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Recombinant Protein Vaccines, a Proven Approach Against Coronavirus Pandemics

Jeroen Pollet, Wen-Hsiang Chen, Ulrich Strych

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Author names and affiliations:

Jeroen Pollet[1,2 †*], Wen-Hsiang Chen[1,2†], Ulrich Strych [1,2†]

† All authors contributed equally

*Corresponding author: Jeroen Pollet, Jeroen.pollet@bcm.edu

[1] Department of Pediatrics, National School of Tropical Medicine, Department of Pediatrics, Baylor College of Medicine, Houston, TX

[2] Texas Children's Hospital Center for Vaccine Development, Baylor College of Medicine, 1102 Bates Street, Houston, TX

Abstract:

With the COVID-19 pandemic now ongoing for close to a year, people all over the world are still waiting for a vaccine to become available. The initial focus of accelerated global research and development efforts to bring a vaccine to market as soon as possible was on novel platform technologies that promised speed but had limited history in the clinic. In contrast, recombinant protein vaccines, with numerous examples in the clinic for many years, missed out on the early wave of investments from government and industry. Emerging data are now surfacing suggesting that recombinant protein vaccines indeed might offer an advantage or complement to the nucleic acid or viral vector vaccines that will likely reach the clinic faster. Here, we summarize the current public information on the nature and on the development status of recombinant subunit antigens and adjuvants targeting SARS-CoV-2 infections.

Keywords:

SARS-CoV-2, COVID-19, Adjuvants, Vaccine Production, Vaccine Delivery, Clinical Trials, Neutralizing Antibodies, Th1

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1. Introduction

More than 10 months into the COVID-19 pandemic, despite unprecedented worldwide efforts the race to develop a vaccine has still not reached the finish line. While Russia was first to approve its Sputnik V vaccine to control SARS-CoV-2 transmission [1], the rest of the world is still awaiting a vaccine that has undergone the traditional testing, review, and approval process [2, 3]. However, with the backing of programs such as the U.S. government's Operation Warp Speed [4] or the WHO's COVAX initiative [5], it is expected that other vaccines will soon reach licensure. With this possibly never-before-seen acceleration of research efforts, some of the frontrunner platform technologies in this vaccine race have not yet been seen in the clinic, such as DNA or mRNA-based vaccines. As of October 2, 2020, the WHO lists 42 candidate vaccines in clinical evaluation [6], among them 12 based on recombinant protein technology (**Table 1**). Arguably, more traditionally produced vaccines such as those based on recombinantly produced subunit proteins are lagging, but this may not necessarily be a reflection of their validity or promise, but rather a reflection of the way the initial funding was directed. Here we will provide a review of the current status of recombinant protein vaccines for COVID-19.

Table 1 Recombinant protein vaccine candidates in clinical trials for COVID-19 as of October 2, 2020 [6]

Antigen	Vaccine developer	Platform/ Technology	Adjuvants	Most advanced clinical stage	References
Full-length S-protein based vaccines					
Trimer	Novavax	Insect cells	Matrix M	Phase 3	[7] [8]
S-protein	Sanofi Pasteur/GSK	Insect cells	2 different adjuvants (likely variants of AS03)	Phase 1-2	[9] [10]
SCB-2019 trimer	Clover Biopharmaceuticals Inc./GSK/Dynavax	CHO cells	Alum +CpG 1018 or AS03	Phase 1	[11] [12]
S-2P (MVC-COV1901)	Medigen Vaccine Biologics Corporation/ NIAID/Dynavax	CHO cells	Alum+ CpG1018	Phase 1	[13] [14]

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Sclamp	University of Queensland/CSL/ Seqirus	CHO cells	MF59	Phase 1	[15] [16]
Covax-19	Vaxine Pty Ltd/ Medytox	Insect cells	AdvaxCpG55.2	Phase 1	[17] [18]
RBD-based vaccines					
AdimrSC-2f	Adimmune	Baculovirus/Sf9	Alum	Phase 1	[19]
FINLAY-FR-1/2	Instituto Finlay de Vacunas, Cuba			Phase 1	[20] [21]
KBP-201	Kentucky Bioprocessing, Inc	Plants		Phase 1-2	[22]
RBD Dimer	Anhui Zhifei Longcom Biopharmaceutical/ Institute of Microbiology, Chinese Academy of Sciences	CHO Cells	Aluminum preparation	Phase 2	[23]
RBD	West China Hospital, Sichuan University P	Insect Cells	Alum	Phase 1	[24] [25]
Multi-epitope vaccines					
Multitope Peptide-based Vaccine (MPV)	COVAXX	Peptides	CpG and alum (AdjuPhos®)	Phase 1	[26, 27]
EpiVacCoron	Vektor Laboratories, Russia	Chemical synthesis	Alum	Phase 1	[28]
CoVac-1	University Hospital Tübingen	Peptides	Montanide ISA51	Phase 1	[29] [30]

2. The spike protein as a vaccine antigen candidate

The ~29.8 kb SARS-CoV-2 genome contains 14 open-reading frames encoding 27 proteins, including the four major structural proteins, E, envelope protein, M, matrix protein, N, nucleocapsid protein, and S, the spike protein [31]. Among these, the immunodominant trimeric S protein is the primary source of all major vaccine antigen targets to date. Other proteins have received considerably less attention as vaccine antigen candidates for various reasons. For instance, while the abundant SARS-CoV-2 N-protein is used in virus diagnostics [32-34], it is not

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included in most COVID-19 vaccine candidates because its SARS-CoV homolog was shown to increase the number of eosinophils within inflammatory infiltrates upon vaccination and subsequent challenge [35]. The S-protein is made up of two subunits, S1 and S2 that fulfill multiple functions related to the initial binding of the virus to its angiotensin-converting enzyme 2 (ACE-2) cell surface receptor and the subsequent endosome mediated entry of the virus into the host cell [36]. In the S-protein trimer, three S1 subunits sit on top of a stem of three S2 subunits. Within S1, a distinct receptor-binding domain (RBD, residues 331-524) and within it, a distinct receptor-binding motif (RBM), is responsible for the initial docking to ACE-2 [37]. Despite each S1 domain having its own functional RBD domain, it appears though that only one at a time is active, folded into the exposed confirmation, while the other two are hidden from the immune system within the trimer [38]. Moreover, there does not appear to be any cooperativity between the three RBDs within the S1 trimer when it comes to ACE-2 binding. Upon RBD/ACE-2 binding and catalyzed by a host protease, transmembrane protease serine 2 (TMPRSS2), S is then cleaved, allowing the S2-fusion peptide to facilitate cell entry. While this process in general is similar to what is observed in SARS-CoV, SARS-CoV-2 is distinguished by the presence of a unique furin cleavage site proximal to the S1/S2 junction that might facilitate cell entry and thus may be responsible for the increased virulence of SARS-CoV-2 over SARS-CoV [39].

SARS-CoV-2 shares extensive sequence homology, as well as structural and functional homologies with prior coronaviruses, namely SARS-CoV, but also MERS-CoV, the causative agent of Middle East Respiratory Syndrome. Early on in the pandemic, it was shown that anti-SARS S-protein antibodies were also capable of inhibiting the binding of SARS-CoV-2 to ACE-2. These observations concentrated vaccine development on antigens derived from the spike protein [40]. While some groups focus on the whole S1 subunit as their primary vaccine antigen candidate, others are using the RBD as their main vaccine antigen. A reason for the focus on the RBD lies in observations with the homologous SARS-CoV S-protein vaccine in mice, made by Drs. Jiang and Tseng [41], who observed lung pathology in mice with the full-length S-protein as their vaccine antigen, but not with the RBD. As a possible underlying cause for this observation, antibody-dependent enhancement (ADE) is considered as a possible contributing factor. In ADE, antibodies present in vaccinated individuals facilitate the entry of virus particles into the host cell through an additional mechanism using the Fc receptor II (Figure 1). In particular, non-neutralizing

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antibodies that do not interfere with the binding of the RBD to ACE-2 might thus increase the risk of ADE. Thus, reducing the size of the antigen to limit exposure to non-neutralizing epitopes might reduce the risk of undesired immunopathology. Notably, though, the majority of ADE data almost exclusively stems from experiments in mice and has not been unequivocally reproduced in, for example, Rhesus models for either SARS-CoV or SARS-CoV-2.

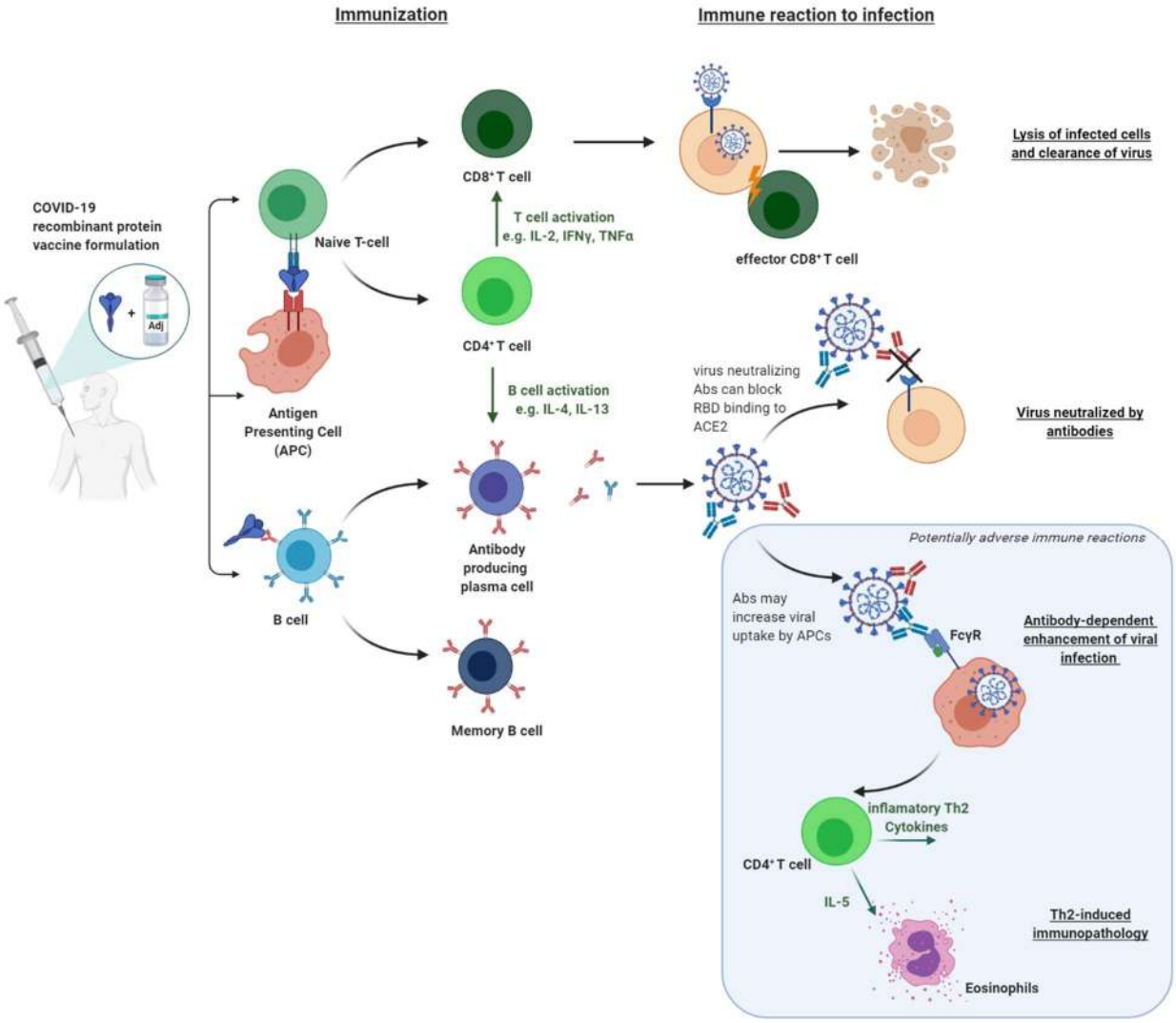

Figure 1: Overview of immune reactions triggered by recombinant protein vaccines and their role in protecting against COVID-19

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2.1. Full-length S-protein based vaccines

The COVID-19 vaccines currently in the clinic, including the recombinant protein vaccines, use various versions of the S-protein as their vaccine antigen component. The NVX-CoV2372 trimeric nanoparticle produced by Novavax is made from the full-length S-protein (GenBank accession number, MN908947; nucleotides 21563–25384). One mutation, 682-QQAQ-685, was introduced at the S1/S2 junction to increase protease resistance, and two other mutations, K986P and V987P, were added to increase the stability of the recombinantly produced vaccine antigen [7]. The antigen for the Sclamp vaccine (M1GSG), developed at the University of Queensland, was selected after systematically screening several hundred antigen candidates for feasibility of expression and recognition by the S-specific CR3022 monoclonal antibody [42]. It comprises nearly the full-length S-protein, including the native signal peptide, with the replacement of residues 680-690 (S1/S2 junction) with a GSG linker and the truncation of aa residue 1204. This construct is expressed as a fusion to a molecular clamp stabilization domain that is intended to generally preserve enveloped virus vaccine antigens in their pre-fusion stage thus improving exposure to the immune system [43]. In the case of SARS-CoV-2 S-protein, the group has shown that the final antigen forms a homotrimer similar to the natural conformation of the spike protein, likewise able to assume both open and closed confirmations [16]. In a similar approach to increasing the stability of the prefusion S-protein antigen (residues 1-1208), Medigen, with support from the NIAID, mutated the furin recognition site at the S1/S2 junction (682-RRAR-685 to GSAS) and exchanged amino acid residues K986 and V987 near the top of the central S-2P helix with two proline residues. The same mutations had also been inserted into S-2 by Wrapp *et al.* [44] to allow the determination of the SARS-CoV-2 structure by cryo-EM and had previously been used with Medigen's MERS-CoV vaccine antigen [45]. In addition, a C-terminal T4 fibritin trimerization domain, an HRV3C cleavage site, an octa-histidine tag as well as a Twin-Strep96 tag were added to the wild-type sequence [14].

A recombinantly produced homotrimer of the full-length S-protein also serves as the antigen in Clover Biopharmaceuticals' S-Trimer vaccine [12]. Using the company's Trimer-tag platform, originally developed for cancer therapeutics, Clover has genetically fused the SARS-COV-2 S-protein (aa residues 1-1211) to human C-propeptide of alpha1(I) collagen. The fusion

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protein self-trimerizes and, as an added advantage, aids purification via affinity chromatography using a collagen-receptor-derived resin [46].

For Vaxine's COVAX-19 candidate [47-54], there is a significant amount of information about the adjuvant component of the vaccine, but details of the S-protein derived antigen [18] have not been published yet. Likewise, the collaboration of Sanofi and GSK [10] has yet to publish details on the nature of their S-protein antigen, currently in Phase 1-2 clinical trials.

2.2. RBD-based vaccines

Among those entities that focus on the RBD of the S-protein, Anhui Zhifei Longcom Biologic Pharmacy Co., Ltd., is developing an RBD-dimer produced in mammalian cells as their vaccine antigen. In addition to expressing an RBD monomer (aa residues 319-541), two copies of the RBD-encoding gene fragment (aa residues 319-537) were cloned in tandem, leading to the expression of a 60 kDa homodimer. Based on published reports, this dimerization increased stability of the vaccine antigen, not just for SARS-CoV-2, but also in similar SARS-CoV and MERS-CoV constructs [55]. A slightly longer RBD (aa residues 319-545) is used in the vaccine candidate from West China Hospital [24]. After the alum-adjuvanted vaccine had shown protection in non-human primates, it is now in Phase 1 clinical trials [25].

For some of the vaccine development efforts, for various reasons, little public information about the nature of the vaccine antigen is available. For example, while it is known that Cuba's Soberana 01 vaccine is based on the RBD antigen, additional details have not yet been widely published, although original news reports suggest that a combination with the proven outer membrane vesicle platform of the Cuban meningococcus B vaccine was planned [21]. AdimrSC-2f is a vaccine candidate developed by Adimmune, with the RBD antigen expressed in insect cells. The vaccine is currently in a phase 1 clinical trial with or without aluminum as the adjuvant [19].

2.3. Multi-epitope vaccines

Many vaccine candidates in the literature employ neither the native viruses full-length S-protein or its RBD as their antigen but instead are engineered multi-epitope vaccines synthesized from peptides. Among the most advanced candidates are COVAXX's COVID-19 vaccine, made from

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epitopes of the RBD, the S2 protein, as well as other SARS-CoV-2 proteins, such as membrane and nucleoprotein regions. In guinea pigs, the company reports seeing neutralizing antibody titers that exceed those in human convalescent serum by a factor of 400 [56]. Also using peptides, and based on studies with convalescent sera, Tübingen University is advancing a multi-peptide vaccine made from HLA class I and HLA-DR T-cell epitopes of the S-protein as a potential COVID-19 vaccine to induce broad T-cell immunity [30], and Vektor Labs' (Russia) EpiVacCorona vaccine is also reportedly composed of chemically synthesized peptides of SARS-CoV-2 epitopes, conjugated to a recombinant carrier protein and adsorbed on aluminum hydroxide [28]. It should have recently completed Phase 1 trials, with no results published yet.

It will be interesting to see how the ongoing studies shift the focus between the full-length S-protein based and the RBD vaccines. The main argument in favor of the S-trimer is certainly the ambition to maintain the nature of the vaccine antigen as close to the natural confirmation as possible, while the interest in the RBD alone likely stems from concerns over adverse immune reactions triggered by full-length spike protein in SARS-CoV and also in RSV [57].

3. Protein production and delivery platforms

Over the last decades, recombinant protein technology has become efficient, relatively inexpensive, and widely available, allowing for cost-effective production of recombinant proteins in microbial and other expression host systems [58, 59]. Among other advantages, since recombinant protein vaccines are non-replicating and lack any of the infectious components of an, albeit attenuated, viral particle, the vaccines are considered a safer approach compared to vaccines derived from live viruses. The technology has been tested widely and in general, these vaccines produce only very mild side-effects [60, 61]. Consequently, multiple recombinant protein vaccines are now in clinical use worldwide [62].

3.1. Protein Production Platforms

3.1.1. Escherichia coli

For the production of recombinant proteins, a variety of expression platforms are now available, including microbial systems, such as *Escherichia coli* and various yeasts, as well as insect cells, mammalian cells, and even plants. Certainly, for non-industrial research purposes, *E. coli* is the

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most widely used system for recombinant protein production due to its rapid growth and general cost-effectivity, as well as the availability of the widest range of molecular manipulation tools. Several vaccine antigens have been produced in *E. coli*, including, in 1998, an FDA approved Lyme disease vaccine, which contained the recombinantly-expressed outer surface lipoprotein, OspA, from *Borrelia burgdorferi.* While this particular vaccine was withdrawn from the market in 2002 due to concerns over adverse side effects [63], an improved version, VLA15, likewise produced in *E. coli,* is now in a Phase 3 clinical trial [64, 65]. Other examples of *E. coli* produced antigens include vaccines against meningococcal serogroup B infections; Trumenba®, developed by Pfizer, uses two variants of the meningococcal factor H-binding protein (fHBP) as antigens [66, 67], while Bexsero®, developed by GSK, uses three immunogenic meningococcal antigens (fHbp, NadA, and NHBA) synthesized in *E. coli [68]*. These two vaccines were approved by the FDA in 2014 and 2017, respectively.

However, *E. coli* expression systems do not typically provide post-translational modifications (PTMs), such as glycosylation, which can affect the nature of the immune response and consequently the functionality of the vaccine. PTMs also affect protein characteristics such as solubility and stability, and therefore it is critical to confirm correct folding and disulfide bond formation. In the case of SARS-CoV-2, depending on the product, the length of the vaccine antigen component ranges from ~200 to ~1,300 amino acids with 4-12 potential disulfide bonds [38]. Due to this complexity, it is difficult to produce these antigens properly folded in *E. coli*, and other production platforms are favored.

3.1.2. Yeasts

Yeasts are another well-known microbial expression platform. Similar to *E. coli* , they grow rapidly and are easy to manipulate genetically. Unlike *E. coli*, yeasts can secrete recombinant proteins extracellularly, which makes the downstream purification process simpler and less costly. The inclusion of certain PTMs in this eukaryotic expression system also often facilitates proper folding of the recombinant protein [69]. Several currently licensed hepatitis B vaccines, such as Engerix-B®, Recombivax HB, and HEPLISAV-B, use recombinant hepatitis B surface antigens (HBsAg) synthesized in yeast [70]. Another licensed vaccine, Gardasil®, uses the major capsid protein, L1, from four human papillomaviruses (HPV L1) as its antigens [71]. While N-linked glycosylation in

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yeast resembles that in higher eukaryotes, a more controlled and humanlike N-glycosylation can be achieved in specialized, genetically engineered strains of the fungus [72].

For the production of the SARS-CoV-2 spike protein, it was discovered that the epitopes which are likely to trigger a potent neutralizing antibody response, are located in the N-terminal domain (NTD, residues 1-290 of S protein) and in the RBD (residues 306-577) of the spike protein [44], where the most potent ones could block ACE-2 binding [73]. With respect to the NTD, there are eight potential N-glycosylation sites within this region [74], making it likely that different glycosylation of a potential recombinant vaccine antigen will affect the ability to trigger neutralizing antibodies within this region. However, no N-glycosylation sites are within or proximal to the ACE-2 binding site, making glycosylation much less of a concern when expressing the antigen in yeast. A yeast-expressed RBD antigen (Residues 332-549 of the spike protein) is currently being pursued by Texas Children's Center for Vaccine Development at Baylor College of Medicine in partnership with Biological E [75]. This follows the prior production of a recombinant SARS-CoV RBD antigen in the same system; formulated with alum, this antigen-induced high neutralizing antibody titers and 100% protection in mice after viral challenge [76, 77].

3.1.3. Mammalian cell culture expression systems

Most current COVID-19 recombinant protein vaccine candidatesare expressed in mammalian cell culture-based expression systems (Table 1) that have been used to produce various biopharmaceuticals in recent years, including enzymes, antibodies, and vaccine antigens. Though more costly, mammalian systems are appreciated for their ability to express glycoproteins with their native structures and PTMs, and thus constitute the majority of the recently approved recombinant biologics [78]. A successful example for this class of vaccines is Shingrix®, the herpes zoster vaccine manufactured by GSK, uses Chinese Hamster Ovary (CHO) cells to produce recombinant glycoprotein E from the virus as its antigen [79].

3.1.4. Insect cells

COVID-19 subunit vaccine candidates, like those from Novavax, Sanofi and Adimmune are produced in a system that uses a baculovirus vector and insect cells as hosts. This system was first developed in 1983 [80] and has since been used for several recombinant proteins [81, 82]. Currently, there are two licensed vaccines in the USA, utilizing insect cell-expressed antigens;

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Cervarix®, an HPV vaccine that uses the recombinant HPV L1 antigen [60], and Flublok®, an influenza vaccine using a recombinant trivalent hemagglutinin antigen [75]. When compared to *E. coli* or yeast, the required growth medium is more costly and the cell growth rate is slower, but insect cells can reach higher densities in a shorter period when compared to mammalian cells [83, 84]. Additionally, like mammalian cells, insect-cell expressed recombinant proteins are usually well-folded, soluble, and often contain the desired PTMs. However, even though this system does not cause hyperglycosylation, N-glycosylation by baculovirus-infected insect cells is not equivalent to those of higher eukaryotes [85], and thus, if sophisticated glycans are required to maintain the function of a recombinant protein, this system may not be the optimal option.

In addition to traditional vaccine manufacturing platforms, alternative expression systems are also being used to produce vaccine antigens. Kentucky BioProcessing and other tobacco growers, for example, are employing tobacco plants to express SARS-CoV-2 vaccine antigens [86]. While the manufacturing of recombinant proteins in tobacco is a proven technology [87-90], controlling cost at the pandemic scale might reserve this expression system to those with access to the necessary capacity.

Generally speaking, for any expression system, production cost will vary depending on the production yield, but based on the general cost comparison analyzed by Owczark *et al.* [91], and the example retail pricing for a few biopharmaceuticals [92], *E. coli* is the least expensive choice for protein production, and while mammalian cells are the most expensive option, the production cost for insect cells and yeasts is generally somewhere in between.

3.2 Vaccine Delivery

3.2.1. Parenteral Vaccination

COVID-19 subunit vaccine candidates currently at an advanced clinical stage of development are being administered either by intramuscular (i.m.) or subcutaneous (s.c.) injection, and while some novel vaccine platforms require specialized administration equipment (e.g. electroporation devices), protein-based vaccines can be administered using conventional low-cost hypodermic needles. Intradermal (i.d.) immunizations could probably generate a stronger immune response [93], because the dermis contains higher numbers of dendritic cells, which will facilitate the uptake of antigens. Local inflammation in the dermis induces the maturation of the dendritic cells

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and stimulates migration into draining lymph nodes [94]. However, i.d. needle injections are technically complex and allow for only small volumes. Therefore, alternative delivery systems for i.d. injection of recombinant protein subunits are being developed. For example, Kim *et al.* have published on an intradermal [95] comprising of two antigens from SARS-CoV-2 spike proteins, rSARS-CoV-2 S1, and rSARS-CoV-2-S1fRS09, that are delivered using a Micro-Needle Array (MNA) technology. This vaccine triggered substantial antigen-specific antibodies in mice when dosing low amounts of antigen.

3.2.2. Mucosal Vaccination

Wang *et al.*, (2020) [96] have designed a strategy to produce an oral vaccine based on the SARS-CoV-2 spike protein. Oral vaccines promise to be particularly suitable for low-and middle-income countries since they can be administered without trained personnel and can be transported and stored without requiring a cold chain. In addition, the vaccine designed in this study in the benign probiotic bacterium *Lactobacillus plantarum* is expected to specifically trigger an enhanced mucosal immune response, desirable for preventing viral respiratory infections such as COVID-19. In their study, the authors cloned the full-length spike protein from strain Wuhan-Hu-1 and confirmed its expression on the bacterial cell surface by Western Blotting. While further evaluations of safety, immunogenicity, and functionality of the vaccine candidate are pending, the authors have shown that that the functionalized bacteria displaying the spike protein were stable in a high temperature, low pH environment as found in the digestive system.

In addition, a first Phase 1 Clinical Trial of an oral COVID-19 vaccine tablet, containing an adenovirus vector expressing the spike protein was started by Vaxart Inc. on October 13, 2020 [97, 98]. Merck, another major player in the vaccine realm, also reports that it is looking at testing an oral COVID-19 vaccine in the clinic [99].

Intranasal vaccination for COVID-19 has also been investigated by many groups, mostly with live attenuated flu viruses that are genetically modified to express the spike protein. These viral mimickers can infect cells in the mucosal layer of the nose through the ACE-2 receptors and induce protection by producing high levels of both mucosal and systemic antibodies as well as by cell-mediated immunity. Approval was granted in China on September 9, 2020, to Hong Kong University, Xiamen University, and Wantai Biological Pharmacy Enterprise Co. Ltd, to initiate the

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first intranasal Phase I clinical trial for COVID-19 [100, 101]. Elsewhere, Coroflu (University of Wisconsin-Madison, FluGen, Bharat Biotech) and Altimmune are developing intranasal COVID-19 vaccine candidates using similar viral platforms, however, to date, no data has been published on any recombinant protein nasal vaccine [102, 103].

4. Adjuvants

Recombinant proteins by themselves generally elicit only a weak immune response, unless they are assembled into larger particles [104]. To augment the immune response and allow for antigen dose sparing, most protein-based COVID-19 vaccines are formulated in combination with adjuvants (**Table 2**). The addition of these immunostimulants can trigger specific cell receptors and induce an innate immune response at the site of injection and in the draining lymph nodes. The innate immune response to the adjuvants then further activates the adaptive immune system by mobilizing antigen-presenting cells (APCs), thus improving antigen presentation to CD4 T helper cells. Depending on the phenotype, the activated T helper cell will stimulate the proliferation of antigen-specific antibody-producing B cells or $CD8^+$ T cells (**Figure 1**).

To protect against COVID-19, high levels of neutralizing antibodies to the spike protein of SARS-CoV-2 are essential. However, similarly to antibody levels in patients that have recovered from SARS-CoV, SARS-CoV-2 antibody responses seem to wane rapidly within months after infection. In addition, while less severe cases of SARS were associated with accelerated induction of a Th1-type immune response, Th2 cell responses have been associated with enhancement of lung disease following infection in mice parenterally vaccinated with inactivated SARS-CoV viral vaccines. Therefore, the FDA specifically stated in their guidelines to the industry from earlier this year that COVID-19 vaccine candidates should preferably elicit a strong Th1-skewed CD4 T cell response, in addition to the induction of high levels of neutralizing antibodies [105].

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Table 2: List of adjuvants used in recombinant protein COVID-19 vaccine candidates currently tested in the clinic.

Name	Components	Receptor/pathway	Disease target tested in the clinic
Alum*	Aluminum salts (aluminum hydroxide, aluminum phosphate)	NLRP3 uric acid, DNA	Anthrax* , Diphtheria*, Tetanus*, Pneumococcus*, hepatitis A * Hepatitis B*, Japanese Encephalitis*, Meningococcal B* and C*, human papillomavirus*, SARS, COVID-19
MF59*, AS03*	Oil-in-water emulsion squalene oil plus surfactants	MyD88, ASC, ATP	Influenza*, COVID-19
CpG 1018*	Synthetic DNA alone or formulated with Alum	TLR9	Hepatitis B *, Malaria, Influenza, Anthrax, Cancer, COVID-19
Matrix M / IscoMatrix	Saponin	Unknown	Hepatitis C, Influenza, HSV, human papillomavirus, Malaria, Cancer, COVID-19
Advax	polysaccharide particle made from delta inulin	Unknown	HIV, Influenza, Hepatitis B, COVID-19

*Adjuvants in licensed vaccines in the USA.

4.1. Aluminum hydroxide (alum)

Semi-crystalline suspensions of aluminum are the most commonly used adjuvants used in vaccine development worldwide [106]. The aluminum salts have a high binding capacity and typically will adsorb the antigens on the surface. Although hundreds of millions of people have been vaccinated with aluminum-based vaccines, there is still discussion on the exact mechanism of action. The most widely accepted explanations include a possible depot effect, enhancement of phagocytosis of the antigen, and activation of the pro-inflammatory NLRP3 pathway [107]. Aluminum-based formulations generally induce a strong humoral response in combination with the secretion of Th2 biased cytokines by T-cells (e.g. IL-4, IL-6, IL-10). In some studies, it was found

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that candidate SARS-CoV vaccines formulated with aluminum induced specific Th2-biased responses which induced lung eosinophilic immunopathology in mice [108]. Other studies found no direct evidence which links aluminum to enhanced eosinophilic immunopathology [77] and the true cause of the undesired immune response remains under discussion [57, 109]. Nonetheless, since the FDA guided the industry toward a Th1 immune response, most COVID-19 recombinant protein vaccine formulations that are formulated with aluminum hydroxide (alum) include a second adjuvant such as CpG, in order to balance the immune response and stimulate proliferation of Th1 type CD4(+) cells. Alum has an excellent safety record and can be produced at a relatively low cost, which could make it an ideal COVID-19 vaccine adjuvant for global health [109, 110].

4.2. MF59

MF59® is an oil-in-water emulsion developed by Novartis. The adjuvant contains squalene oil and two surfactants, Tween 80 and Span 85, emulsified in a citric acid buffer [111]. MF59 has been deemed safe and is well-tolerated in humans. MF59-adjuvanted vaccines have been approved for pandemic and seasonal influenza in over 38 countries worldwide. Fluad®, an MF59-adjuvanted seasonal influenza vaccine, has been licensed since 1997. However, in the United States, FLUAD and FLUAD Quadrivalent are licensed only for persons over the age of 65 years. [112] MF59 was also added to vaccines against the pandemic flu strain H1N1 (Focetria® and Celtura®). Clinical evaluation in broad study groups, including children, adults, and the elderly, proved that the MF59-adjuvanted vaccines are both safe and potent [113]. Within oil-in-water formulations, the antigen remains typically in the water phase and does not interact with the oil droplets. It provides neither direct transport nor depot effect for the antigen. Antigens and MF59 are taken up by neutrophils and monocytes, and later followed by dendritic cells (DCs) and B cells, and moved to draining lymph nodes [114]. MF59 effects the apoptosis-associated speck-like protein containing a caspase recruitment domain (ACS) and stimulates IL-4, and Stat-6 signaling, while being independent of any type-1 interferon or inflammasome pathways. The emulsion has further been shown to significantly increase the IL-5 and IL-6 levels [115]. MF59 has been selected as a COVID-19 vaccine adjuvant because it has proven to induce fast priming of

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antigen-specific CD4(+) T-cell responses, to induce strong and long-lasting memory T- and B-cell responses, and to broaden the immune response against the vaccine antigens [116].

4.3. CpG

CpG adjuvants are synthetic DNA sequences containing unmethylated CpG sequences. These oligonucleotides are potent stimulators of the innate immune system through activation of Toll-like receptor-9. TLR9 agonists directly induce the activation and maturation of plasmacytoid dendritic cells and enhance differentiation of B cells into antibody-secreting plasma cells [117]. As a vaccine adjuvant, CpG augments the induction of vaccine-specific cellular and humoral responses. Dynavax Technologies has developed a short CpG-containing oligonucleotide sequence named CpG 1018 and progressed it through clinical testing as an adjuvant for immunization against hepatitis B virus (HEPLISAV-B) [118]. The immunostimulatory effects of CpG are optimized by keeping the oligonucleotide and the vaccine antigen in close proximity. Driven by electrostatic interaction CpG 1018 binds well to aluminum hydroxide and is therefore co-formulated with aluminum-based subunit vaccines against COVID-19 [12].

4.4. AS03

AS03 is a squalene-based oil-in-water emulsion produced by GSK. It has been tested extensively in the clinic and is used for the H1N1 pandemic flu vaccine Pandemrix [119]. It is also in Arepanrix and the new Q-pan for H5N1 influenza [120]. Similar to MF59, AS03 can induce proinflammatory cytokines and chemokines, including CXCL10, but independently of type-1 interferon. This pro-inflammatory response is associated with improved recruitment, activation, and maturation of antigen-presenting cells at the injection site [121].

4.5. Matrix-M

Novavax's proprietary Matrix-M adjuvant consists of two individually nanosized particles, made with a different saponin fraction (Fraction-A and Fraction-C). The saponin particles are stabilized with cholesterol and phospholipid [122]. As a part of different vaccine formulations, Matrix-M has been proven to augment both Th1 and Th2 type responses, inducing high levels of neutralizing antibodies, and enhancing immune cell trafficking [123, 124]. Based on clinical data

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from over a dozen studies Matrix-M is considered safe and potent [125-129], however, it has not yet been part of a commercially available vaccine.

4.6. Advax

Advax made by Vaxine (Australia) is a microcrystalline polysaccharide particle composed of delta inulin [51]. In published studies covering many years of research, delta inulin has been shown to provide a robust humoral and cellular immune response when formulated with recombinant vaccine antigens. Advax adjuvant has recently also successfully been tested in several human trials including vaccine studies to prevent seasonal and pandemic influenza, hepatitis B, and hyperallergic reactions to insect venom [130]. Compared to the controls, the Advax adjuvant [52, 130] seems to improve antibody and T-cell responses, while being safe and well-tolerated [50]. It should however be noted that Advax is still a relatively new adjuvant, which has only been tested on small groups of patients and has not yet been part of a marketed vaccine.

5. Recombinant protein vaccine frontrunners for COVID-19

In this review, we generally focused on those recombinant protein vaccines for COVID-19 that, according to the WHO have reached human clinical trial status. With these clinical trials now reporting data, we will briefly summarize the available information for the leading vaccine candidates. We note that this is a fast-moving field, so it is understood that by the time this review is published, new data will likely have been made available, including from partners that are yet to show results to the public.

5.1. Novavax

In its Phase 1/2 study, Novavax's NVX-CoV2373 vaccine, formulated with Matrix-M, elicited a Th1-biased immune response with two injections on day 0 and day 21 of two different protein doses (5 and 25 µg) [7]. Additionally, both 5 and 25 µg doses of antigen were able to induce high neutralizing antibody titers (IC99 = 3906 and 3309, respectively), which exceeded those seen in human convalescent serum (IC99 = 983) was observed [7]. This immunogenicity profile fulfilled the FDA guideline for an ideal COVID-19 vaccine candidate [105]. In addition, no serious adverse effects were reported. Novavax has initiated a Phase 3 clinical trial in the UK [131] and is

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continuing Phase 2/3 studies in Australia and the US, as well as a Phase 2b trial in South Africa that will also include adults infected with HIV [132]. To prepare for global distribution, Novavax has made manufacturing agreements with multiple manufacturers including Emergent, Fujifilm, AGC Biologics, and the Serum Institute of India to produce 2 billion doses annually, with production slated to start in 2021 [133].

5.2. Sclamp (University of Queensland)

While still without data from human clinical trials, results from the evaluation of Sclamp with Mf59C-1 have now been pre-published. With the large antigen, as expected antibodies to the RBD, the S protein, and the clamp were generated; with respect to neutralizing antibodies, interestingly, they were found to bind to not only bind to the RBD but also to other parts of the spike protein. Using MF59C-1 as the adjuvant, the study also demonstrated the presence of a higher number of IFN-γ, TNF-α, and IL-2 expressing CD4+ and CD8+ cells compared to IL-4 or Il-13 expressing cells, indicating the desired Th1 bias. In addition to the strong IgG response in mice, one or two doses of the vaccine significantly reduced peak viral loads in the throats of a hamster model and all but one animal showed improved lung protection and significant protection against rhinitis, tracheitis, bronchitis, bronchiolitis, and alveolitis [16].

5.3. Clover biopharmaceuticals

Clover Biopharmaceuticals' vaccine candidate, SCB-2019, was shown to trigger a robust immune response in their non-human primate study. In the study, 30 µg of the S-trimer adjuvanted with either AS03 or CpG1018/alum were used to immunize Rhesus macaques on Day 0 and Day 21. On day 35, neutralizing antibody titers in the AS03-adjuvanted S-Trimer group (IC50 = 20,234) were significantly higher than CpG 1018 plus alum group (IC50 = 11,682), however, the lymphocyte response seems to sustain in the CpG 1018 plus alum group longer [12]. Clover biopharmaceuticals have partnered with GlaxoSmithKline to produce the vaccine for the current Phase 1 study [134] and have formed an advisory board for global vaccine development and access [135].

5.4. West China Hospital

West China Hospital in collaboration with Sichuan University is developing an insect cell-based RBD vaccine and has so far published its testing in Rhesus macaques. By formulating 20 or 40 µg

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RBD with alum, and using two injections on days 0 and 7, the vaccine was triggered neutralizing antibody titers of approximately 100 on the 35th day post-vaccination. While this neutralizing antibody titer appears to be on the lower range of published SARS-CoV-2 vaccines, we acknowledge that there is still no unified way to determine neutralizing antibody titers across different laboratories. West China Hospital also reports that vaccinated non-human primates were protected against viral challenge, and with this encouraging data, the vaccine is currently in a phase I clinical trial [136].

6. Conclusions and outlook

In an earlier assessment of Operation Warp Speed, Moore and Klasse noted that while recombinant proteins are "by far the most immunogenic vaccine candidates for antibody responses", they were not included in the first wave of vaccine candidates [137]. DNA and mRNA vaccines inactivated viruses, as well as vector-based strategies, were able to attract more attention (and funding). Early on, these platforms offered a faster time to the clinic and the ability to produce the necessary quantities of vaccine. In the meantime, of course, a recombinant protein antigen-based vaccine has been added to the government-supported OWS portfolio and initial data from human clinical trials has begun to enter the public domain. Based on self-reporting by HHS [138], OWS continues to aim for the initial doses of a vaccine to be available by January 2021. Whether this first-generation vaccine will have the necessary efficacy to prevent infection in humans [139], and whether its, likely, new vaccine technology, will be received well by an increasingly vaccine-hesitant public remains to be seen [140]. Arguably, while recombinant protein vaccines may lag in development, they may offer the better solution in the long run [141], in particular with respect to transferring a proven vaccine technology to low- and middle-income countries, where facilities to reproduce the new production platforms are unavailable [110], or where the infrastructure to distribute fastidious nucleic acid vaccines, e.g. storage required at -94° F [142], is out of reach. Moreover, the true efficacy of nucleic acid-based vaccines remains unproven in humans and vector-based vaccines carry the risk of immunity to the vector which would make booster vaccinations challenging [141].

Newly emerging data from the initial human trials of COVID-19 vaccines suggest that the most rapidly produced vaccines (i.e., nucleic acids and virus vectors) may not be the most capable

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of eliciting high titers of total antibodies and neutralizing antibodies (**Table 3**). In the absence of a standardized virus neutralization assay and the fact that ELISAs are run using varying protocols, the absolute numbers need to be reviewed cautiously, but although all vaccine candidates appear to elicit neutralization that is equivalent to that in human convalescent sera, there appear to be significantly higher neutralizing antibody titers in the only recombinant protein vaccine (NVX-CoV2373) tested so far.

Table 3: Reported neutralizing antibody titers for a selection of COVID-19 vaccines that have been tested in Phase 1 and Phase 1-2 human clinical trials.

Vaccine candidate	Category	Doses	Neutralizing AB titers[1]	Ref.
ChAdOx1 nCoV-19	Vectored Vaccine	2	Ic50: 451[2]	[143]
Ad26.COV2.S	Vectored Vaccine	1	Ic50: 243[3]	[144]
mRNA-1273	RNA	2	Ic50: 374[4]	[145]
BNT162b2	RNA	2	Ic50: 363[5]	[146]
NVX-CoV2373	Recombinant Protein	2	Ic99: 3,906[6]	[7]

Highest reported value in the referenced publication
[2] 50% neutralization titer, $5x10^9$ viral particles, 42 days post first vaccination
[3] 50% neutralization titer, $1x10^{11}$ viral particles, 29 days post first vaccination
[4] Ic50, 250 μg, 36 days post first vaccination
[5] 50% neutralization titer, 20 μg RNA vaccine, 28 days post first vaccination
[6] Wild-type SARS-CoV-2 microneutralization, inhibitory concentration greater than 99% (MN IC>99%) titer response, 5 μg adjuvanted protein, 35 days post first vaccination

So, as we continue to struggle to contain the pandemic, the question remains, whether the first next-generation vaccines to the clinic will also be the best vaccine in the clinic in the long-term, or, whether recombinant proteins will not catch up and provide the more efficacious long-term solution.

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Abbreviations:

aa:	Amino acid
ACE-2:	Angiotensin-converting enzyme 2
ADE:	Antibody-dependent enhancement
Alum:	Aluminum hydroxide
APC:	Antigen-presenting cell
cGMP:	Current good manufacturing practices
CoV:	Coronavirus
COVID:	Coronavirus disease 2019
FDA:	US Federal Drug Administration
HHS:	US Department of Health and Human Services
MERS:	Middle East respiratory syndrome
MNA:	Micro-needle array
NTD:	N-terminal domain
OWS:	Operation Warp Speed
PTM:	Post-translational modification
RBD:	Receptor binding domain
RBM:	Receptor binding motif
RSV:	Respiratory syncytial virus
S-protein:	Spike protein (S1/S2)
SARS:	Severe acute respiratory syndrome
TMPRSS2:	Transmembrane protease serine 2
WHO:	World Health Organization

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