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Review Article

The COVID-19 Vaccine Race: Challenges and Opportunities in Vaccine Formulation

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Abstract. In the race for a safe and effective vaccine against coronavirus disease (COVID)-19, pharmaceutical formulation science plays a critical role throughout the development, manufacturing, distribution, and vaccination phases. The proper choice of the type of vaccine, carrier or vector, adjuvant, excipients, dosage form, and route of administration can directly impact not only the immune responses induced and the resultant efficacy against COVID-19, but also the logistics of manufacturing, storing and distributing the vaccine, and mass vaccination. In this review, we described the COVID-19 vaccines that are currently tested in clinical trials and provided in-depth insight into the various types of vaccines, their compositions, advantages, and potential limitations. We also addressed how challenges in vaccine distribution and administration may be alleviated by applying vaccine-stabilization strategies and the use of specific mucosal immune response-inducing, non-invasive routes of administration, which must be considered early in the development process.

KEY WORDS: coronavirus; vaccine; adjuvant; route of administration; mucosal vaccination.

INTRODUCTION

First reported in late 2019, the COVID-19 has become a pandemic across the world. As of the time of writing this review (May 29, 2020), there are more than 5 million confirmed cases and over 357,000 deaths due to COVID-19. The elderly have a higher mortality rate than other age groups. The pathogen that causes COVID-19 is the severe acute respiratory syndrome coronavirus (SARS-CoV-2), a betacoronavirus that is genetically homologous to the SARS coronavirus from the 2003 outbreak (SARS-CoV) (1). Initial work suggests that the SARS-CoV-2 enters the host cells by binding to the angiotensin-converting enzyme 2 (ACE2), similar to the case SARS-CoV (2). Potential therapeutics including antiviral medications, protease inhibitors, and monoclonal antibodies are being developed or in clinical trials. Remdesivir, a nucleoside analog prodrug developed by Gilead, is the most advanced antiviral drug undergoing clinical investigation against COVID-19 (NCT04292899, NCT04292730, NCT04280705, NCT04315948, NCT04257656). In a recent placebo-controlled, randomized clinical trial with 1063 patients, remdesivir shortened the medium recovery time by 4 days, from 15 to 11 days, and reduced the mortality by 14 days from 11.9 to 7.1% (3). In a separate study, remdesivir was shown to be ineffective for patients with severe COVID-19, however (4). Data from an open-label, randomized phase 2 clinical trial showed that the triple combination of interferon-beta-1b, oral lopinavir-ritonavir (protease inhibitors), and oral ribavirin (a nucleoside analog), when given within 7 days of symptom onset, was more effective in suppressing virus shedding and alleviated symptoms, as compared to lopinavir-ritonavir alone (5). Despite the fact that there are hundreds of clinical trials initiated since the outbreak of COVID-19, an antiviral drug effective in all patient groups is yet to be developed and evaluated. Therefore, the need for vaccinating the entire population against the SARS-CoV-2 virus is urgent, and vaccination will likely be the most effective way to control the pandemic. This task is highly challenging because we first need to develop a safe and effective vaccine, and then manufacture, distribute, and administer it to the vulnerable population within a short timeframe. Development of a safe and effective COVID-19 vaccine is not easy, but manufacturing, distribution, and administering the vaccine could potentially face extraordinary challenges as well, especially in developing countries and if the vaccine must be injected, and the cold chain is required to maintain its stability and activity. Currently, there are more than 100 COVID-19 vaccine candidates under development and that number is still increasing. As of May 29, 2020, thirteen vaccine candidates are undergoing clinical evaluation (see Table I).

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Table I. Landscape of COVID-19 Vaccine Development

Vaccine Category	Sponsor	Vaccine Candidate	Ref/registration number	Preclinical	Phase I	Phase II	Phase III/IV
mRNA	Moderna/NIH	mRNA-1273	NCT04283461				
mRNA	Pfizer/BioNTech	BNT162	EudraCT 2020-001038-36, NCT04380701				
Adenovirus-based	CanSino Biologics/Academy of Military Medical Sciences	AD5-nCoV	ChiCTR2000031781, ChiCTR2000030906				
Inactivated	Wuhan Institute of Biological Products	COVID-19 vaccine (Vero cells)	ChiCTR2000031809				
Inactivated	Sinovac Biotech	PiCoVacc	NCT04352608				
Adenovirus vector	Oxford University/AstraZeneca	ChAdOx1 nCoV-19	NCT04324606				
Inactivated	Beijing Institute of Biological Products	Inactivated SARS-CoV-2 vaccine (Vero cells)	ChiCTR2000032459				
Lentivirus vector	Shenzhen Genoimmune Medical Institute	Covid-19/aAPC	NCT04299724				
Lentivirus vector	Shenzhen Genoimmune Medical Institute	LV-SMENP-DC	NCT04276896				
DNA plasmid	Inovio/Beijing Advaccine/Ology	INO-4800	NCT04336410				
Recombinant protein	Novavax	NVX-CoV2373	NCT04368988				
bacterial vector	Symvivo Corporation	bacTRL-Spike	NCT04334980				
Trained Immunity-Based	Merck	Bacille Calmette-Guerin (BCG)	NCT04328441, NCT04362124, NCT04379336, NCT04350931, NCT04327206, NCT04369794, NCT04373291, NCT04348370				

These diverse types of vaccine candidates face a variety of challenges that are related to development, manufacturing, storage, and distribution, to mass vaccination.

Overview of the Types of Vaccine Candidates Against COVID-19

Vaccine candidates against COVID-19 have diverse compositions, from traditional whole-pathogen vaccines to various new-generation vaccines (see Table II). Traditional whole-pathogen vaccines consist of live-attenuated vaccines (live pathogens with reduced virulence) and inactivated vaccines (thermally or chemically inactivated pathogens), both are relatively straightforward in their development processes (6). Live-attenuated vaccines introduce a mild infection that resembles the real infection, leading to a strong immune response and the immunological memory can last for years. The main drawback associated with live-attenuated vaccines is potential safety concerns. Live-attenuated vaccines often have higher reactogenicity compared to recombinant protein-

Table II. Overview of the Types of Vaccine Candidates Against SARS-CoV-2

Vaccine type	Mechanism features	Development and production features
Live-attenuated vaccines	Elicit strong immune response, the protection is long-lasting, causes reactogenicity	Product development and manufacturing process is highly established but requires handling live virus
Inactivated vaccines	Less reactogenicity, also weaker immune response than live-attenuated vaccines, requiring multiple dosages and adjuvants	Product development and manufacturing process is highly established but requires handling live virus
Recombinant protein-based and vector-based vaccines	Safe, induce a precise immune response, weak immunogenicity, and may require the addition of adjuvants	Epitope selection, antigen design, and vehicle development are not straightforward. Some new-generation vaccine types were not produced on large scale before.
Trained immunity-based vaccine	May boost the innate immunity against a wide range of infectious agent, the efficacy, and mechanisms are still under study	Current available across the world, but each country has its version. Not the traditional specific adaptive immunity-inducing vaccine.

based vaccines, and the live-attenuated viruses have the potential to infect people with compromised immune systems or reverse back to virulent strain (7,8). The inactivated vaccines are relatively safer as live pathogens are not involved, but they can be lower in immunogenicity and often require multiple doses to establish immune memory. Although the vaccine itself is safe conceptually, a defect in the manufacturing process may cause a disease outbreak, as happened in the Cutter incident, in which the defective polio vaccine manufactured by Cutter Laboratories caused 40,000 new cases of polio, including 200 cases of paralysis and 10 deaths (9).

The new-generation vaccines, including recombinant protein vaccines and vector-based vaccines, only incorporate a specific antigen or antigens from the pathogen, instead of the whole pathogen, giving a better safety profile (10). Designing a successful new-generation vaccine requires a thorough understanding of the structure and the immunopathogenesis of the pathogen. Therefore, it may take a longer time to initiate the development of new-generation vaccines for novel pathogens. Fortunately, the SARS-CoV-2 virus is homologous to SARS-CoV and the Middle East Respiratory Syndrome Coronavirus (MERSCoV), which have been studied for years. Based on the carrier of the antigen, the new-generation vaccines for COVID-19 can be classified into recombinant protein-based vaccines and vector-based vaccines, *e.g.*, Messenger RNA (mRNA) vaccines, plasmid DNA vaccines, viral vector-based vaccines, and non-pathogenic bacterial vector-based vaccines. Some critical features of new-generation vaccines are listed in Table III. Other vaccine types such as toxoid vaccines and polysaccharide conjugate vaccines are mainly used for bacterial infection and therefore will not be discussed here.

The S Protein Is the Main Target for the Recombinant Protein and Vector-Based Vaccines

The selection of the target antigen for a new-generation vaccine is based on the structural and pathobiology information of the SARS-CoV-2 virus. The genome of the SARS-CoV-2 is a single-stranded, positive-sense RNA (2). SARS-CoV-2 has four main structural proteins including spike protein (S), envelope protein (E), membrane protein (M), and nucleocapsid (N) protein. The S proteins are located at the outer surface of the virus particles and can bind to ACE2 on the cell surface, allowing receptor-mediated endocytosis of the virus (2). Based on crystallography, ACE2 binding patterns between SARS-CoV-2 and SARS-CoV are highly homologous (2,11) (Fig. 1). The ACE2-dependent mechanism also suggests animal models that express human ACE2 equivalents should be used in challenge studies to evaluate the efficacy of a vaccine (12,13).

The SARS-CoV S protein can use CD209 and CD209L as alternative receptors, but it has not been reported if SARS-CoV-2 can also use these receptors (14). Since the S protein plays a critical role in the virus life cycle, most COVID-19 vaccine candidates use the S protein as the antigen. Previously in SARS-CoV vaccine development process, liver damage was observed in the animal model when the full-length S protein was used as the vaccine antigen (15). Therefore, using an S protein fragment, such as the receptor-binding domain (RBD), as vaccine antigen might be a safer choice for COVID-19 vaccine candidates.

Information Technology Accelerate COVID-19 Vaccine Development Process

In a fast-paced research environment like the COVID-19 pandemic, high-speed genomic sequencing technology allows early identification of the pathogen, and the online database and preprint platforms allow researchers to share the latest data and opinions without the time-consuming publishing process. Using samples collected in December 2019, the complete genome sequence of SARS-CoV-2 was posted on January 10, 2020, on virological.org by Edward C. Holmes on behalf of a consortium led by Yong-Zhen Zhang (16) and later on GenBank (GenBank: MN908947.1). The fast sequencing and data publishing process allow early initiation of vaccine development. The United States National Institutes of Health (NIH) even started to develop COVID-19 vaccines on January 11, 2020, the next day after the genome sequence was available (17). In theory, a recombinant proteinvaccine or a vector-based vaccinecan be developed solely from the sequence information. The binding pattern of the SARS-CoV-2 protein to ACE2 receptor was first published in preprint server BioRxiv on February 19, 2020 (18) before it was published in a peer-reviewed journal on March 30, 2020 (2).

Recently, applications of computational approaches including machine learning, deep learning, and molecular dynamics (MD) have been expanded to vaccine antigen constructs. MD simulation has been applied in epitope-carrier fusion construction in HIV vaccine and malaria vaccine (19,20). The machine learning tool was used to predict the antigen-specific immune signatures in vaccines based on immune profiling data (21,22). Ong *et al.* (2020) predicted possible vaccine targets of COVID-19 using a machine learning tool, including the non-structural protein (nsp3), a novel target that has not been tested for vaccines (23). The *in silico* tools can reduce the time and cost associated with vaccine development. Although using computational approaches in vaccine development is a relatively new area, it can be foreseen that they will play a more important role in the future.

It is noted that the computational tools, especially the MD simulation, demand high computing power. The deployment of cloud-based computing allows researchers to conduct such research without investment in expensive information technology infrastructures. This unique advantage became available after the last SARS-CoV pandemic. For example, Amazon Web Services, Google Cloud Platform, and Microsoft Azure were launched in 2006, 2008, and 2010, respectively.

DELIVERY SYSTEMS OF NEW-GENERATION VACCINES

Recombinant Protein Vaccines

The recombinant protein vaccine uses a part of the whole protein or a protein fragment such as the RBD or fusion of RBD with a carrier protein as the antigen (24).

Table III. Delivery Systems for Next-Generation Vaccines Against COVID-19

Vaccine type	Vaccine vehicle	Adjuvant	Example of commercial vaccines	In development for Covid-19	Advantages	Disadvantages
Recombinant protein vaccines	Delivery systems can be used but not required	Alum, QS-21, AS03, $AS01_B$, AS04, CpG1018, MF59 are used in commercial vaccines	Hepatitis B, HPV, *etc.*	NVX-CoV2373	Most studied strategy, having lower immunoreactivity than whole-pathogen vaccines	Immunogenicity is weak and requires multiple vaccine dosages and/or adjuvants
mRNA vaccines	Naked mRNA or nanoparticles (NPs)	mRNA itself has adjuvant effect, but additional adjuvant can be added	In clinical trials	mRNA-1273, BNT162	No risk of host DNA integration, production platform is highly flexible	Stability and half-live of mRNA are short, transfection efficacy is at least ten-fold lower than viral vector, have never been commercialized before
plasmid DNA vaccines	*Ex vivo* transfection or NPs	The vehicle serves as adjuvant	for veterinary use only	INO-4800	Easy to produce, chemically stable	Requires an intradermal electroporation device to transfect the cells
Viral vector-based vaccines	Virus	The virus serves as adjuvant	LUXTURNA®	ChAdOx1 nCoV-19, Covid-19/aAPC, LV-SMENP-DC	High transfection efficacy, the viral vector imitates the natural process of infection	Some population have existing antibodies against the vehicle virus, thermostability is poor
Non-pathogenic bacterial vector-based vaccines	Lactic acid bacteria (LAB)	The vehicle serves as adjuvant	All in clinical trails	bacTRL-Spike	The easiest platform for scale-up production, better thermo-stability than other platforms	The immunogenicity is generally weak, no existing product using this approach

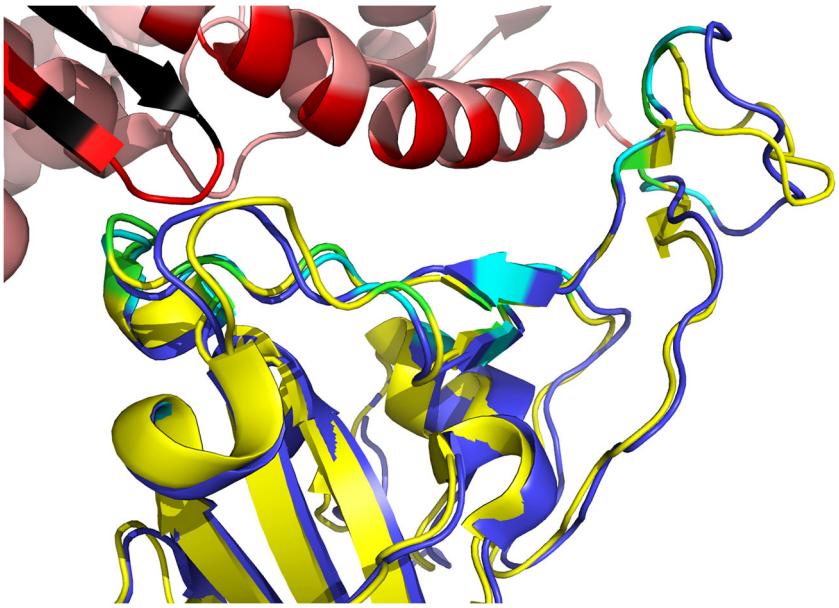

Fig. 1. Overlay comparisons between SARS-CoV-2 RBD (yellow, PDB ID: 6VW1) and SARS-CoV RBD (blue, PDB ID: 3D0H) bind to ACE2 (red, PDB ID: 6VW1). Residues close to the interface are highlighted in green for SARS-CoV-2 RBD and cyan for SARS-CoV RBD. Image of 6VW1 (Shang, J., Ye, G., Shi, K., Wan, Y., Luo, C., Aihara, H., Geng, Q., Auerbach, A., Li, F., 2020. Structural basis of receptor recognition by SARS-CoV-2. Nature 581, 221–224.) and 3D0H (Li, F., 2008. Structural Analysis of Major Species Barriers between Humans and Palm Civets for Severe Acute Respiratory Syndrome Coronavirus Infections. J. Virol. 82, 6984–6991.) are visualized using the PyMOL Molecular Graphics System

Once taken by the antigen-presenting cells (APC), the antigen protein is digested in the endosome, while a small fraction of the digested fragments is trimmed and presented to the major histocompatibility complex (MHC) II molecules, triggering downstream immune responses. For SARS-CoV, it was shown that animals immunized with recombinant protein vaccine candidates can produce neutralizing antibodies (24,25). The main disadvantage of the recombinant protein vaccine is that it usually only induces specific humoral immune responses and sometimes only provides partial protection to viral infections (26,27). Therefore, recombinant protein vaccines often require an adjuvant in the formulation to increase the immunogenicity. For example, vaccine candidate NVX-CoV2373 for COVID-19 uses Matrix-M as the adjuvant (28).

Viral Vector-Based Vaccines

In viral vector-based vaccines, the antigen is cloned into a viral vector that lacks the ability to reproduce. Common vectors include lentivirus, adenovirus, and adeno-associated virus (AAV). The viral vector imitates viral infection disease state and therefore can produce stronger cellular immune responses as compared to the recombinant protein vaccine. Previously, a SARS-CoV vaccine candidate was developed using the AAV vector (29).

Bacterial Vector-Based Vaccines

Bacterial vector is another option for vector-based vaccines. Among them, the non-pathogenic lactic acid bacteria (LAB) are the most promising (30). Symvivo's COVID-19 vaccine candidate, bacTRL-Spike, uses LAB as the vector and is currently in the clinical trial. The LAB vaccine vector has some advantages: LAB is generally recognized as safe (GRAS) as a food or food additive, the manufacturing cost is low, and it can be lyophilized to provide better stability profile (31).

Plasmid DNA Vaccines

DNA vaccine eliminates the need for using live viruses hence having a better safety profile. The manufacturing process of plasmid DNA is relatively straightforward, and the double-strand DNA molecules are more stable than virus, protein, and mRNA, and can be freeze-dried for long-term storage. The main prohibitory factor for the plasmid DNA vaccine is the low transfection efficacy, requiring transfection modalities. For example, the Inovio's COVID-19 vaccine candidate, INO-4800, uses a handheld electroporation device, CELLECTRA (32). The vaccine is injected intradermally along with electrodes, then an electric pulse is applied to open the cell membrane, allowing the plasmid to enter the cells. Using an established device allows fast launch in clinical trials, but it also introduces additional hurdles in mass vaccination.

Messenger RNA Vaccines

The mRNA vaccine is the newest generation of vaccines in which all components can be produced *via* chemical synthesis. Since antigen expression from mRNA is a transient

process, the risk of host DNA integration is negligible. The elimination of using live materials is an advantage from a quality control standpoint and allows quick product switching in manufacturing facilities. This is because different proteins differ only in the sequence of the RNA molecules, which can be easily modified in the solid phase synthesis process. Being fully-synthetic also eliminates the risk of disease transmissions from the manufacturing facility, especially for high-risk pathogens like Ebola (33).

Naturally occurring mRNA molecules have low apparent transfection efficacy. Therefore, lipid nanoparticles (LNPs) are often used to incorporate the mRNA molecules for transfection purposes (34). A typical LNP formulation consists of an RNA condensing lipid to form a complex with the mRNA molecule, helper lipids to provide the structural rigidity, and lipidized polymer coating to modify the surface properties of the particles (35). Once phagocytosed by a cell, the LNPs are exposed to a low pH environment in the endosome, and the RNA condensing lipid can puncture the endosome and allow the mRNA molecule tobe released in the cytosol. Therefore, the RNA condensing lipid is the key component of this platform. The 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and dilinoleylmethyl-4-dimethylaminobutyrate (DLin-MC3-DMA) are the two common commercially available positively charged lipids for this purpose (35). Moderna's COVID-19 vaccine candidate mRNA-1273 is an LNP-encapsulated mRNA vaccine that encodes the S protein, given as two doses by intramuscular (IM) injection (36).

Trained Immunity-Based Vaccines

Conventional vaccines activate the adaptive immune system, providing pathogen-specific protection. In contrast, the trained immunity-based vaccines (TibV) stimulate the innate immune system, providing protection to unrelated pathogens (37,38). The Bacille Calmette-Guerin (BCG), a vaccine for tuberculosis disease, is currently under clinical evaluation for its ability to induce trained immunity against COVID-19, which will take time to prove (39). Even if the BCG vaccine is effective against COVID-19, it has a unique challenge that the manufacturing standards of BCG vaccine differ across different countries, and it is unclear if certain quality criteria should be required to provide protection against COVID-19 (40).

Inactivated and Some of the Vector-Based Vaccines Require Adjuvants to Boost Their Immunogenicity

Establishing an antigen-specific immune response requires triggering of the innate immune system to detect the antigen as foreign objects. However, the inactivated virus and recombinant protein antigen are often weakly immunogenic and require an adjuvant to boost the immunogenicity. Viral vector-based vaccines and bacterial vector-based vaccines do not require adjuvants. In the COVID-19 vaccine development race, the inactivated COVID-19 vaccine candidate from Sinovac uses $Al(OH)_3$ as the adjuvant (41). Introduced in the 1930s, aluminum salts, known as "alum," are the first adjuvant used in commercialized human vaccines and are still used in about 80% of adjuvanted vaccines today (42). Once injected, the insoluble alum particles activate cascade 1 by activating NLRP3 inflammasome, followed by releasing proinflammatory downstream cytokines including IL-1β, IL-18, and IL-33 (43–47).

Alum induces the recruitment of monocytes to the site of injection, which then move to the draining lymph node and differentiate into $CD11c^+$ MHC class II^+ peritoneal dendritic cells (DCs) (43). These monocytic DC precursor cells were shown to be responsible for priming naive $CD4^+$ T cells. The release of IL-1β leads to T helper type 2 (Th2) $CD4^+$ T cell differentiation, which mediates the differentiation of B cells that secrete antibody isotypes IgG1 and IgE. The bias towards Th2 response explains that alum is not efficient in inducing cell-mediated immune responses, especially cytotoxic T cell responses (48). For an efficacious COVID-19 vaccine, if specific antibodies alone cannot stop the viral infection and prevent the disease, then an adjuvant other than alum that can help induce a strong cellular immune response might be needed. For example, the monophosphoryl lipid A (MPL) is a Toll-like receptor (TLR-4) agonist used in multiple adjuvants systems. TLR-4 activation promotes IFN-γ production and differentiation of $CD4^+$ T cells with the Th1 profile, enhancing cellular immune response (49). Among the few vaccine adjuvants in human vaccines, CpG1808, AS-04, and $AS\text{-}01_B$ may be tested.

Messenger RNA molecules have intrinsic immunogenicity that is analogous to RNA virus infection, often referred to as "self-adjuvant" in mRNA vaccines (50). For mRNA vaccines, proinflammatory responses may be from the mRNA molecules *per se* and the delivery vehicles such as the LNPs. Chemical modifications of the mRNA molecules may alter their proinflammatory activity, but the delivery vehicles and the mRNA condensing lipids can both induce unwanted proinflammatory responses (34,51).

Scaling-Up Viral Vector-Based Vaccines Has to Balance Between Cost and Quality

Viral vector-based vaccines are replication-defective and lack the essential components for viral replication in normal cells (52). In the manufacturing process, the viruses are replicated in a complementing cell line contains the missing components. To avoid producing viral particles with replication-competent, in complementing cell lines, the missing components are separated into three or four plasmids (53).

Although the commercial manufacturing process of viral vectors for gene therapy had been developed decades ago, the complexity of viruses requires optimization for specific products to fulfill the requirements of quality and cost-efficiency. Scaling-up of viral production capacity is a bottleneck of viral vector manufacturing (54,55). Therefore, the current challenges of virus vector-based vaccines still lie in manufacturing with a good balance between high recovery yield and impurity clearance and the reduction of cost (54,56). Conventionally, transient transfection of adherent cell lines (*e.g.*, HEK293T cell) is used for viral vector production, but this method has a limitation of production capacity (57). Scaling-up can be achieved by applying a suspension expression system culturing in a bioreactor to replace the adherent cells grown in cell stacks (54,58). However, the suspension systems in which cells and virus particles are mixed in the liquid together may generate more challenges to downstream processing where the virus particles are purified from the media. Virus particles adhere to the cell debris, cell

membranes, and/or host cell impurities (*e.g.*, DNA and proteins) resulting in loss of virus particles in clarification steps if small pore size filters are chosen, or insufficient removal of process-related impurities if related large pore size filters are chosen (59). Effective removal of impurities, maintaining high recovery yield, and reducing the price per dose rely on the downstream process optimization. Centrifugation, clarification, ion exchange chromatography, size exclusion chromatography, DNA digestion, and tangential flow filtration are the common methods for downstream process (60,61). Although most supplies for those processes are commercially available, each product requires process optimization to meet the quality standards, making process development time-consuming and labor-intensive. The clearance of residual host cell DNA and host cell proteins is another challenge of suspension viral production process as host cell DNA and proteins may adhere to viral particles tightly, and co-elute in chromatography processes (61,62). The biophysical properties of the individual virus type and the relationship between each step should be considered in manufacturing process development. For example, lentiviral vector (LVV) is sensitive to shear force, temperature, and pressure (61); the sheer force generated in the tangential flow filtration process can damage the capsids of LVV and lead to virus aggregation. The aggregated LVV will then clog the membrane of the filter during the consequent sterile filtration process, resulting in a low recovery yield.

LACK OF THERMOSTABILITY IS A MAJOR BURDEN AND BARRIER LIMITING WORLDWIDE DISTRIBUTION

The ideal vaccine will be in a ready-to-use dosage form that can be stored at ambient temperatures with a long shelf life (see Fig. 2). Our current reality is far from ideal because commonly used vaccines today require refrigerated storage at between +2 and +8°C (63). Some biologics even requires a lower temperature to maintain stability. For example, ZOLGENSMA® is an adeno-associated virus (AAV) vector-based gene therapy product that requires −60°C storage condition (64). Maintaining vaccines in a cold chain is challenging in both developed and developing countries (65,66). It is estimated that cold chain alone contributes to as high as 80% of all vaccination costs, and the lack of vaccine knowledge during transportation and storage often causes the exposure of vaccines to a temperature below +2°C (67). Vaccines in liquid form should not be frozen because the slow freezing creates tremendous stress to the colloids. During the freezing process, nucleation of water pushes the solutes or particles into small volumes between the water crystal, which causes irreversible aggregations (68).

Although formulation technology cannot address the cold chain capacity and equipment issues, the thermostability of vaccines can be improved by formulation technologies. As a starting point, the solution pH, ionic strength, redox potential impact both the chemical stability of the antigen, as well as the colloidal stability of the suspension. If changing these parameters alone cannot provide enough stabilization, additional stabilizers can be used. For example, arginine is a commonly used stabilizer for protein-containing formulations, preventing aggregation. The excipients can also stabilize the active ingredient during the manufacturing process. For example, sugars are often used in live-attenuated vaccines as the cryoprotectant to protect the viability during the lyophilization process (69). For vaccines using modifiable delivery vehicles such as mRNA in LNP vehicles, surface modification to the LNP can also stabilize the particles. For example, PEGylation is commonly applied to nanomedicines to prevent aggregation and enhance penetration through bio-

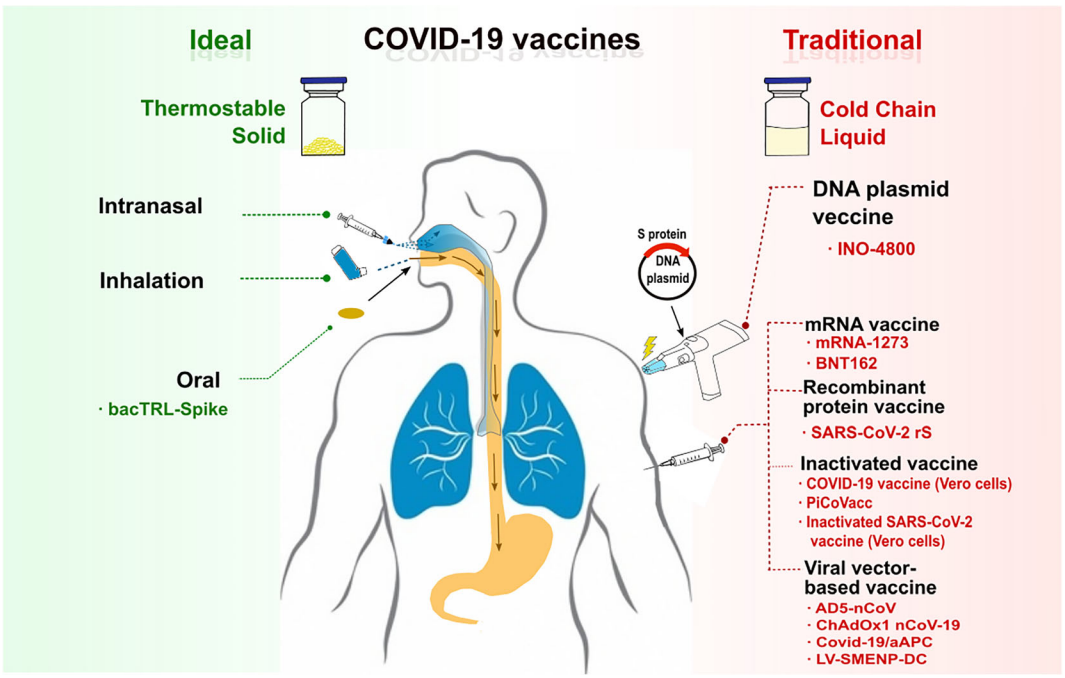

Fig. 2. A comparison of routes of administration between the ideal vaccines and the current COVID-19 vaccine candidates

logical barriers by providing water dispersibility and steric repulsion (70,71). Phenol, 2-phenoxyethanol, and thimerosal are used in some vaccines as preservatives (72). Note that the selection of the excipients still mostly relies on empirical knowledge with limited rationalization. For example, although arginine has been used for years as a stabilizer for protein products, the mechanism is not well understood (73–75). Systematic approaches such as Quality by Design (QbD) are often used to identify suitable excipients and their concentrations (76).

Lyophilized Vaccine Has a Better Thermostability

Lyophilized biological products have better stability compared to biological products in liquid form in general. The lyophilized powder can be reconstituted before injection or used directly for inhalation or intranasal vaccination if it has good aerosol performance properties. As mentioned previously, many vaccines, especially those containing aluminum salts, cannot tolerate slow freezing conditions. Previously, we reported that an ultra-rapid thin-film freezing (TFF) technology can be used to address this problem (77,78). In the TFF process, droplets of the vaccine suspension are dropped onto a frozen surface, and the small droplets are frozen in the order of milliseconds (~ 10^2 K/s). The frozen pallets are then dried using a standard lyophilizer. Compared to slow freezing (*e.g.*, 1 K/min) where large ice crystals are formed, smaller ice crystals are formed in rapid freezing conditions (Fig. 3). The direction of heat transfer, the thickness, and volume of the thin-film may also play a key role in reducing the sheer force generated during the freezing process (79). Using TFF technology, we prepared dry powder of alum adjuvanted vaccine without causing aggregation or loss of immunogenicity (78). The dry powder vaccine can be stored at ambient temperature while maintaining its immunogenicity (80). Importantly, the highly porous, brittle matrix nature of the thin-film freeze-dried powder, with good aerosol performance properties, enables needle-free intranasal administration of the vaccine powder (81,82). For live bacterial and viral-based vaccines that are unstable in liquid formulations, lyophilization is often used to preserve the viability, such as BCG and MMR vaccines (83). Thin-film freeze-drying can also be applied to prepare aerosolizable dry powder of viruses or bacteria (84).

For dry powder vaccine intended for hypodermic needle-based injection after reconstitution, main challenges include potential contamination in the reconstitution step, errors made during reconstitution, and the extra time needed to reconstitute the vaccine and fill it into a syringe, which may significantly slow down the rate of mass immunization in the case of vaccinating against COVID-19. In the project management perspective, perhaps the biggest challenge to the COVID-19 vaccine formulation is the time constrain. COVID-19 vaccine development demands quick product launch, leaving little time for optimizing formulation as the stability study is time-consuming. The understanding of the degradation pathway, aggregation process, and antigen-adjuvant interactions is critical for rational experimental design, reducing trial-and-error failures.

Route of Administration Plays an Important Role in the Vaccination Outcome

The vaccine dosage form design must take into consideration of the route of administration. The vaccine has the unique property that the route of administration can affect the extent and quality of immune responses that are independent of the administered dose (85,86). Since COVID-19 is primarily a respiratory disease, establishing a mucosal immune protection is critical, indicating that mucosal

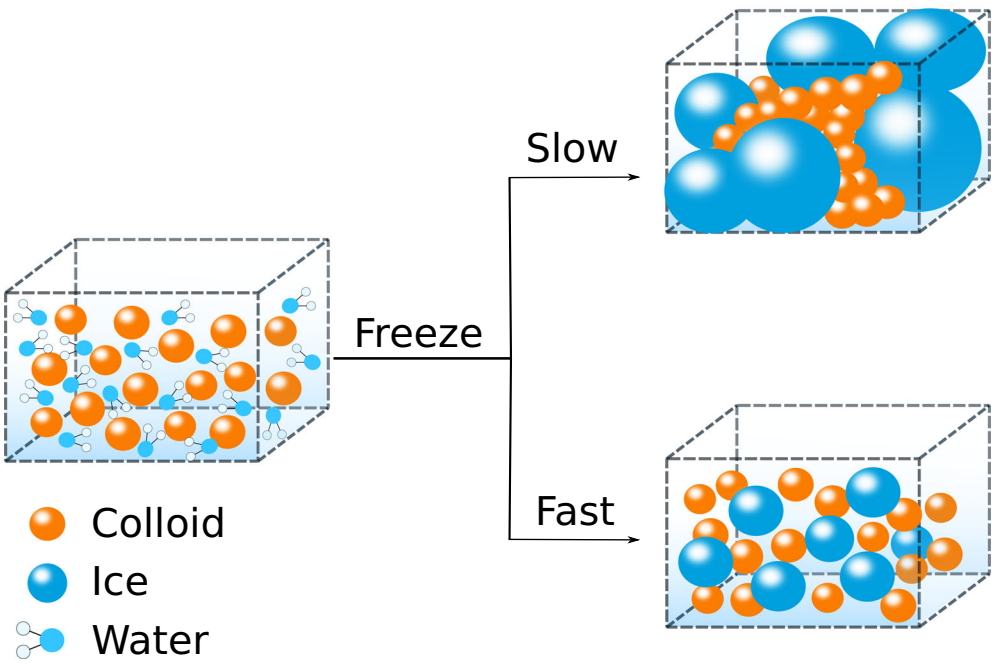

Fig. 3. A comparison between slow and fast freezing process

Table IV. Routes of Administration for Commercial Vaccines and COVID-19 Vaccine Candidates

	Route of administration	Examples of commercial vaccines (non-COVID-19)	Vaccine candidates under clinical investigation for COVID-19
Mucosal vaccinations	Intranasal	Flumist®, Fluenz®, NasoVac®	None
	Oral	Oral polio vaccine (OPV), RotaTeq® (RV5), Vaxchora®	bacTRL-Spike
Parenteral vaccinations	Surface electroporation	None	INO-4800
	Intramuscular injection	BioThrax®, ERVEBO®, HEPLISAV-B®, Menveo®, PNEUMOVAX® 23, Pentacel®, GARDASIL® 9, Fluzone®	mRNA-1273, BNT162, AD5-nCoV, PiCoVacc, Covid-19/aAPC, ChAdOx1 nCoV-19
	Intravenous infusion	None	LV-SMENP-DC
	Intradermal	BCG Vaccine, Intanza®, Fluzone®	BCG vaccine
	Subcutaneous injection	BioThrax®, DENGVAXIA®	LV-SMENP-DC

vaccination (*e.g.*, intranasal, pulmonary, oral) might be superior to parenteral vaccination. Some vaccines induce different immune responses when administered *via* different mucosal routes. Tomar *et al*. (2019) demonstrated that hepatitis B vaccine can induce high IgG titers when delivered to the deep lung but no titers when delivered intranasally, in contrast to the influenza vaccine where no difference in titers was observed between lung delivery *versus* nasal delivery (87). Previously, Du and coworkers developed an intranasal SAR-CoV vaccine candidate (29), indicating that an intranasal COVID-19 vaccine is feasible. However, most of the human vaccines approved by the United States Food and Drug Administration (FDA) are administered parentally, which induce systemic immunity only. Currently, there are no commercial vaccines that use the pulmonary delivery route, and therefore, intranasal and oral vaccinations are the most straight forward solution to satisfy the mucosal immunity need. There are three intranasal vaccines already on the market: Flumist®, Fluenz®, and NasoVac®. FluMist® is a live-attenuated trivalent/quadrivalent influenza virus vaccine (LAIV) that consists of the influenza A strains H1N1 and H3N2 and two influenza B strains, protecting against seasonal influenza infections. European Medicines Agency-approved Fluenz® is also an intranasal influenza vaccine consisting of four live-attenuated strains (H1N1, H3N2, and two influenza B virus) developed to prevent influenza infection in individuals older than 24 months until the age of 18. NasoVac®, another intranasal vaccine, was developed in India for the sudden threat of a pandemic during the year 2009. It contains a live-attenuated monovalent strain of pandemic A/California/7/2009 (H1N1) influenza. Noted that these existing intranasal influenza vaccines are all cold adapted, but heat sensitive, allowing the viral replication in the nostrils but not in the lungs. Furthermore, the vaccines also display a good safety profile (88). Nasal vaccines based on inactivated or subunit vaccines remain difficult to commercialize. For example, the subunit Nasalflu Berna® adjuvanted with an active heat-labile toxin (LT) from *Escherichia coli* had to be taken off the market shortly after its launch because it was linked to several cases of Bell's palsy, a transient facial paralysis (89). It was suggested that the adjuvant in the formulation caused the side effects (90,91). Finally, when dosed intranasally, a vaccine is intrinsically prone to inducing Th17 immune responses (92), which may not be ideal for the clearance of SARS-CoV-2 viral particles from the lung. Another limiting factor for a nasal or pulmonary COVID-19 vaccine is the need for a special delivery device, and the delivery device, while costly, may also exert pressure on the vaccine formulation. For example, loss of a virus titer was observed when using a nebulizer to deliver a live virus formulation (93). Therefore, it is not surprising that almost all COVID-19 vaccine candidates made to clinical trials are given by injection (see Table IV), although they may not induce specific mucosal immunity.

CONCLUSION

The COVID-19 pandemic now exerts tremendous pressure to scientists to develop safe and effective vaccines. In this race to develop a vaccine, its formulation serves as a bridge between stages of product development and must be considered throughout the development process. In the early stage, selecting the appropriate antigen, adjuvant, and the delivery vehicle is the most critical task in the formulation, which requires an understanding of the working mechanisms and biophysical characteristics of the vaccine antigen. Then, the formulation emphasis moves to formulate the vaccine in a specific dosage form that is stable and feasible for large-scale production. Aspects of product distribution and route of administration should be considered at this stage as well. Needle-free mucosal vaccination by the intranasal or pulmonary route offers advantages for COVID-19 protection as well as for mass immunization. Formulating vaccines into dry powder can help improve the thermostability of the vaccines and ultimately reduce the costs of vaccination. Technologies are available to transform vaccines from liquid to thermally stable solid powder for needle-free intranasal or pulmonary vaccination. Even if they are not adopted in the current race for safe and effective COVID-19 vaccines, it is not too early for governmental agencies and not-for-profit organizations around the world to support the commercialization of these technologies to prepare for the need of rapid mass vaccination for the whole world in the next pandemic.

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REFERENCES

1. Wu F, Zhao S, Yu B, Chen Y, Wang W, Song Z, et al. A new coronavirus associated with human respiratory disease in China. Nature [Internet]. 2020;579(7798):265–9 Available from: http://www.nature.com/articles/s41586-020-2008-3.
2. Lan J, Ge J, Yu J, Shan S, Zhou H, Fan S, et al. Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2 receptor. Nature [Internet]. 2020;581(7807):215–20. Available from:. https://doi.org/10.1038/s41586-020-2180-5.
3. Beigel JH, Tomashek KM, Dodd LE, Mehta AK, Zingman BS, Kalil AC, et al. Remdesivir for the treatment of Covid-19—preliminary report. N Engl J Med [Internet]. 2020;22:NEJMoa2007764. Available from:. https://doi.org/10.1056/NEJMoa2007764.
4. Wang Y, Zhang D, Du G, Du R, Zhao J, Jin Y, et al. Remdesivir in adults with severe COVID-19: a randomised, double-blind, placebo-controlled, multicentre trial. Lancet [Internet]. 2020; Available from: https://linkinghub.elsevier.com/retrieve/pii/S0140673620310229.
5. Hung IF-N, Lung K-C, Tso EY-K, Liu R, Chung TW-H, Chu M-Y, et al. Triple combination of interferon beta-1b, lopinavir–ritonavir, and ribavirin in the treatment of patients admitted to hospital with COVID-19: an open-label, randomised, phase 2 trial. Lancet [Internet]. 2020;395(10238):1695–704 Available from: https://linkinghub.elsevier.com/retrieve/pii/S0140673620310424.
6. Murphy K, Casey W. Janeway's immunobiology. 9th ed. New York: Garland Science; 2017. p. 729–41.
7. Zepp F. Principles of vaccine design—lessons from nature. Vaccine [Internet]. 2010;28:C14–24 Available from: http://linkinghub.elsevier.com/retrieve/pii/S0264410X10010030.
0. Hanley KA. The double-edged sword: how evolution can make or break a live-attenuated virus vaccine. Evol Educ Outreach [Internet]. 2011;4(4):635–43. https://doi.org/10.1007/s12052-011-0365-y (Available from:).
9. Offit PA. The cutter incident, 50 years later. N Engl J Med [Internet]. 2005;352(14):1411–2. Available from:. https://doi.org/10.1056/NEJMp048180.
10. Vartak A, Sucheck S. Recent advances in subunit vaccine carriers. Vaccines [Internet]. 2016;4(2):12 Available from: http://www.mdpi.com/2076-393X/4/2/12.
0. Li F, Li W, Farzan M, Harrison SC. Structural biology: structure of SARS coronavirus spike receptor-binding domain complexed with receptor. Science. 2005;309(5742):1864–8.
12. McCray PB, Pewe L, Wohlford-Lenane C, Hickey M, Manzel L, Shi L, et al. Lethal infection of K18-hACE2 mice infected with severe acute respiratory syndrome coronavirus. J Virol [Internet]. 2007;81(2):813–21 Available from: https://jvi.asm.org/content/81/2/813.
13. Gretebeck LM, Subbarao K. Animal models for SARS and MERS coronaviruses. Curr Opin Virol [Internet]. 2015;13:123–9 Available from: https://linkinghub.elsevier.com/retrieve/pii/S187962571500098X.
14. Marzi A, Gramberg T, Simmons G, Möller P, Rennekamp AJ, Krumbiegel M, et al. DC-SIGN and DC-SIGNR interact with the glycoprotein of Marburg virus and the S protein of severe acute respiratory syndrome coronavirus. J Virol [Internet]. 2004;78(21):12090–5 Available from: https://jvi.asm.org/content/78/21/12090.
15. Weingartl H, Czub M, Czub S, Neufeld J, Marszal P, Gren J, et al. Immunization with modified Vaccinia virus Ankara-based recombinant vaccine against severe acute respiratory syndrome is associated with enhanced hepatitis in ferrets. J Virol [Internet]. 2004;78(22):12672–6 Available from: https://jvi.asm.org/content/78/22/12672.
16. Holmes EC, Zhang Y-Z. Novel 2019 coronavirus genome [Internet]. virological.org. 2020 [cited 2020 May 26]. Available from: http://virological.org/t/novel-2019-coronavirus-genome/319
17. Garden R. Remarks by President Trump on vaccine development [Internet]. 2020 [cited 2020 May 25]. Available from: https://www.whitehouse.gov/briefings-statements/remarks-president-trump-vaccine-development/
18. Lan J, Ge J, Yu J, Shan S, Zhou H, Fan S, et al. Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2 receptor. bioRxiv. 2020.
19. Lapelosa M, Gallicchio E, Arnold GF, Arnold E, Levy RM. In silico vaccine design based on molecular simulations of rhinovirus chimeras presenting HIV-1 gp41 epitopes. J Mol Biol [Internet]. 2009;385(2):675–91 Available from: https://linkinghub.elsevier.com/retrieve/pii/S0022283608014022.
20. Shamriz S, Ofoghi H. Design, structure prediction and molecular dynamics simulation of a fusion construct containing malaria pre-erythrocytic vaccine candidate, PfCelTOS, and human interleukin 2 as adjuvant. BMC Bioinformatics [Internet]. 2016;17(1):–71 Available from: http://www.biomedcentral.com/1471-2105/17/71.
21. Chaudhury S, Duncan EH, Atre T, Storme CK, Beck K, Kaba SA, et al. Identification of immune signatures of novel adjuvant formulations using machine learning. Sci Rep [Internet]. 2018;8(1):17508 Available from: http://www.nature.com/articles/s41598-018-35452-x.
22. Chaudhury S, Duncan EH, Atre T, Dutta S, Spring MD, Leitner WW, et al. Combining immunoprofiling with machine learning to assess the effects of adjuvant formulation on human vaccine-induced immunity. Hum Vaccin Immunother [Internet]. 2020;16(2):400–11. Available from:. https://doi.org/10.1080/21645515.2019.1654807.
23. Ong E, Wong MU, Huffman A, He Y. COVID-19 coronavirus vaccine design using reverse vaccinology and machine learning. bioRxiv [Internet]. 2020 ;2020.03.20.000141. Available from: http://biorxiv.org/content/early/2020/03/21/2020.03.20.000141.abstract
24. He Y, Zhou Y, Liu S, Kou Z, Li W, Farzan M, et al. Receptor-binding domain of SARS-CoV spike protein induces highly potent neutralizing antibodies: implication for developing subunit vaccine. Biochem Biophys Res Commun [Internet]. 2004;324(2):773–81 Available from: https://linkinghub.elsevier.com/retrieve/pii/S0006291X04021588.
25. Du L, Zhao G, He Y, Guo Y, Zheng B-J, Jiang S, et al. Receptor-binding domain of SARS-CoV spike protein induces long-term protective immunity in an animal model. Vaccine [Internet]. 2007;25(15):2832–8 Available from: https://linkinghub.elsevier.com/retrieve/pii/S0264410X06011534.
26. Fang M, Cheng H, Dai Z, Bu Z, Sigal LJ. Immunization with a single extracellular enveloped virus protein produced in bacteria provides partial protection from a lethal orthopoxvirus infection in a natural host. Virology [Internet]. 2006;345(1):231–43 Available from: https://linkinghub.elsevier.com/retrieve/pii/S0042682205006306.
27. Galmiche MC, Goenaga J, Wittek R, Rindisbacher L. Neutralizing and protective antibodies directed against Vaccinia virus envelope antigens. Virology [Internet]. 1999;254(1):71–80 Available from: https://linkinghub.elsevier.com/retrieve/pii/S0042682298995162.
28. Novavax. Novavax to present COVID-19 vaccine candidate progress in World Vaccine Congress Webinar Series [Internet]. 2020 [cited 2020 May 25]. Available from**:** http://ir.novavax.com/news-releases/news-release-details/novavax-present-covid-19-vaccine-candidate-progress-world
29. Du L, Zhao G, Lin Y, Sui H, Chan C, Ma S, et al. Intranasal vaccination of recombinant adeno-associated virus encoding receptor-binding domain of severe acute respiratory syndrome coronavirus (SARS-CoV) spike protein induces strong mucosal immune responses and provides long-term protection against SARS. J Immunol [Internet]. 2008;180(2):948–56. Available from:. https://doi.org/10.4049/jimmunol.180.2.948.
30. Detmer A, Glenting J. Live bacterial vaccines—a review and identification of potential hazards. Microb Cell Fact. 2006;5(iii):1–12.

31. Montel Mendoza G, Pasteris SE, Otero MC, Fatima Nader-Macías ME. Survival and beneficial properties of lactic acid bacteria from raniculture subjected to freeze-drying and storage. J Appl Microbiol. 2014;116(1):157–66.
32. INOVIO Expands manufacturing of COVID-19 DNA vaccine INO-4800 With New Funding from CEPI [Internet]. [cited 2020 May 18]. Available from: http://ir.inovio.com/news-releases/news-releases-details/2020/INOVIO-Expands-Manufacturing-of-COVID-19-DNA-Vaccine-INO-4800-With-New-Funding-from-CEPI/default.aspx
33. Thi EP, Mire CE, Lee ACH, Geisbert JB, Zhou JZ, Agans KN, et al. Lipid nanoparticle siRNA treatment of Ebola-virus-Makona-infected nonhuman primates. Nature [Internet]. 2015;521(7552):362–5 Available from: http://www.nature.com/articles/nature14442.
34. Schlake T, Thess A, Fotin-Mleczek M, Kallen KJ. Developing mRNA-vaccine technologies. RNA Biol. 2012;9(11):1319–30.
35. Kowalski PS, Rudra A, Miao L, Anderson DG. Delivering the messenger: advances in technologies for therapeutic mRNA delivery. Mol Ther [Internet]. 2019;27(4):710–28. Available from:. https://doi.org/10.1016/j.ymthe.2019.02.012.
36. Safety and immunogenicity study of 2019-nCoV vaccine (mRNA-1273) for prophylaxis of SARS-CoV-2 Infection (COVID-19) [Internet]. 2020 [cited 2020 May 20]. Available from: https://clinicaltrials.gov/ct2/show/NCT04283461
37. Sánchez-Ramón S, Conejero L, Netea MG, Sancho D, Palomares Ó, Subiza JL. Trained immunity-based vaccines: a new paradigm for the development of broad-Spectrum anti-infectious formulations. Front Immunol. 2018;9(December):2936.
38. Quintin J, Saeed S, Martens JHA, Giamarellos-Bourboulis EJ, Ifrim DC, Logie C, et al. Candida albicans infection affords protection against reinfection via functional reprogramming of monocytes. Cell Host Microbe [Internet]. 2012;12(2):223–32 Available from: https://linkinghub.elsevier.com/retrieve/pii/S1931312812002326.
39. Texas A&M University. BCG vaccine for health care workers as defense against COVID 19 (BADAS) [Internet]. 2020 [cited 2020 May 20]. Available from: https://clinicaltrials.gov/ct2/show/NCT04348370
40. Angelidou A, Conti MG, Diray-Arce J, Benn CS, Shann F, Netea MG, et al. Licensed Bacille Calmette-Guérin (BCG) formulations differ markedly in bacterial viability, RNA content and innate immune activation. Vaccine [Internet]. 2020;38(9):2229–40. Available from:. https://doi.org/10.1016/j.vaccine.2019.11.060.
41. Gao Q, Bao L, Mao H, Wang L, Xu K, Yang M, et al. Development of an inactivated vaccine candidate for SARS-CoV-2. Science (80-) [Internet]. 2020;6:eabc1932. Available from:. https://doi.org/10.1126/science.abc1932.
42. Editor CBF, Walker JM. Vaccine adjuvants [Internet]. In: Fox CB, editor. Methods in Molecular Biology, vol. 1494). Available from:. New York: Springer New York; 2017. https://doi.org/10.1007/978-1-4939-6445-1.
43. Kool M, Soullié T, van Nimwegen M, Willart MAM, Muskens F, Jung S, et al. Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. J Exp Med [Internet]. 2008;205(4):869–82. Available from:. https://doi.org/10.1084/jem.20071087.
44. Kool M, Petrilli V, De Smedt T, Rolaz A, Hammad H, van Nimwegen M, et al. Cutting edge: alum adjuvant stimulates inflammatory dendritic cells through activation of the NALP3 inflammasome. J Immunol [Internet]. 2008;181(6):3755–9. Available from:. https://doi.org/10.4049/jimmunol.181.6.3755.
45. Eisenbarth SC, Colegio OR, O'Connor W, Sutterwala FS, Flavell RA. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. Nature. 2008;453(7198):1122–6.
46. Mannhalter JW, Neychev HO, Zlabinger GJ, Ahmad R, Eibl MM. Modulation of the human immune response by the non-toxic and non-pyrogenic adjuvant aluminium hydroxide: effect on antigen uptake and antigen presentation. Clin Exp Immunol. 1985.
47. Ulanova M, Tarkowski A, Hahn-Zoric M, Hanson LÅ. The common vaccine adjuvant aluminum hydroxide up-regulates accessory properties of human monocytes via an interleukin-4-dependent mechanism. Infect Immun. 2001;69(2):1151–9.
48. Khurana S, Coyle EM, Manischewitz J, King LR, Gao J, Germain RN, *et al.* AS03-adjuvanted H5N1 vaccine promotes antibody diversity and affinity maturation, NAI titers, cross-clade H5N1 neutralization, but not H1N1 cross-subtype neutralization. NPJ vaccines [Internet]. 2018;3(March):40. Available from: https://www.ncbi.nlm.nih.gov/pubmed/30302282%0A http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC6167326.
49. Didierlaurent AM, Morel S, Lockman L, Giannini SL, Bisteau M, Carlsen H, et al. AS04, an aluminum salt- and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity. J Immunol [Internet]. 2009;183(10):6186–97. Available from:. https://doi.org/10.4049/jimmunol.0901474.
50. Schell B, Braedel S, Probst J, Carralot JP, Wagner H, Schild H, et al. Immunostimulating capacities of stabilized RNA molecules. Eur J Immunol. 2004;34(2):537–47.
51. Edwards DK, Jasny E, Yoon H, Horscroft N, Schanen B, Geter T, et al. Adjuvant effects of a sequence-engineered mRNA vaccine: translational profiling demonstrates similar human and murine innate response. J Transl Med. 2017;15(1):1–18.
52. Dudek T, Knipe DM. Replication-defective viruses as vaccines and vaccine vectors. Virology [Internet]. 2006;344(1):230–9 Available from: https://linkinghub.elsevier.com/retrieve/pii/S0042682205005891.
53. Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, et al. A third-generation lentivirus vector with a conditional packaging system. J Virol. 1998.
54. Faust N. Addressing the challenges of commercial-scale viral vector production. Cell Gene Ther Insights. 2018;4(2):31–6.
55. Clément N, Grieger JC. Manufacturing of recombinant adeno-associated viral vectors for clinical trials. Mol Ther Methods Clin Dev. 2016;3:16002.
56. Vellinga J, Smith JP, Lipiec A, Majhen D, Lemckert A, van Ooij M, et al. Challenges in manufacturing adenoviral vectors for global vaccine product deployment. Hum Gene Ther. 2014;25(4):318–27.
57. Moleirinho MG, Silva RJS, Alves PM, Carrondo MJT, Peixoto C. Current challenges in biotherapeutic particles manufacturing. Expert Opin Biol Ther. 2020;20(5):451–65.
58. Terova O, Soltys S, Hermans P, De Rooij J, Detmers F. Overcoming downstream purification challenges for viral vector manufacturing: enabling advancement of gene therapies in the clinic. Cell Gene Ther Insights. 2018;4(2):101–11.
59. Hernandez Bort JA. Challenges in the downstream process of gene therapy products. Am Pharm Rev. 2019;22(4).
60. Merten O-W, Schweizer M, Chahal P, Kamen A. Manufacturing of viral vectors: part II. Downstream processing and safety aspects. Pharm Bioprocess. 2014.
61. Bandeira V, Peixoto C, Rodrigues AF, Cruz PE, Alves PM, Coroadinha AS, et al. Downstream processing of lentiviral vectors: releasing bottlenecks. Hum Gene Ther Methods. 2012;23:255–63.
62. Gramer MJ. Product quality considerations for mammalian cell culture process development and manufacturing. Adv Biochem Eng Biotechnol. 2014.
63. Temperature sensitivity of vaccines [Internet]. World Health Organization; 2006. Available from: https://apps.who.int/iris/handle/10665/69387
64. ZOLGENSMA. Package insert. Bannockburn: AveXis, Inc.; 2019.
65. Murhekar MV, Dutta S, Kapoor AN, Bitragunta S, Dodum R, Ghosh P, et al. Frequent exposure to suboptimal temperatures in vaccine cold-chain system in India: results of temperature monitoring in 10 states. Bull World Health Organ [Internet]. 2013;91(12):906–13 Available from: http://www.who.int/entity/bulletin/volumes/91/12/13-119974.pdf.
66. Hanson CM, George AM, Sawadogo A, Schreiber B. Is freezing in the vaccine cold chain an ongoing issue? A literature review. Vaccine [Internet]. 2017;35(17):2127–33 Available from: https://linkinghub.elsevier.com/retrieve/pii/S0264410X16309471.
67. Matthias DM, Robertson J, Garrison MM, Newland S, Nelson C. Freezing temperatures in the vaccine cold chain: a systematic literature review. Vaccine [Internet]. 2007;25(20):3980–6

Available from: https://linkinghub.elsevier.com/retrieve/pii/S0264410X07002289.
68. Niu L, Panyam J. Freeze concentration-induced PLGA and polystyrene nanoparticle aggregation: Imaging and rational design of lyoprotection. J Control Release [Internet]. 2017;248:125–132. Available from: https://doi.org/10.1016/j.jconrel.2017.01.019
69. Pastorino B, Baronti C, Gould EA, Charrel RN, de Lamballerie X. Effect of Chemical Stabilizers on the Thermostability and Infectivity of a Representative Panel of Freeze Dried Viruses. Digard P, editor. PLoS One [Internet]. 2015;10(4):e0118963. Available from:. https://doi.org/10.1371/journal.pone.0118963.
70. Zhang Y, Zhang J. Surface modification of monodisperse magnetite nanoparticles for improved intracellular uptake to breast cancer cells. J Colloid Interface Sci [Internet]. 2005;283(2):352–7 Available from: https://linkinghub.elsevier.com/retrieve/pii/S0021979704009592.
71. Acar HY c, Garaas RS, Syud F, Bonitatebus P, Kulkarni AM. Superparamagnetic nanoparticles stabilized by polymerized PEGylated coatings. J Magn Magn Mater [Internet]. 2005;293(1):1–7. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0304885305001009.
72. Offit PA, Jew RK. Addressing parents' concerns: do vaccines contain harmful preservatives, adjuvants, additives, or residuals? Pediatrics [Internet]. 2003;112(6):1394–7. Available from:. https://doi.org/10.1542/peds.112.6.1394.
73. Tischer A, Lilie H, Rudolph R, Lange C. L-arginine hydrochloride increases the solubility of folded and unfolded recombinant plasminogen activator rPA. Protein Sci [Internet]. 2010;19(9):1783–95. Available from:. https://doi.org/10.1002/pro.465.
74. Baynes BM, Wang DIC, Trout BL. Role of arginine in the stabilization of proteins against aggregation. Biochem Int. 2005;44(12):4919–25. Available from:. https://doi.org/10.1021/bi047528r.
75. Das U, Hariprasad G, Ethayathulla AS, Manral P, Das TK, Pasha S, et al. Inhibition of protein aggregation: supramolecular assemblies of arginine hold the key. Rutherford S, editor. PLoS One [Internet]. 2007;2(11):e1176. Available from:. https://doi.org/10.1371/journal.pone.0001176.
76. Morefield GL. A rational, systematic approach for the development of vaccine formulations. AAPS J. 2011;13(2):191–200.
77. Engstrom JD, Lai ES, Ludher BS, Chen B, Milner TE, Williams RO, et al. Formation of stable submicron protein particles by thin film freezing. Pharm Res. 2008;25(6):1334–46.
78. Li X, Thakkar SG, Ruwona TB, Williams RO, Cui Z. A method of lyophilizing vaccines containing aluminum salts into a dry powder without causing particle aggregation or decreasing the immunogenicity following reconstitution. J Control Release [Internet]. 2015;204:38–50. Available from:. https://doi.org/10.1016/j.jconrel.2015.02.035.
79. Aso Y, Yoshioka S. Effect of freezing rate on physical stability of lyophilized cationic liposomes. Chem Pharm Bull (Tokyo) [Internet]. 2005;53(3):301–4 Available from: http://joi.jlc.jst.go.jp/JST.JSTAGE/cpb/53.301?from=CrossRef.
80. Thakkar SG, Ruwona TB, Williams RO, Cui Z. The immunogenicity of thin-film freeze-dried, aluminum salt-adjuvanted vaccine when exposed to different temperatures. Hum Vaccines Immunother [Internet]. 2017;13(4):936–46. Available from:. https://doi.org/10.1080/21645515.2016.1259042.
81. Xu H, Ruwona TB, Thakkar SG, Chen Y, Zeng M, Cui Z. Nasal aluminum (oxy)hydroxide enables adsorbed antigens to induce specific systemic and mucosal immune responses. Hum Vaccines Immunother. 2017;13(11):2688–94.
82. Thakkar SG, Warnken ZN, Alzhrani RF, Valdes SA, Aldayel AM, Xu H, et al. Intranasal immunization with aluminum salt-adjuvanted dry powder vaccine. J Control Release [Internet]. 2018;292:111–8. Available from:. https://doi.org/10.1016/j.jconrel.2018.10.020.
83. BCG vaccine [Package insert] [Internet]. Roseland, NJ: Merck & Co; 2019. Available from: https://nctr-crs.fda.gov/fdalabel/services/spl/set-ids/a83f0b99-9038-4c5a-aaac-8792b32838fe/spl-doc?hl=BCG
84. Smyth H, Zhang H, Cui Z, Wang J, Xu H, Zhang Y, *et al.* Biologically active dry powder compositions and method of their manufacture and use,. U.S. Patent Application No. 63/012,792, 2020.
85. Belyakov IM, Ahlers JD. What role does the route of immunization play in the generation of protective immunity against mucosal pathogens? J Immunol. 2009;183(11):6883–92.
86. Mohanan D, Slütter B, Henriksen-Lacey M, Jiskoot W, Bouwstra JA, Perrie Y, et al. Administration routes affect the quality of immune responses: a cross-sectional evaluation of particulate antigen-delivery systems. J Control Release [Internet]. 2010;147(3):342–9. Available from:. https://doi.org/10.1016/j.jconrel.2010.08.012.
87. Tomar J, Tonnis WF, Patil HP, de boer AH, Hagedoorn P, Vanbever R, et al. Pulmonary immunization: deposition site is of minor relevance for influenza vaccination but deep lung deposition is crucial for hepatitis B vaccination. Acta Pharm Sin B [Internet]. 2019;9(6):1231–40. Available from:. https://doi.org/10.1016/j.apsb.2019.05.003.
88. Kulkarni PS, Raut SK, Dhere RM. A post-marketing surveillance study of a human live-virus pandemic influenza A (H1N1) vaccine (Nasovac ®) in India. Hum Vaccin Immunother [Internet]. 2013;9(1):122–4. Available from:. https://doi.org/10.4161/hv.22317.
89. Mutsch M, Zhou W, Rhodes P, Bopp M, Chen RT, Linder T, et al. Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. N Engl J Med [Internet]. 2004;350(9):896–903. Available from:. https://doi.org/10.1056/NEJMoa030595.
90. Miller E, Andrews N, Stellitano L, Stowe J, Winstone AM, Shneerson J, et al. Risk of narcolepsy in children and young people receiving AS03 adjuvanted pandemic A/H1N1 2009 influenza vaccine: retrospective analysis. BMJ [Internet]. 2013;346:f794. Available from:. https://doi.org/10.1136/bmj.f794.
91. Connell TD. Cholera toxin, LT-I, LT-IIa and LT-IIb: the critical role of ganglioside binding in immunomodulation by type I and type II heat-labile enterotoxins. Expert Rev Vaccines [Internet]. 2007;6(5):821–34. Available from:. https://doi.org/10.1586/14760584.6.5.821.
92. Linehan JL, Dileepan T, Kashem SW, Kaplan DH, Cleary P, Jenkins MK. Generation of Th17 cells in response to intranasal infection requires TGF-β1 from dendritic cells and IL-6 from CD301b + dendritic cells. Proc Natl Acad Sci [Internet]. 2015;112(41):12782–7. Available from:. https://doi.org/10.1073/pnas.1513532112.
93. Astudillo A, Leung SSY, Kutter E, Morales S, Chan H-K. Nebulization effects on structural stability of bacteriophage PEV 44. Eur J Pharm Biopharm [Internet]. 2018;125(December 2017):124–30 Available from: https://linkinghub.elsevier.com/retrieve/pii/S093964111731007X.