], gene delivery, and a promising nanomaterial for vaccine delivery by taking advantage of phage display technology [193]. The virus like particle COVID-19 vaccines from Medicago Inc. is classified to be in this type of nanocarrier by expressing recombinant viral proteins and assembly in the plant expression system.

4.1.1.5 Inorganic nanoparticles

Inorganic nanoparticles such as gold nanoparticles (AuNP), silver nanoparticles (AgNP), iron oxide (Fe_3O_4), and carbon nanotubes (CNTs) are used for various biomedical applications including imaging, targeting, antimicrobial agents, and drug delivery [194, 195]. Gold nanoparticles are promising inorganic nanoparticles for vaccine delivery of various type of antigens including nucleic acids, peptide, and proteins. AuNPs are less toxic to cells and biocompatible, high surface to volume ratio, inert and stable at room temperature. The size and shape of AuNP coated with antigens induce the different outcome of immune responses *in vivo*. It was found that 40 nm spherical AuNPs are the most effective vaccine delivery platform by enhancing antibody production compared to the cube or rod shape [196].

4.1.2 Strategies to develop immuno-enhanced COVID-19 vaccine

4.1.2.1 Deliver antigens to the pivotal cell of immunology

Prior to the 1980s, mechanisms involved in conferring immunity were unknown. Most vaccines used live attenuated virus. A

discovery of dendritic cells in murine spleen cells is the big progress in cellular immunology [197]. The defend mechanism is classified into innate immunity and adaptive immunity. Innate immune is maintained by antigen presenting cells (APCs) (also called accessory cells), and their surfaces display an antigen with major histocompatibility complexes (MHCs). Recognition of an antigen by T lymphocytes occurs when the antigen is presented on the surface of the APCs, which include DCs, macrophages, and B-lymphocytes (B cells). DCs are the “professional” of APCs that initiate primary immune responses and mediated the adaptive immune responses [198] by using synthetic peptides or soluble protein antigens to sensitize naive T cells *in vitro* both CD4⁺ [199] and CD8⁺ [200]. The theory of antigen delivery to specific receptor of DCs was proposed by Ralph Marvin Steinman. These advantages are low dose of injected protein antigen and high dose of antigen-specific T cells [201]. The *in vivo* experiments for various antigen delivery targeted to DCs were demonstrated [202–204].

4.1.2.2 A brief story of protection from antigens to immune responses

Immature DCs (iDCs) possess a strong ability of capturing and phagocytosing antigen. iDCs can take up exogenous antigens by phagocytosis [205], macropinocytosis [206], and C-type lectin family receptor at peripheral sites [207] (Fig. 4). Once the antigens internalized to iDCs and sensed from TLR [208], they can be activated and differentiated into mature DCs. Mature DCs processed in MHC class I or MHC class II pathways at the secondary lymphoid tissues. The exogenous antigens are processed in MHC class I while intercellular antigens are processed in MHC class II pathways [209]. The process stimulates T cells into cytotoxic T cells (CD8⁺ cells), or T helper (Th) cells (CD4⁺ cells) in Thymus [210–212]. CD4⁺ plays critical roles in mediating adaptive immunity by helping B cells make antibody. Once naive CD4⁺ cells are processed through MHC class II pathways, the release cytokine elicits Th cell differentiation into various effector subsets including Th1, Th2, Th17, Treg, and Tfh [213]. The cellular immune response to protect viruses, bacteria, and microorganism infection is mediated by IL-2, TNF- β , and IFN- γ from Th1 cells. The Humoral immune response is mediated by Th2 cells, which increase the antibody and promote the production of IgG2a and IgG1/IgE in B cells [214]. Thus, DCs are pivotal cells of immunology by linking innate immunity with

adaptive immunity, and the critical role is the activation of T cells from naive stage. To develop immuno-enhanced Covid-19 vaccines, the key factors are (1) how to deliver the antigen effectively; (2) DCs maturation [215]; and (3) the pathways of antigen processing [216–218]. Nanotechnology-based platform offers efficient antigens delivery formulation. Nanoparticles in the range of 20–45 nm are easily uptaken into lymph node APCs *in vivo*. Limiting size of particles to be below 70 nm, they can remain at the site of injection [219].

4.2 Advantages of nanovaccine

Nanovaccine refers to the vaccine that has the particle size in nano scale. The particle in the range of 1–100 nm is easy to uptake into the cell while the particle size larger than 150 nm will be eliminate by the reticuloendothelial system [220–222]. Nanovaccine was engineered in particle as carrier the antigens [223, 224]. Viruses regarded as naturally nanomaterials which were engineered to deliver the antigens by infecting into the human cells [225]. Viruses based vaccine may compromise to the person who has the weak immune system. Nanovaccines mimic to the viruses in size and surface properties, but lack of nucleoprotein structure [226, 227] and unable to infect into the human cells will be described in this section. Comparing to the live attenuated and inactivated virus vaccine, nanovaccines have many advantages including: (1) Nanovaccine can shorten the time and save the cost for the development of a new vaccine. Nanovaccine can use the same formulation materials that were passed safety in the clinical trials to deliver new antigens of pathogen. Development of a conventional vaccine from concept to licensure nowadays takes at least 15 years [4]. For most emerging COVID-19, the obstacle is the time for developing and testing the new vaccine. Nanotechnology-based formulations of new generation vaccines can shorten the usual timeline from 15 to 20 year to 12 to 18 months to licensure of vaccines for COVID-19 (FDA-2020-D-1137). (2) Nanovaccine can be administered via IM injection, intranasal spray application, and oral administration. (3) Nanovaccine can be customized on nano-carrier surfaces such as adjuvant property to increase the stimulation of the immune systems, and peptide target specific to APCs. (4) Nanovaccine removes undesired characteristics for safety by mimicking the overall structure of authentic virus particles without containing genome of virus, or unable to replicate itself in the vaccinated

Figure 4 Surface receptors on antigen presenting cell and T-Cell activation. Adapted with permission from Ref. [213], © The American Physiological Society 2013 and Ref. [442], © Apostolopoulos, V. et al. 2013. (a). The antigens can be captured by several receptors including DEC205, Mannose receptor, and DC-SIGN. Once the antigens internalized to iDCs and sensed from TLR, they can be activated and differentiated into mature DCs. Mature DCs processed in MHC class I or MHC class II pathways at the secondary lymphoid tissues. (b) Antigen presenting cell has MHC bound with TCR. TCR is a protein complex composed of two chains, which interact with a complex CD3 membrane protein. CD3 molecules transmit activation signals to the T cell.

person. Live virus vaccine has a safety issue of transmitted to contacts of the unvaccinated individuals [103] while inactivated vaccine to boost the immunity requires multiple doses. (5) Nanovaccine has a protective function. The shield of nanocarriers protects the antigen from degradation in the blood circulation system before release in the target site. (6) Nanovaccine co-delivery of antigens and adjuvants result in enhancing immune activation [228].

4.3 Nanoparticle-based antigen delivery systems

4.3.1 Nanomaterials properties

Nanomaterials design is important for vaccine development to overcome biological barriers such as nonspecific distribution [229], protein corona that affects materials uptake in biological environments [230, 231], and inappropriate dose threshold of antigen concentration [232] that may cause antibody dependent enhancement (ADE) [233]. The adjuvants property has increased the magnitude of an antigen-specific immune response by vaccination. Nanoparticles hold great promise for modern vaccine. NPs are defined as materials with the length of 1–1,000 nm in at least one dimension [234]. NPs can enter into the cells depending on its properties by using four routes: phagocytosis, macropinocytosis, clathrin-mediated endocytosis (CME), and caveolin-dependent endocytosis (CvME) [235]. However, NPs can be absorbed into the cells mainly through endocytosis because their polarity cannot cross the cell membrane, which is mostly favorable to small and non-polar molecules [236, 237]. The particle sizes of materials that achieve targeting and reduced toxicity *in vivo* are diameters less than 100 nm [238]. Thus, particle size, surface charge, and shape play key roles in clearance of these nanomaterials from the body. Larger NPs are filtered out by the macrophage system after injection and accumulated in liver, spleen, and lymph nodes [239, 240]. Toxicity of materials is another point for safety consideration. Since 2016, the number of nanomaterials that pass the safety certify by FDA for clinical trial has increased. Most of which are lipid-based nanoparticles. Different formulations in liposome composition have been synthesized to be antigen carriers, which include ligands for specific receptors, or stimuli responsive compounds on the surface [174, 241–243].

4.3.2 Nanomaterial load antigens

A key to success of the vaccine is to find the right immunogen to trigger an immunity with the lowest adverse effects. The term antigens ("antibody generators") and later called "immunogen" were first proposed by Paul Ehrlich. He termed antibodies are made an accidental good fit to antigens by natural body [244]. Vaccine antigens could be polysaccharides or protein of pathogen components, mRNA, DNA, or the attenuate whole live pathogens [245]. Antigens can be loaded in nanocarriers by several mechanisms such as physical adsorption [246, 247], encapsulation [248, 249], encapsulation with coating [250, 251], encapsulation with targeting mechanisms [252, 253], chemical conjugation [254, 255], and conjugation with targeting mechanisms [256, 257]. The release of antigens can be designed by chemical properties of NPs. When acid-sensitive NPs were uptaken into the cell membrane, encapsulated antigens were released within acidified early endosomes. In contrast, acid-resistant NPs can escape endosomal processing events. Encapsulated antigens were released and degraded only upon delivery to lysosome [258].

4.3.3 Targeted delivery of antigens

The antigens can be captured by several receptors including DEC205 receptor [259], Mannose receptor [260], transferrin receptor [261], Fcγ receptors [262], and B cell receptor (BCR)

[263]. The nanoparticles previously reported uptake into APCs such as virus-like particles [264–266], liposomes [171, 267, 268], and AuNPs [269–271]. NPs delivery of plasmid DNA encoded SARS-CoV nucleocapsid (N) protein targeted to nasal dendritic cell DEC-205 receptor can reduce the amount of immunogen approximately 500-fold and administer through a non-invasive intranasal route, enhancing both humoral and cellular immune responses [272].

4.4 Nucleic acid-based antigens

Nucleic acid (DNA or mRNA) vaccines for COVID-19 have become a new attraction in the field of biopharmaceuticals. DNA vaccines target to the nucleus while mRNA vaccine targets to the cytosol. Thus, the mRNA vaccine approach is more rapid because it does not need to process in the nucleus. However, naked nucleic acid is a large hydrophilic negatively charged molecule. Therefore, it cannot cross the hydrophobic lipid membrane of the cells. Nucleic acid encoded antigens require an efficient delivery platform for degradation protection and cellular uptake. Non-viral vectors have been modified for nucleic acid-based antigens delivery, including cationic liposomes (lipids), cationic polymers, and cationic proteins (peptides). Key properties of synthetic delivery nanoparticles that incorporated nucleic acid are surface properties, biodistribution, and toxicity.

4.4.1 mRNA vaccines

The mRNA was first discovered in 1961. It has a role in intermediate template between protein-encoding DNA and protein translation process in the cytoplasm [273]. mRNA vaccines were reported to be effective for direct gene transfer *in vivo* for the first time by Wolff et al. [274]. However, the instability of "naked" mRNA due to easily degraded is an obstacle to develop it to be a vaccine [275, 276]. Antigen-encoding RNA could be optimized in its stability and translatability by modifying in 5' and 3' UTR elements, the addition of a 5' cap, and optimizing the length of polyadenylated tail (poly(A) tail) [277]. Recent advance in nanomaterials mRNA vaccine has become one of the most attractive approaches in the field of biopharmaceuticals. The mRNA vaccine has many advantages: (1) Safety. mRNA does not integrate itself into the host genome. (2) Rapid process. mRNA does not require crossing the nuclear membrane. Once mRNA reaches the cytosol, it uses the host cell machinery for translation of their sequence into the corresponding protein. (3) Production is fast, scalable, and standardized. mRNA for clinical applications does not involve cell culture or fermentation. It was synthesized and purified using a high-performance liquid chromatography (HPLC) according to good manufacturing practice (GMP) production [278]. The two types of mRNA vaccine in clinical trial include (1) non-replicating mRNA (conventional) [279] and (2) self-amplifying RNA (sa-RNA) based on an alphavirus that enables intracellular RNA amplification [280].

4.4.1.1 Strategies to effectively design mRNA antigens

A conventional mRNA antigen structure is composed of 5' cap, 5'UTR, antigen-encoding sequence, 3'UTR, and poly(A) tail [279, 281]. To optimize the translation and stability of mRNA antigen *in vivo*, Yizhou Dong et al., engineered "the NASAR mRNAs" which were modified in the 5' UTRs and 3'UTRs regions. They demonstrated the effects of UTRs on antigen expression. For example, (1) the optimal length of the composition at the 5'UTR is 70-nucleotides (nt); (2) Kozak sequence (GCCACC) should be included in 5' UTR for reliable starting codon recognition in mammalian mRNAs; (3) The sequence AUG within 5' UTR should be excluded for avoiding alternate translation initiation, and (4) avoiding high GC base composition in 5'UTR to decrease

formation of secondary structures. The RNA secondary structure could be predicted from the model of free energy, thermodynamic, and deep learning neural networks [282–285]. However, the 3'UTRs modified regions in their studies did not improve the translation capacity. The NASAR mRNAs derived the 5'UTR sequence from murine Rps27a gene which has the highest translation capacity per mRNA molecules, resulting in improved 5- to 10-fold more efficient translation than the control UTRs [286].

4.4.2 Non-replicating mRNA (conventional) vaccine

4.4.2.1 mRNA-1273 vaccine

The mRNA-1273 vaccine is an mRNA-based antigen formulated in lipid nanoparticle (mRNA/LNP). The antigen encoding the full-length Spike (S) protein which is prefusion stabilized in amino acid positions 986 and 987 in the S2 subunit by two consecutive proline substitutions is named as S-2P [287]. The mRNA is encapsulated in LNP using modified ethanol-drop nanoprecipitation [288]. The LNP is formulated from SM-102, cholesterol, DSPC, and PEG2000 DMG. The rapid development of mRNA-1273 takes advantages from innovative structure-based vaccine antigen design [289] and a lipid-nanoparticle delivery system. The mRNA-1273 vaccine took 63 days from sequence selection to enter Phase I clinical trial on 16 March 2020 (ClinicalTrials.gov: NCT04283461). The Phase I clinical trial aimed to validate the vaccine in a small population. 155 volunteers who were in good health were enrolled into one of thirteen groups by age and dosage. Participants received the candidate vaccine two times on days 0 and 28 by an intramuscular injection. After the first vaccination, IgG antibodies were detected at day 15 by ELISA anti-S-2, and ELISA anti-receptor-binding domain in all 45 subjects (age: 18–55 years; each dose $N = 15$; dosage: 25, 100, or 250 μg). The dosage 100 and 250 μg had antibody dose-dependent responses. After 4 weeks of two-dose vaccinations, neutralizing antibody was measured in all subjects by using PsVNA and plaque reduction neutralization test (PRNT) assay. However, solicited adverse symptoms including headache, fatigue, and muscle pain were observed over half of the participants. At the dose of 250 μg , 21% participants had severe adverse effects [290]. The mRNA-1273 dosage of 100 μg was selected to enter Phase 3 clinical trial on 11 June 2020 (ClinicalTrials.gov: NCT04470427). The Phase 3 clinical trial enrolled 30,000 volunteers to compare the two shots of the vaccine or saline placebo to evaluate the prevention of COVID-19 for two years after the booster doses. The mRNA-1273 was 94.1% vaccine efficacy to prevent COVID-19 (Wuhan-2019 strain) [291]. However, the recent study showed that the NABs titers in vaccinated person with mRNA-1273 vaccine were reduced significantly against the SARS-CoV-2 variant in South Africa (B.1.351), UK (B.1.1.7), Brazil (P.1), and USA (CAL.20C) [292, 293]. Currently, mRNA-1273 vaccine for variant strain has been evaluated in the clinical trial (NCT04785144). The mRNA-1273 vaccine was developed by Moderna, Inc. and the U.S. National Institute of Allergy and Infectious Diseases (NIAID).

4.4.2.2 BNT162 vaccine

BNT162 is an mRNA/LNP vaccine encoding trimerized spike protein. mRNA vaccines were designed in 4 formats (BNT162a1, BNT162b1, BNT162b2, and BNT162c2) to enhance the immunogenicity in dendritic cells. Antigen-encoding RNA was modified in 3' of the coding region and poly(A) tail to increase the stability [277], and superior mRNA translation *in vivo* by modifying nucleosides from uridine to 1-methylpseudouridine [294]. BNT162b1 and BNT162b2 showed promising results as a candidate COVID-19 vaccine by inducing neutralizing antibody,

and specific T cell responses in various animal models. BNT162b1 vaccine encodes foldon trimerized RBD which is optimized for increasing immunogenicity by fusing with a 27-amino acid phage T4 fibrin domain (Foldon: RBD-Fd) [295], while BNT162b2 vaccine expressed two proline substitutions (2P) in full-length spike glycoprotein [287]. The lipid nanoparticle formula was previously developed by BioNTech to deliver mRNA targeting DCs using N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) and 1,2-dioleoyl-sn-glycero-3-phosphatidyl-ethanolamine (DOPE) containing lipoplexes (RNA-LPX) [296]. The clinical trial NCT04368728 varied dosage administered of BNT162b1 or BNT162b2 via intramuscular injection two times: 10 or 30 μg doses (booster doses 3 weeks apart) or one time 100 μg dose in healthy adults (age 18–55 years; 65 to 85 years). The results showed that both vaccines elicited NABs titres depended on the dosage. Two-time 30- μg dose of BNT162b1 had the highest RBD-binding IgG and neutralizing antibodies in younger adults aged 18–55 years [297]. However, the mild to moderate systemic events (fatigue and headache) were observed after 7 days of vaccination. BNT162b2 had an advantage for the elderly (aged 65 to 85 years) with the protection and lower systemic events when compared to the BNT162b1 [298]. The BNT162b vaccine from Pfizer and BioNTech was 95% VE against Wuhan 2019 strain [299]. The comparative study of sera samples from the vaccinated with BNT162b vaccine to the neutralization activity in SARS-CoV-2 variant found that the level of NABs in vaccinee was reduced 3 folds in B.1.1.7, less sensitive to B.1.351 strain [300].

4.4.3 Self-amplifying RNA vaccine

The RNA molecule which has an ability to make multiple identical copies of positive-sense single-stranded RNA (+ssRNA), resulting in exponentially more copies of RNA is sa-RNA. Most of sa-RNA vaccine vector was developed from three well-studied alphaviruses such as SIN virus, SFV virus, and VEE virus [301]. Self-amplifying RNA was engineered to solve the major problem of the conventional mRNA vaccine *in vivo* such as easy to degrade, short time, and the expression level of antigen-encoding mRNA is relatively low [280]. sa-RNA vaccine vectors consist of four non-structural protein genes (nsP1–4) encoding viral replication derived from Alphavirus, subgenomic promoter, and capsid protein-encoding genes are replaced by antigen-encoding genes, thereby rendering the mRNA incapable of packaging into virus particles [302–304]. Naked sa-RNA was demonstrated as a potential nucleic acid vaccine for the first time *in vivo* by Peter Liljestrom [305]. Self-amplifying RNA has many advantages: (1) sa-RNA vaccines generated high-titre IgG responses to expressed antigens but at much lower doses (micrograms) of mRNA than non-replicating mRNA vaccine [306]; (2) sa-RNA gave high-level, transient expression proteins lasting up to two months *in vivo* from a single dose injection [307]. However, naked sa-RNA is a large (9–10 kb) negatively charged molecule and it requires a delivery vehicle for protection from blood nucleases, extended blood circulation, and efficient cellular uptake. Therefore, chemical modifications of delivery material such as mannosylation of lipid nanoparticles (MLNP) can potentially enhance uptake by APCs [308].

4.4.3.1 LNP-nCoVsaRNA

The LNP-nCoVsaRNA is an encapsulation of sa-RNA in lipid nanoparticle (sa-RNA/LNP) vaccine. The mRNA antigens encode the full-length spike proteins (GenBank: QHD43416.1) that amino acid position 968 and 969 in the S2 subunit were mutated by two consecutive proline substitutions (named K968P and V969P) [309]. To generate vaccine vector, sa-RNA based on VEE

alphavirus [310] encoding prefusion spike was synthesized and cloned into pDNA. Self-amplifying RNAs were transcribed *in vitro* using template from pDNA via an enzymatic reaction. An aqueous solution of RNA was self-assembled with lipids dissolved in ethanol at pH 4 [311]. The lipid nanoparticle capsule was formulated from Acuitas lipid, cholesterol, phosphatidylcholine, and PEG-lipid. The lipid nanoparticle encapsulated self-amplifying RNA was measured to be ~ 75 nm in diameter. The pre-clinical study in animals showed that mice received two intramuscular injections, 4 weeks apart. 50- μ L doses of 1 and 10 μ g of LNP nCoVsaRNA generated specific IgG antibodies more than the group that injected with pDNA at the same amount at 6 weeks. The LNP-nCoVsaRNA vaccine candidate induced Th-1 and high IFN- γ secretion [312]. Currently, the candidate vaccine reaches Phase I clinical trial (ISRCTN17072692). The volunteers who are in good health aged 18–45 years old will receive the dose escalation and evaluation of vaccine. Subjects will be followed up for 52 weeks in total. LNP-nCoVsaRNA vaccine was developed by Imperial College London, funded by Medical Research Council (UK) and UK Research and Innovation (UK).

4.4.3.2 LION/repRNA-CoV2S vaccine

The LION/repRNA-CoV2S vaccine (also called HDT-301) is formulated from lipid in organic nanoparticle (LION) and encapsulated self-amplifying RNA (sa-RNA/LION). The sa-RNA encodes full-length spike protein which was optimized codon for highly express immunogen. To generate vaccine vector, the nucleotide sequence strain Wuhan-Hu-1 position range 21,536–25,384 (GenBank: MN908947.3) was synthesized with c-terminal tag and ligated to pDNA VEE strain TC-83 vector [313]. Self-amplifying RNA was transcribed *in vitro* using template from pDNA via an enzymatic reaction. sa-RNA was encapsulated in LION by using electrostatic interaction between the cationic from LION and anionic from sa-RNA. The LION was composed of lipid 1,2-dioleoyloxy-3-(trimethylammonium)propane (DOTAP) chloride and inorganic superparamagnetic iron oxide (SPIO) nanoparticle within a squalene oil-based emulsion. Squalene oil-based emulsion enhanced the immunity as adjuvant [314] and SPIO used for imaging, targeting, and therapy applications *in vivo* [315]. The LION encapsulated self-amplifying RNA vaccine was 52 nm in diameter. The pre-clinical study in BALB/c mice showed single IM injection of 1 or 10 μ g in dosage generated specific IgG antibody at 2 weeks. Moreover, both doses induced neutralizing antibodies NABs and induced Th1 immune responses. The study in pigtail macaques with the comparative two administered LION/repRNA-CoV2S vaccine methods: (1) second shot of 50 μ g at days 0 and 28, or (2) prime dose 250- μ g intramuscular injection. The results suggest that both methods are enough to protect non-human primates model from SARS-CoV-2 infection [316]. Currently, the candidate vaccine reaches pre-clinical. The LION/repRNA-CoV2S vaccine was developed by the University of Washington and the National Primate Research Center, and the National Institutes of Health and HDT Bio Corp.

4.4.4 DNA vaccine

DNA vaccine is a DNA-encoding immunogen delivered to the nucleus without using a viral vector. DNA vaccine mostly produced from plasmid DNA (pDNA) vector or minicircles (MC) vector. pDNA consists of circular DNA containing an expression cassette with the genes encoding antigens and regulatory sequence as a promoter and polyadenylation signals, and the sequences needed for propagation and selection in the bacteria. In order to improve safety, minicircles MC were developed as the next generation of DNA vectors without any selection of a marker [317, 318]. DNA vaccines were administered into the body via

various routes including direct injection, intranasal spray, and oral administration [319–321]. Once DNA vaccine reaches the nucleus, DNA sequence of antigens is transcribed to mRNAs. mRNAs are translated to proteins, and proteins induced an antigen-specific MHC, resulting in specific cellular and humoral immune responses [322]. DNA vaccine began in the 1990s. It was first demonstrated *in vivo* by injecting pDNA encoding an antigen leading to generated specific antibody and protective from a subsequent challenge of virus [323]. DNA vaccine has many advantages: (1) Stable at room temperature, which is necessary for transport and storage vaccine [324]; (2) easy to produce and relatively inexpensive; (3) suitable for encapsulated with various nano platforms including gold nanoparticles adsorbed on gold beads into skin [325, 326]. Liposome-entrapped DNA facilitated their uptake by APCs in the lymphoid tissues [327]. However, DNA vaccine has a concern in a safety evaluation. It was found that DNA vector integrated into the chromosome of the received host [328].

4.4.4.1 INO-4800 vaccine

INO-4800 (also called pGX9501) vaccine is a naked pDNA vector, which uses synthetic DNA-based vaccine approach to accelerate developmental timelines. INO-4800 was administered via intramuscular injection and used electroporation (EP) technique to deliver the DNA vector into the cell. The EP uses millisecond (ms) electric pulses to create a reversible pore on the cell membrane that allows pDNA vector to enter in different kinds of cells including muscle, skin, and APCs. By receiving electroporation, the vaccine efficacy could increase up to 100-fold [329, 330]. To generate pDNA vaccine vector, the four genomic sequences annotated of SARS-CoV-2 spike glycoprotein were retrieved from GISAID database and aligned. Three genome sequences were similar identity, and one was 98.6% matched identity with the others (named: outlier). The fully matched sequence was added of the N-terminal IgE leader sequence to enhanced targeting Trans-Golgi network (TGN) [331], then used a codon optimization tool to generate DNA sequences that enhanced protein expression. The optimized codon was synthesized and ligated into the expression cassettes consisting of CMV promoter and bgh-PolyA tail, resulting in INO-4800 vector. The outlier was constructed with the same methods, designated as pGX9503. The pre-clinical study in mice model after immunization with twice shot of vaccine on days 0 and 14 (2.5 μ g, or 10 μ g) showed that IgG binding against viral spike protein in the RBD regions, and S1 + S2 regions were detected at 2 weeks. Moreover, specific T cells were detected at day 7 and superior at dosage 10 μ g on day 10. PRNT assay showed that sera from mice at day 21 could neutralize the viruses, suggesting a successful humoral response [332]. INO-4800 vaccine was developed by Inovio Pharmaceuticals. Currently, the INO-4800 candidate vaccine reaches Phase I/II clinical trial (NCT04336410).

4.5 Virus-like particle

The structures that composed of multiprotein mimic the authentic virus particles, but exclude the genetic material of the virus is termed virus-like particles [333]. VLPs are monodisperse, defined by the surface chemistry and the VLPs self-assembly from nanoparticles subunits to form high-order hierarchical structures. The idea of protein engineering has started since 1980s with the purposes to synthesize enzymes, redesign antibodies, analyze molecular recognition, and determine structure–activity relationships [334]. Due to the fact that virus like particles are self-assembled structural proteins in the heterologous host expression system, VLPs have antigenic and morphology properties comparable with the native virus. To study the mechanism of viral

infection, VLPs are used as a model for interaction between viruses and cell tropism [335, 336]. The capsid of a virus has been engineered as nano-containers including drug delivery [337], constrained polymer synthesis, and catalysis [338]. The interest in VLPs was inspired by the successful development of recombinant VLPs carrying hepatitis B virus surface antigen (HBsAg) which formed 22 nm non-infectious particles in yeast [339].

4.5.1 Advantages of virus-like particle vaccines

Virus-like particles have many benefits as an antigens delivery platform. First, VLPs are safer than live virus because they are mimic the authentic virus without the genetic material (DNA/RNA) making them unable to replicate or cause infection. Second, VLPs have nano size between 22–150 nm which are easy to enter the APCs to stimulate B cell and T cell responses [340]. Third, the epitopes of VLPs display as native of viral particles resulting in the NABs are stimulated precisely. Fourth, VLP-based vaccines can be produced in large scale [341].

4.5.2 Strategies for SARS-CoV-2 VLPs engineering

Coronaviruses are among the largest enveloped RNA viruses, and SARS-CoV-2 are measured to be 60–140 nm in diameter. Virus particles have quite distinctive spikes, about 9–12 nm [342]. Coronavirus virus-like particles (CoV VLPs) based-vaccine is constructed by recombinant expression of the structural proteins: Spike (S), Envelope (E), Membrane (M), and Nucleocapsid (N) proteins in baculovirus expression system [343], mammalian cells [344], and plant cells (Medicago Inc.). The baculovirus expression system in insect cells is the most suitable for efficient expression of complex protein structures that require posttranslational modification such as glycosylation, phosphorylation, and acylation [345, 346]. Dissimilar to other enveloped viruses, the coronavirus assembles and buds intracellularly at the ERGIC (Fig. 2). The SARS-VLPs are isolated from cell lysates [347].

4.5.3 Component proteins for SARS-CoV-2 VLP

The minimal requirements for the assembly of SARS-VLPs are membrane (M) protein, envelope (E) protein, and nucleocapsid (N) proteins [348]. To generate SARS-CoV-2 VLPs based-vaccine, the minimal system for efficient formation and release VLPs are M and E proteins [344]. Nevertheless, S proteins and N proteins have been used as the leading target antigen in vaccine development.

S protein: The Spike glycoprotein of the viral has the major role in triggering the immune responses of host cells including neutralizing antibody and cellular immunity against SAR-CoV [349] and SAR-CoV-2 [350]. However, IgG and neutralizing antibodies against SARS-CoV do not persist and drop within 3 years [351] whereas N proteins are highly immunogenic and inducing specific T cells could be presented up to 11 years [158].

N protein: Although nucleocapsid proteins are shared 90% similarity on amino acid sequences among coronaviruses, new reports have shown that SARS-CoV-2 adjusts in the electrostatic surface characteristics [159]. Moreover, specific T-cells were assayed frequently targeting nucleocapsid (N) proteins as well as non-structural proteins NSP7 and NSP13 [352].

M protein: The membrane proteins interact with nucleocapsid and spike protein during the assembly of viruses. Mutation in membrane proteins has effects on VLPs assembly [353].

E protein: The envelope protein has the tiny size, but it has the major role on virion assembly. The study by constructing SARS-CoV-VLP lacking E protein significantly reduces viral titres and lowers the number of mature virions [354, 355].

4.5.4 VLPs display antigens

There are four strategies for engineering VLPs display antigens.

(1) Gene fusion technique

The advantage of this strategy is displayed epitope in quaternary structure, requiring less processing, the capsid self assembles *in vivo*, and homogeneous protein nanoparticles. VLPs allow the fusion of foreign antigens at N or C terminal to display the epitope with high immunogenicity [356]. VLPs can also act as effective platforms targeting DCs *in vivo* by fused lymphocytic choriomeningitis virus (LCMV) peptide gp33 [KAVYNFATM] [357, 358]. The SARS-CoV-VLPs can be fused with foreigner peptide by removing amino acid (aa) 1,196–1,255 in spike (S) protein (GenBank: AAP13441) and replacing with amino acid from influenza hemagglutinin. The fused SARS-CoV-VLPs increased significantly yield [359].

(2) Chemical coupling of antigens to the VLP surface

A common approach is through cross-linking of lysine residues on the VLP surface to cysteine residues present into the antigen by using *n*-hydroxysuccinimide (NHS) ester reactions [360]. Various antigens such as peptides, proteins, carbohydrates, and lipoproteins were coupled to the VLPs surface through chemical reaction and bonding lysine [361]. Surface mannosylation of VLPs by conjugating the mannoside or dimannoside to the lysine residues can significantly enhance binding and internalization by APCs, leading to improved immune responses [264]. However, the VLP surface often contains multiple lysine residues. Modification by NHS ester reactions may lead to an uneven antigen distribution and a little control over antigen orientation or may cause antigen misfolding.

(3) Affinity-based conjugation

This approach uses high-affinity binding interactions between biotin and streptavidin, or interactions between His-tag and Ni-NTA. Affinity-based conjugation has an advantage to display large and complex vaccine antigens without compromising VLP formation and stability. To generate recombinant proteins biotinylated, an amino acid sequence GLNDIFEAQKIEWHE was added in the region of coating protein that was not compromised VLP-assembly, allowing VLPs surface to dock monovalent streptavidin fused antigen to the biotin [362]. Biotinylated fused spike protein was constructed by fused Spike (S) protein (GenBank: QHD43416.1) amino acid position Val 16–Arg 685 with poly-His and AviTag™ sequence.

(4) Peptide-protein (Tag/Catcher) conjugation

The Tag/Catcher conjugation was developed based on a split protein CnaB2 from *S. pyogenes* (named: SPY). CnaB2 splits into two fragments to enable peptide–protein ligation. The small fragment named SpyTag has short peptide 13 aa, and protein binding partner named SpyCatcher has 138 aa. Once mixed into a solution, the SpyTag and SpyCatcher rapidly covalently conjugate to each other through an isopeptide bond [363]. In order to decorate peptide on VLPs, the SpyCatcher protein is engineered to form covalent bond to the SpyTag peptide. SpyCatcher was fused to coating protein and produced SpyCatcher-VLPs in an expression system. SpyTag peptide was fused at the C-terminus of antigen for example anti-DEC205 scFv. By simple mixing SpyTag/SpyCatcher, resulted in display targeting DCs peptide on VLPs [364, 365].

4.5.5 Expression systems for VLP production

Viral capsid has produced in various expression systems such as bacteria [366, 367], yeast [368], plant [369, 370], insect cells [346, 371], mammalian cells [372, 373], and cell-free platform [374]. To generate immunogenic virus-like particles, popular choices of eukaryotic expression systems are insect cells, mammalian cells, and plant cells.

In baculovirus/insect cell system, it can provide

posttranslational modifications (PTM) for SARS-CoV-2 protein such as N-glycosylation [375, 376]. However, a disadvantage of this system is contaminating baculovirus particles. For safety, inactivation of baculoviruses and purification for VLP are required [377].

In mammalian cells expression system, VLP has PTM and assembly similar to authentic virus [378]. Nevertheless, the production cost of mammalian cells at large scale deployment is higher than the other systems.

In plant expression systems, the advantage is the low risk of introducing adventitious human pathogens and cost-effectiveness for large-scale deployment. However, the drawback of this system is low VLP expression and PTM unlike authentic virus due to plant-specific N-glycosylation of glycoproteins [379, 380]. Thus, the choice of the host cell expression system has consequences both in terms of the structure of the resulting VLP and the cost of production process [381].

4.5.6 Virus-like particle vaccine for SARS-CoV-2

4.5.6.1 Plant-derived virus-like particle

The idea to use plants as mini factories to produce vaccine started in 1980s. It was inspired by the success expressed recombinant antibodies in tobacco [382]. Australian tobacco (*Nicotiana benthamiana*) is most widely used as susceptible host due to the good efficiency in genetically transformation. Ability to express foreign genes from a plant virus vector and yield of purified protein were acceptable [383]. The first plant-derived virus-like particle vaccine displayed hepatitis B surface antigen with an average diameter of 22 nm [384]. Plant VLPs received increasing attention in clinical trials because the nano particles were biocompatible, biodegradable, low-risk of introducing human pathogens, and can be chemically and genetically engineered to target [385, 386] and imaging [387].

4.5.6.2 CoVLP vaccine

CoVLP vaccine is a plant-derived SARS-CoV-2 virus-like particle. It was developed based on the transient *Agrobacterium*-mediated expression of viral structural proteins in tobacco (*N. benthamiana*). This approach was used to produce influenza vaccines [388, 389]. To generate vaccine, genes encoding SARS-CoV-2 structural proteins were synthesized and cloned into plasmid. The recombinant plasmid was transferred into bacteria *Agrobacterium tumefaciens* as a vector to create transgenic plants. *N. benthamiana* were vacuum infiltrated in batches with an *Agrobacterium* inoculum containing a SARS-CoV-2 expression cassette. Upon bacteria inside the tissue, *Agrobacterium* transfers recombinant DNA to the nucleus. Inside the nucleus, the T-DNA was transcribed into mRNAs without integrated into the plant genome [390, 391]. mRNAs can be exported into the cytoplasm whereas transient expression protein and self-assembly into VLP. Tobacco was incubated up to eleven days before harvested plant leaves and purified. The production process was only 20 days to obtain vaccine. CoVLP had ~ 100 nm in diameter. Currently, the candidate vaccine reaches Phase I clinical trial (NCT04450004). CoVLP was developed by Medicago Inc. and Laval University Infectious Disease Research Center in Canada.

4.5.6.3 Mammalian-derived virus-like particle

SARS-CoV-2 VLP was constructed by expressed viral structural proteins in mammalian cells. To generate plasmid, the genes encoding spike protein (GenBank: QHD43416.1), membrane protein (GenBank: QHD43419.1), envelop protein (GenBank: QHD43418.1), and nucleocapsid protein (GenBank: QHD43423.2) of SARS-CoV-2 with C-terminal tag, and

restriction bases at 5' and 3' terminal were synthesized and cloned into expression plasmid. The four plasmids were co-transfected with commercial transfection reagent in mammalian cell line 293T or Vero E6. Mammalian-derived virus-like particles were separated by sucrose gradients and ultra-centrifuged. Xu et al. reported the necessary two structural proteins for assembly SARS-CoV-2 VLPs were membrane protein (M) and envelope proteins (E). Moreover, this study reported that VLPs produced from Vero E6 cells had a diameter ~ 71 nm, smaller than that in HEK-293T cells (diameter ~ 90 nm), but the spike (S) from Vero E6 was precisely shape of trimeric spikes. Their results suggested that Vero E6 cells were appropriate mammalian cells to produce VLPs [344]. Mammalian-derived SARS-CoV-2 virus-like particle was developed by the China Academy of Chinese Medical Sciences.

4.6 Subunit vaccine

A fragment of an antigen protein, typically Spike in SARS-CoV-2 used to stimulate an immune response without introducing overall structure of virus particles is termed subunit vaccine. To produce a subunit vaccine containing only spike (S) protein, the conventional approach used a detergent as sodium dodecyl sulphate (SDS) to solubilize proteins from membranes. However, the conventional approach was more difficult to achieve [392]. Recent advance in biotechnology has accelerated the subunit vaccine development including modification protein solubility, protein stability, and improved immunogenicity by fused foreigner peptide or proteins [393]. The advantage of subunit vaccine is low risk. However, individual subunit vaccine is less effective than VLP vaccine due to their rarely present epitopes in their native conformation [379, 394]. Moreover, subunit vaccine often requires multidose, followed by booster doses, as well as codelivery of adjuvants to elicit the necessary immune responses [395]. In order to enhance immunogenicity and targeted of protein-based vaccines, numerous biodegradable polymers have been designed to deliver subunit antigens including polymeric micelles [396, 397], polyacrylate dendritic polymer [398], and PLGA particles [399].

4.6.1 NVX-CoV2373

The vaccine composing of spike subunit protein and adjuvant Matrix-M. To generate vaccine, the nucleotide sequence of spike gene (GenBank: MN908947.3) was codon optimized to express in Sf9 cell. The optimized sequence was synthesized and cloned into baculovirus transfer vector, designated as wild type (WT). NVX-CoV2373 baculovirus transfer vector was generated by double mutant in WT: (1) Mutation at furin cleavage site using site directly mutagenesis from amino acid RRAR to QQAQ at the position 682–685, resulting in furin protease resistance [25] and (2) mutated the amino acid from K to P at position 986 and V to P position 987 (2P) to stabilize spike protein in a prefusion conformation [287]. Double mutant Spike proteins were produced using a recombinant baculovirus infected Sf9 cells and harvested at 72 h post infection. Purified NVX-CoV2373 Spike proteins forming 27.2 nm nanoparticles increase the affinity to bind the ACE2 receptor, thermostable and tolerated to the proteolytic cleavage [228]. The adjuvant Matrix-M composed of saponin oil from *Quillaja saponins*, phospholipid, and cholesterol encapsulated in nanoparticles. Matrix-M has demonstrated induction lymphocyte cells and substantially greater levels of neutralizing antibodies *in vivo* both in animal [400, 401] and clinical trial [228, 402]. However, the Matrix-M can only administrate via IM injection [403]. NVX-CoV2373 vaccine from Novavax composed of polysorbate 80 (PS80) as a core to hold the subunit proteins with hydrophobic interaction then further co-formulated with the saponin adjuvant which is able to dissolve in

lipid and water [404]. The adjuvant Matrix-M was mixed with NVX-CoV2373 prior vaccine. The study in healthy adults aged 18–59 years segmented by dosage (Placebo–Saline, 5 and 25 µg) and Matrix-M adjuvant dosage (0 and 50 µg) administered vaccine or placebo twice doses on day 0 and day 21 by an intramuscular injection (0.6 mL) showed that two-dose NVX-CoV2373 5 µg with adjuvanted Matrix-M induced anti-spike IgG and neutralization antibodies that higher than the convalescent from recovered patients. No adverse events were observed. The vaccinated participants have mild reactogenicity with the average of 2 days [228]. NVX-CoV2373 was 49.4% VE against COVID-19 in the clinical trials at South Africa which major strain is B.1.351 [404]. The safety and efficacy of vaccine to prevent COVID-19 in pediatric (12–18 years) is currently study (NCT04611802).

4.6.2 COVID19 recombinant spike protein with Advax-SM adjuvant (COVAX-19)

COVAX-19 vaccine composed of SARS-CoV-2 spike subunit protein with ADVAX adjuvant. COVAX-19 has also used the same approach that developed vaccines against SARS-CoV. SARS-CoV subunit vaccine lacks the transmembrane and cytoplasmic domains of spike [405–407]. To generate vaccine, nucleotide sequence encoding truncated spike was optimized to express recombinant protein using baculovirus system. Advax adjuvant was formulated with antigen by simple mixture immediately prior to immunization. Advax adjuvant is plant-based polysaccharide comprised of inulin polymer. The crystalline (gamma) inulin has ability to increase C3 deposition on antigen-presenting cell, resulting in enhanced antibody and T-cell immune responses [408, 409]. Currently, the candidate vaccine reaches Phase 1 clinical trial (NCT04453852). Forty volunteers who are in good health, aged between 18 and 65, will receive two doses of vaccine 25 µg with adjuvant, or placebo (saline) by intramuscular injections. COVAX-19 was developed by Vaxine and Flinders University in Australia.

4.7 Phage-based vaccines

Bacteriophage therapy has been used to combat bacterial infections in human since the discovery in 1940 because their safe and high specificity towards the target and have no effect on non-target. Phage can be administered by oral, intraperitoneal injection, intramuscular injection, subcutaneous injection, and medullary injection. M13 bacteriophage (phage) was classified in the genus *Inovirus* (family *Inoviridae*). Phage has long, thin filamentous shape with nano-size [410]. Recent advances in the genetic engineering pave a way to develop phage as novel nanobiomaterials to deliver nucleic acid as therapeutic agents for silencing expression of genes or restoring the expression of non-functional gene with highly specific, less toxicity, and low-cost production. Phage genome can be engineered to display functional peptide motif on their minor (pIII) and major coat proteins (pVIII), encoding the antigen of interest for vaccines and immunotherapies. The advantage of phage genome is the ability to directly display between genotype and phenotype. This technology demonstrates a new strategy for nano-vaccine delivery platforms. Previously, AAVP vector has demonstrated to display foreigner peptide on the minor coated protein [411] and major coated protein [412]. There are two strategies to develop phage-based vaccine: (1) Phage-based vaccine can mimic to display antigens on their coated protein surface, or (2) display peptide target to the antigen presenting cells and deliver DNA-encoded antigens. Phage-based vaccine has a potential for immunotherapy including cancer [413, 414], malaria [415, 416], and Alzheimer [417].

5 Promising candidate COVID-19 vaccines

Clinical trials are experimental conditions with randomization designed and controlled by scientists to answer the specific questions such as dosage, timing, safety, and effectiveness of vaccines, drugs, or devices in human subjects [418]. There are 5 stages (phases) of the study before commercial approval by FDA. (1) Pre-clinical evaluates the safety and doses equivalent of vaccine in animal studies. For small animal models, it was found that SARS-CoV-2 could not infect through wild type mouse ACE2 receptor. There are three ways of testing vaccine efficacy: using transgenic mouse replaced with human ACE2 gene [419]; using adapted strain for mice (MASCp6) [420]; using Syrian hamster which is an alternative small animal model [421]. For large animals models, ferrets were used to prove direct contact [422] and airborne transmission [423]. The most popular model for testing vaccine in the non-human primate model is Rhesus macaque, which provides valuable data of protective efficacy after challenge with SARS-CoV-2 [424]. (2) Phase I trial (dose-escalation) is the first study in a small group of humans (20–100 subjects; at least 3 placebo) to find recommended dose (RD), the maximum tolerable dosage (MTD), and investigate the outcome protective immunity and dose-limiting toxicity (DLT) [425]. (3) Phase II trial (therapeutic exploratory) is in larger human subjects than Phase I trial (500 subjects; at least 125 placebo) to test pharmacokinetics, pharmacodynamics, and safety. It was designed to find dose frequencies and optimal dose for planning the Phase III trial [129]. (4) Phase III trial (therapeutic confirmatory) aims to confirm the therapeutic efficacy in large number of human subjects (300–3,000 subjects randomly assigned 1:1). (5) Phase IV trial (post marketing study) aims to conducted vaccine safety profile after vaccine was approved by FDA for commercial [426]. The amount of subjects has an important role in statistical power to detect the adverse reaction no less than 1 subject in 100 subjects after vaccination [418, 427, 428]. Vaccine efficacy is the critical outcome of FDA approval (FDA-2020-D-1137) that should not be less than 50% to prevent COVID-19 [429]. Nano-based vaccines have shown the most promising result in Phase III trial higher than the FDA standard. The lipid nanoparticle encapsulated mRNA-based (mRNA/LNP) BNT162 vaccine from Pfizer and BioNTech reported 95% efficacy. The vaccine efficacy was calculated from the 38,955 subjects who received the vaccine and placebo in the randomize design 1:1. The data were collected from 170 subjects that later confirmed positive SARS-CoV-2. The risk of disease after vaccination was 0.041% (8 positive subjects from 19,478 subjects), while the risk of disease in non-vaccinates was 0.832% (162 positive subjects from 19,477 subjects). The risk ratio was 0.049 (0.041% risk of disease in vaccinates: 0.832% risk of disease in non vaccinates). Thus, the vaccine efficacy was approximately 95%. However, the challenge for the mRNA vaccine in clinical application was a need for cold chain storage and transportation. The BNT162 vaccine must be kept at -70°C for 6 months and $2-8^{\circ}\text{C}$ in refrigerator up to 5 days before vaccination to ensure vaccine potency (Pfizer Inc). The mRNA-1273 vaccine from Moderna had 94.5% vaccine efficacy, which was calculated from 30,000 subjects. The vaccinated group had 5 positive subjects while the placebo group had 90 positive subjects. The lipid nanoparticle encapsulated mRNA-based (mRNA/LNP) formulation mRNA-1273 vaccine could be kept at -20°C for 6 months and $2-8^{\circ}\text{C}$ in refrigerator up to 30 days (Moderna Inc). AZD1222 (ChAdOx1 nCoV-19 vaccine) reported 90% efficacy when administered with 2-time doses with a prime-boost at lower doses and boost with standard doses at 28 days apart ($n = 2,741$) while administering 2 times with the prime and boost standard doses decreased the vaccine efficacy to 62% ($n = 8,895$). The

explanation of dose-dependent administered was not clear, however, AZD1222 had an average efficacy of 70% ($n = 11,636$), which passed the requirement of the FDA standard. A single shot of vaccine from Johnson & Johnson had an average efficacy of 66.9% (116 positives in the 19,630 vaccinees subjects vs. 348 positives in the 19,691 placebo subjects). Ad5-nCoV from CanSinoBio had an average efficacy of 65.7% ($n = 30,000$), which is similar to Ad26.COV2.S. PiCoVacc, which is an inactivated virus vaccine from Sinovac Biotech reported far less effective than the others, around 50% efficacy (85 positives in the 13,000 vaccinees subjects vs. 167 positives in the 13,000 placebo subjects) in the Brazilian clinical trial. The 2 nano-based mRNA/LNP vaccines (mRNA-1273, BNT162) have reached the positive interim results without doubt in the vaccine safety and efficacy. Interestingly, these will be the first RNA vaccines for human. The application of nanocarriers for the COVID-19 vaccine development has proved its advantages such as accelerating vaccine development, minimal adverse effect (biocompatible, biodegradable, less toxic to the cells), superior vaccine efficacy, and high public trust in vaccine safety. Nevertheless, the duration of vaccine immune response will have to be monitored. It should be noted that the conventional vaccines based on Chimpanzee adenoviral vector (AZD1222) and inactivated virus (PiCoVacc) reached the primary efficacy endpoint but had the vaccine efficacy lower than mRNA/LNP vaccines. The BNT162b2 from Pfizer-BioNTech was the first mRNA COVID-19 vaccine that was authorized for an emergency use in human in the UK on 2 December 2020, authorized in the USA on 11 December 2020, and authorized by the European Medicines Agency (EMA) in all EU countries on 21 December 2020. The mRNA-1273 from Moderna was the second mRNA COVID-19 that was authorized by the U.S. Food and Drug Administration on December 18, 2020.

Nevertheless, the rapid mutation of SARS-CoV-2 strain in South Africa (B.1.351), UK (B.1.1.7), Brazil (P.1), and USA (CAL.20C) delayed neutralization antibodies and decreased the vaccine efficacy to prevent the COVID-19 including the individual whom vaccinated with the mRNA vaccine from Pfizer and Moderna, subunit proteins NVX-CoV2373, and viral-based vaccine ChAdOx1, Ad26.COV2.S [138, 292, 293, 430]. The comparative study of the third doses of vaccine against SARS-CoV-2 in the transplant recipients showed that the patients who received the boost doses mRNA vaccine from Moderna had the highest antibody titers [431]. In the responses to the variant outbreak, the mRNA vaccine from Moderna which modified in the mRNA antigens to prevent B.1.351 strain has been tested in the clinical trial (NCT04785144). Various platforms of COVID-19 vaccine are beneficial for the consumers in terms of choice for selection, cost, and acceptable adverse effects after vaccinated.

6 Conclusions

The vaccines for COVID-19 remain a critical issue. Nanotechnology presents promising strategies for improving the immunogenicity of a vaccine antigen. Numerous vaccines and biodegradable nanoparticle-based delivery vehicles are being evaluated in clinical trials. Within 63 days of genomic sequence of SARS-CoV-2 donated in GenBank, the first nanovaccine candidate entered into the clinical trial Phase I. Today, the scientific community is waiting for Phase III clinical efficacy data. However, there is still plenty of room for further enhancement of COVID-19 vaccines. As shown in the immune-enhanced strategies, the format and uptake of immunogens into dendritic cells are critical parameters for stimulus immunity. Moreover, the vaccine combination with adjuvants can enhance the humoral and

cellular immune responses. An ideal candidate COVID-19 vaccine should be a single-dose vaccination, resulting in a long-term and broad immune protection for variant SARS-CoV-2, stable vaccine at room temperature and ability to administer through a noninvasive route. Challenges for nanomaterial-based vaccines that limit the clinical transformation are as follows: (1) design of antigens, which is important for inducing immune responses. The best design of RNA encoded antigens with increased stability and *in vivo* translation will lead to improved vaccine efficacy; (2) design of carriers, which is important to achieve the target cells and increase the time for storage. Different lipid nanoparticle formulation has different clinical outcomes. For example, the BNT162 vaccine must keep at -70°C , while mRNA-1273 vaccine could be kept at -20°C up to 6 months; (3) selection of appropriate animal models for vaccine testing. SARS-CoV-2 cannot infect the wild type of mice and requires special mice and a special strain of SARS-CoV-2. Immune responses to properties of nanomaterials should be considered in the planning of pre-clinical stage evaluation [432–434]. A promising vaccine from the efficacy reported of Phase 3 clinical trial is mRNA/LNP. The mRNA/LNP vaccines (mRNA-1273, BNT162) showed a high safety profile with superior vaccine efficacy. However, a drawback of mRNA/LNP vaccines is the cold chain limitation, which requires expensive equipment for storage (-70°C or -20°C) and requires two doses administered to boost the immunogenicity. In contrast, the adenoviral based vaccine could be kept in the $2-8^{\circ}\text{C}$ refrigerator and requires only single dose vaccination. Nevertheless, the viral-based vector previously shows the risk of getting public trust (AZD1222 and Ad26.COV2.S) and the experiments in Phase 3 are still on-going. For the next generation of immune-enhanced COVID-19 vaccine, self-assembled macromolecules (phage, protein-based platform) are attractive nanomaterial. The phage-based vaccine can enhance immune responses by phage display technology. The virus-like particle is a protein-based platform, which mimics the overall structure of SARS-CoV-2 particles without infectious genomic RNA. It is very safe and adjustable. Further improvements in the next generation nanoparticle-based COVID-19 vaccine include: (1) design for match variants strain including wildtype (D614), mutated (G614) that become a worldwide dominance, and further prediction of virus strains by computational modeling [435, 436], resulting in broad protection of variant SARS-CoV-2. (2) Single dose vaccination with minimum dose exposure of antigens. Nanoparticle-based vaccine targeting APCs delivery of the self-amplifying RNA or pDNA could lower administered dose, give high-level transient expression proteins, and elicit robust neutralizing antibody. The low amount requirement of nucleic acid-based antigens also saves costs of large scale production of vaccine [437]. (3) Stable vaccines at room temperature and ability to be administered through a noninvasive route. Nanoparticle-based formulations could offer diminish cold-chain for storage and ease of transportation such as dry-powder VLPs suitable for nasal delivery [438], and gold nanoparticle encapsulated pDNA, and protein for skin vaccination [326, 439]. Several nanomaterials have been synthesized as antigen carriers with ligands for specific receptors, or stimuli responsive compounds on the surface, expecting to be more efficient than the conventional vaccines. In addition, these nanoparticles can efficiently deliver antigens due to their nanoscale size, chemical properties on their surface, and ability to protect the antigens along with adjuvants. However, the cost of vaccine and public trust in vaccine safety are two of the key factors for the success of vaccination.

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