

REVIEW



DNA vaccines for SARS-CoV-2: toward third-generation vaccination era

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ABSTRACT

Introduction: Coronavirus outbreak 2019 (COVID-19) has affected all the corners of the globe and created chaos to human life. In order to put some control on the pandemic, vaccines are urgently required that are safe, cost effective, easy to produce, and most importantly induce appropriate immune responses and protection against viral infection. DNA vaccines possess all these features and are promising candidates for providing protection against SARS-CoV-2.

Area covered: Current understanding and advances in DNA vaccines toward COVID-19, especially those under various stages of clinical trials.

Expert opinion: Through DNA vaccines, host cells are momentarily transformed into factories that produce proteins of the SARS-CoV-2. The host immune system detects these proteins to develop antibodies that neutralize and prevent the infection. This vaccine platform has additional benefits compared to traditional vaccination strategies like strong cellular immune response, higher safety margin, a simple production process as per cGMP norms, lack of any infectious agent, and a robust platform for large-scale production.

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1. Introduction – background of COVID-19

COVID-19 is a new infectious disease that is impacting the globe. The SARS-CoV-2 is responsible for such devastating conditions globally [1,2]. It is an enveloped, non-segmented, positive sense RNA virus which belongs to the subgenus of *sarbecovirus*, *orthocoronavirus* sub-family, and is present in humans and other mammals. Its size is 63–125 nm in diameter, encompassing single strands of RNA (Figure 1) [3]. SARS-CoV-2 contains four structural proteins, spike S1 and S2 subunits (S), envelope protein, membrane protein, and nucleocapsid protein, as well as sixteen non-structural proteins (Nsp). Notably, Nsp1 is involved in RNA production and replication. Nsp2 on the other hand involves in the host cell's survival signaling process and Nsp3 ensures separation of the translated protein [4].

The S1 subunit of the viral spike glycoprotein is having a receptor binding domain (RBD). Initially an RBD binds with the target cell's receptor which is then transferred into endosomes [5,6]. The RNA-dependent RNA polymerase (Nsp12) and helicase (Nsp13) accelerate SARS-CoV-2 genome expansion and mRNA transcription upon entering the host cell [7,8]. At endoplasmic reticulum (ER)-golgi pathway transcription and translation process of viral mRNA lead to the formation of viral structural proteins that are essential for viral assembly formation along with other non-structural proteins and enzymes and once viral assembly is engineered the virus exits the cell through exocytosis [9,10] going on to infect other host cells. As the virus is foreign to the host, antigen presenting cells

(dendritic cells, macrophages) present viral proteins to CD4 + T-helper (Th) cells via the major histocompatibility complex (MHC) class 1, resulting in the secretion of interleukin-(IL)-12 and interferon- γ to further trigger Th1 and natural killer cells [11,12]. In addition there is activation of the NF- κ B signaling pathway resulting in pro-inflammatory cytokines (IL-1 β , IL-6, TNF- α , IL-17, IL-8) [13,14] which aid to attract neutrophils and monocytes which stimulate other pro-inflammatory cytokines and chemokines including monocyte chemoattractant protein-1 (MCP-1) [15,16]. Concurrently, IgM antibodies are generated lasting up to 12 weeks followed by IgG antibodies providing long lasting protection as a result of viral invasion [17]. Further, sensitization to the virus promotes the stimulation of CD8+ memory cells which are long term [18,19]. As such, a vast amount of research has been focussed in the last year for COVID-19 vaccines development to stimulate primarily antibody responses, but also T cell responses.

2. COVID-19 vaccines – A global picture

The effect of vaccination on human wellbeing is one of the brightest chapters in the history of medicine. Although the concept of vaccinations dates back thousands of years, the first formal vaccine was developed over 300 years ago. Vaccines include either damaged, live, or destroyed microorganisms or virus, as well as proteins or toxins from the organism. They help escape illness from infectious diseases by boosting innate and adaptive immune systems [20].

Article highlights

- DNA vaccine platform has many benefits over traditional vaccination strategies like a strong cellular immune response along with generation of neutralizing antibodies, higher safety margin, a simple production process as per cGMP norms, lack of any infectious agent, and a robust platform for large-scale production.
- The short amount of time needed from design to clinical trials is a significant benefit of current advances in DNA vaccine development. As a result, this could eventually be able to test alternative antigen variants that cover ubiquitous mutations in the very same vaccination.
- In human clinical trials in healthy individuals, DNA-based COVID-19 vaccines have shown better safety profile, and a strong immune response generation in the investigated time frames.
- This manuscript covers information related to all DNA vaccines for COVID-19 including EUA vaccine from Zydus.

Vaccination was a widely used term instead of the immunization because it was first extracted from a virus that afflicted cows. Edward Jenner developed the smallpox vaccine in 1796 [21] and through Louis Pasteur's work in microbiology, advanced the idea [22].

Vaccine fundamental research was kick-started after 1950s. New methods for generating viruses resulted in rapid findings and breakthroughs vaccinations [23]. Critical pediatric infections were the focus, such as measles, mumps, and rubella,

and vaccinations for these have greatly reduced burden of disease [24].

2.1. Whole inactivated (dead) vaccines

The first step is to inactivate or destroy the disease-carrying virus or bacterium, or one that is somewhat close to it, using chemicals, heat, or radiation. This technique employs technology that is effective in humans – this is how flu and polio vaccines were produced with great safety profiles [25]. By using the inactivated vaccine platform, 17 vaccine candidates against SARS-CoV-2 are in progress. The Chinese pharmaceutical company, Sinovac Research and Development Co., is conducting a phase 4 clinical trial for CoronaVac® [26]. The company has produced 200 million doses worldwide and can generate 2 billion doses each year to meet global demand (NCT04383574). Covaxin®, India's first COVID-19 vaccine produced in-house, is a two-dose injection that uses an inactivated version of the virus. In a joint statement from Bharat biotech and co-developers, the Indian Council of Medical Research (ICMR), Covaxin® has demonstrated 100% efficacy against severe COVID-19 and hospitalization [27]. According to Phase III clinical data, Covaxin® showed 81% efficacy in preventing mild, moderate, and severe COVID-19 (NCT04918797). The serum samples from the patients who received this vaccine were able to neutralize alpha variant, gamma variant, zeta variant, kappa variant, and delta variant [28,29].

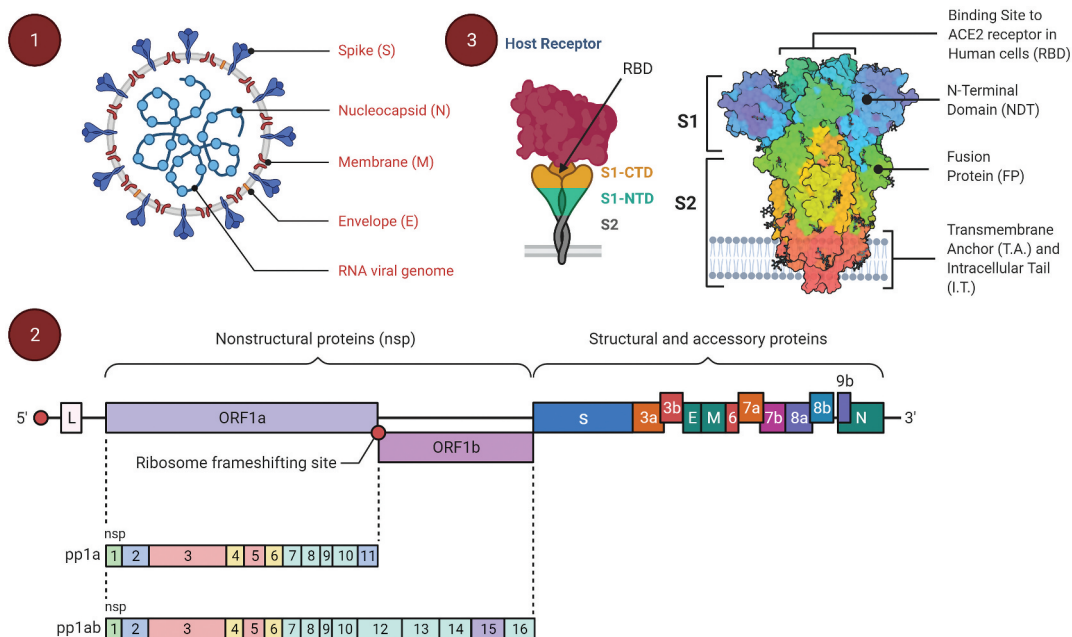


Figure 1. The genomic organization of SARS-CoV-2 and spike protein structure. (1) The structure of SARS-CoV-2 containing an RNA as genetic material with four distinguished structural proteins like spike glycoprotein (S), membrane protein (M), envelop protein (E), and nucleocapsid (N), (2) the genomic organization of the SARS-CoV-2 with 16 Nsps along with the structural and the accessory proteins gene (from 5' end to 3' end), and (3) spike glycoprotein structure with two subunits S1 and S2 that are targeted by the human enzyme Furin, and it may also cause the development of a syncytium (cell fusion) where S1 contains a receptor binding domain (RBD); S1 has a C-terminal domain (CTD) and an N-terminal domain (NTD).

2.2. Live-attenuated vaccines

Live attenuated vaccines comprised living but a damaged form of the virus or very similar to it. This form of vaccine has been used in some traditional vaccines such as, measles mumps rubella (MMR) vaccine as well as the chickenpox and shingles vaccines [30]. Like the inactivated vaccine, this method employs similar technologies and can easily be produced in large scale. However, such vaccinations may not be appropriate for people with weakened immune systems [31]. As such, COVI-VAC, developed by Serum Institute of India in partnership with Codagenix (USA), is in phase I clinical trial (NCT04619628). Meissa Cannines, Inc. is currently conducting a phase 1 clinical trial with MV-014-212, a live attenuated vaccine for respiratory syncytial virus which expresses the S protein of SARS-CoV-2 (NCT04798001).

2.3. Recombinant viral vectors

Recombinant viral vectors comprise an unrelated transformed virus such as adenovirus, incorporating an antigen of interest. The antigen is delivered into cells in the same way that an actual virus would enter, inducing robust antigen-specific cellular and humoral immune responses on their own, eliminating the need for external adjuvants [32]. There are total of six vaccine candidates approved for emergency use based on non-replicating viral vector for vaccine delivery while 20 vaccine candidates are under different stages of clinical development using same platform [33]. Additionally, there are nine vaccine candidates under clinical development based on replicating viral vectors. Russia's Sputnik V vaccine (also known as Gam-COVID-Vac) was the first to be approved prematurely for COVID-19 therapy and uses two different engineered adenovirus vectors rAd26 and rAd5, encoding recombinant SARS-CoV-2 S protein (NCT04530396) for first and second doses respectively. The use of two varying serotypes at an interval of 21 days overcomes already prevailing adenovirus immunity in the population [34]. AZD1222 (Covishield) is presently authorized for emergency use in many countries including Europe and India. However, severe blood clotting adverse reactions have been reported in several countries, raising safety concerns [35,36]. The serum samples from the patients who received this vaccine were able to neutralize variants of the B.1.1.7 (alpha variant), P.1- B.1.1.28 (Gamma), and B.1.617 (delta variant) lineages. Another promising candidate developed using this technology is Ad26.COV.S, a single dose vaccine developed by Janssen Pharmaceuticals showing 66% efficacy in preventing COVID-19 disease in interim phase 1/2a data [37]. It is currently under global phase 3 clinical trial (NCT04505722). The serum samples from the patients who received this vaccine were able to neutralize alpha variant, gamma variant, zeta variant, kappa variant, and delta variant [38–40].

2.4. Subunit protein vaccines

Subunit vaccines include small regions of a virus (proteins) delivered with appropriate adjuvants to induce an immune response. The majority of the vaccines on the pediatric list are

subunit vaccines including, whooping cough, tetanus, and diphtheria [41]. The disadvantage of protein subunit vaccines is that they have reduced immunogenic power and so incorporation of immunostimulatory molecules or adjuvants is needed to address this problem. Also, multiple doses are required to be given for achieving long-term immunity. A number of vaccines have been developed using this technology against SARS-CoV-2, to be precise 45 vaccine candidates [33]. Notably, the Novavax vaccine is in phase 3 clinical trial which uses spike protein made using moth cells and an adjuvant Matrix-M based on a saponin extracted from the soapbark tree *Quillaja Saponaria* (NCT04611802). The adjuvant Matrix-M helps in achieving a higher immune response with a smaller dose of spike protein. Sanofi Pasteur in collaboration with GlaxoSmithKline have also generated a subunit vaccine, VAT00002, which is currently in clinical trial (NCT04762680).

2.5. Virus like particles

Virus like particles (VLPs) are viral structural proteins that self-assemble to resemble the conformation of native viruses, although they lack the viral genome. When compared to protein subunit vaccines, VLP vaccines present the epitope in a more native virus-like conformation, resulting in more robust immune responses [42]. VLP vaccines with firmly repeated antigenic units on its surface aid in inducing a more robust antibody response by effectively cross-linking B cell surface receptors. Medicago (Canada), in collaboration with GlaxoSmithKline, is developing an adjuvanted plant-based VLP vaccine consisting of S recombinant protein and 'coronavirus-like-particle' technology [43]. In addition there are five more vaccine candidates that are under clinical development based on VLP platform [33].

2.6. Nucleic acid vaccines

Nucleic acid vaccines use DNA or mRNA, which encode specific pathogenic antigens to induce an immune response. Until the emergence of the COVID-19 pandemic, no vaccine was approved for human use developed with this technology. Despite the unknown safety of this platform, two COVID-19 vaccines, mRNA-1273 and BNT162b2, have received emergency use authorization in the US [26]. Nucleic acid vaccines are simple to create since they only include a genetic code for the pathogenic antigen and a delivery method. DNA is more durable than mRNA and can therefore be processed at 4°C for a longer period of time [44]. DNA can be supplied directly to dendritic cells to stimulate T cells. Proteins that have been synthesized can also enter various tissues and specifically activate local B cells [45]. ZyCoV-D from Zydus Cadila (CTRI/2021/01/030416), Takeda by Osaka University (NCT04655625), and INO-4800 from Inovio Pharmaceuticals (NCT04447781, NCT04642638 and ChiCTR2000040146) are three DNA vaccine candidates for COVID-19 under the phase 3 clinical development. There are five more DNA vaccine candidates in phase 2 development while three vaccine candidates in phase 1 clinical trial [33]. In case of mRNA vaccine, a delivery platform such as lipid nanoparticles delivers mRNA into the host cell cytoplasm for protein translation. The synthesized protein then

stimulates T cell and antibody responses [46]. There are three vaccine candidates with emergency use approval (EUA) viz. TAK-919 (Moderna formulation developed by Japan based company Takeda), BNT162b2; Tozinameran or Comirnaty (Pfizer/BioNTech), and Spikevax/mRNA-1273 from Moderna. A total of 24 number of candidate vaccines have been developed for COVID-19 and are under different stages of clinical development [33,47].

3. DNA vaccines

Enzo Paoletti and Dennis Panicali of the New York department of health established a way to make recombinant DNA vaccines between 1979 to 1983 by employing genetic engineering to change regular smallpox vaccination into vaccinations that may be capable of preventing other illnesses [48–50]. Consequently, plasmid DNA was noted to activate immune responses to encoded antigens when administered into the skin or muscle of mice [51,52]. Since then, there has been steady improvement in understanding the mechanism of this technology, improving its immunogenic potential [51,53] and attenuating their toxicity [54,55].

3.1. Mode of eliciting host immunity

DNA vaccines are responsible for eliciting both humoral as well as cellular immunity. It consists of delivering DNA plasmids encoding immunogenic antigens and a mammalian promoter [56]. They are administered via intramuscular, subcutaneous, mucosal, or transdermal routes (Figure 2) [57]. Myocytes and antigen-presenting cells are the primary targets of DNA vaccine [58]. Here, the plasmid encoding the antigen of interest is delivered into the host cell's nucleus, where they undergo transcription and translation process with the help of host cell machinery (Figure 2). Once proteins and peptides are formed, they are presented via MHC class I and class II to stimulate T cells [59,60]. In addition, the unmethylated CpG sequences present in the plasmid vector stimulate the innate immune response [61] and synergize with the host's adaptive immune response. In the case of the COVID-19, DNA vaccines encoding the S protein have been developed [62]. Following administration of a DNA vaccine via the intra-muscular route, myocytes take up the plasmid vector and activate Th1 cells, IL-2 and IFN-gamma. If delivered through a gene gun or electroporation technology into the skin, Th2 immune responses have been shown [63,64]. Using DNA vaccine technology,

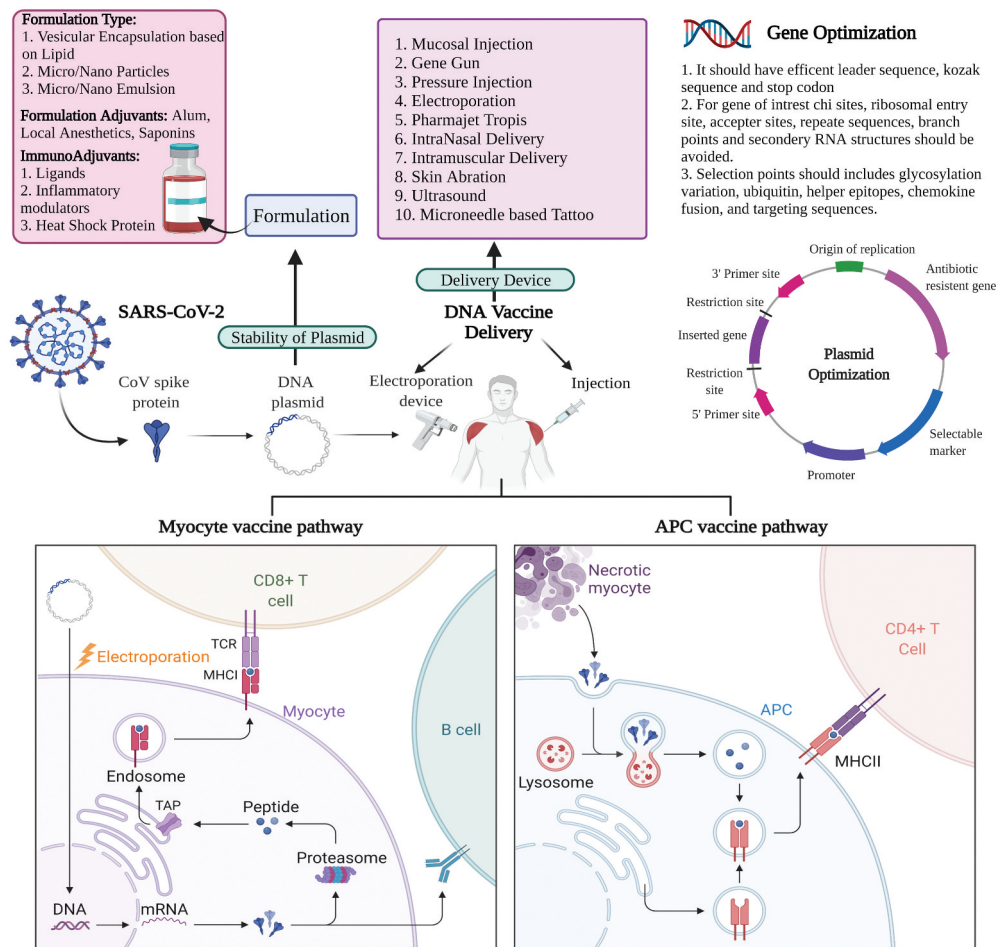


Figure 2. Mechanism of action of DNA based vaccines for SARS-CoV-2. To make the DNA vaccine, single-stranded RNA (ssRNA) of SARS-CoV-2 spike protein (as an example) is extracted, synthesized into double-stranded (dsDNA), and cloned into a plasmid. This plasmid is then injected intramuscularly in conjunction with an electroporation device to facilitate uptake. Within the muscles, myocytes take up the plasmid and express the protein of interest. This will lead to either CD8 + T cell activation through MHC class I or B cell activation. Antigen presenting cells like macrophages will endocytose spike proteins from necrotizing myocytes and activate CD4 + T cells through MHC class II presentation. Overall, leading to the recruitment of multiple immune subsets.

CD4+ Th1, Th2, CD8 + T cells and antibodies have been shown to be induced to a number of antigens [63,65,66]. Thus, DNA vaccines provide balanced Th1/Th2 responses. If more Th2 based immune response is obtained then possibility of vaccine-associated enhanced respiratory disease (VAERD) increases. The alum-adsorbed inactivated SARS-CoV vaccine in animals has caused VAERD [67,68], though no such data is obtained when used on human beings. Thus, DNA vaccine is safe and have potential for providing effective immunity.

3.2. DNA vaccine features

In this pandemic era, the rapid development of vaccines is a critical need. DNA vaccines that use plasmids to deliver antigen of interest are more straightforward to construct than the complex processes required in live, attenuated or recombinant vaccines [69]. Here, exposure to live pathogens is avoided as the constructs can be synthesized chemically to be considered safe and non-dangerous (Table 1). These plasmids are reasonably stable at room temperature, and there is no need for cold storage for transportation world-wide [63,70].

The plasmid constructs can be made to contain the proteins of interest, with improved immunogenicity and safety profile [71]. An essential aspect for manufacturing DNA vaccines is the selection of the promoter region in the plasmid. It is mainly responsible for expressing the gene of interest. The most commonly used promoter is the mammalian CMV promoter, although other host tissue-specific promoters can also be a choice of interest. For proper completion of the transcription process, a termination site or poly (A) signal site is also incorporated, thereby releasing the formed mRNA into the cytosol [72,73]. The most commonly used termination sequences are bovine growth hormone terminator sequence or sequences that downstream from the ORF of gene of interest [59].

Formulation of DNA vaccines need to address two issues. One is protection from degradation, and the second is to increase its ability to transfect the cells *in vivo* [74]. Both these problems can be overcome by delivering the vaccine through the use of devices like mucosal injections, gene guns, electroporation techniques, pressure needle-free injections [75,76]. Injecting through gene guns or Pharmajet devices like Tropis and Stratis directly results in the transfection of skin or muscle cells [77]. Using the electroporation technique, pores are formed in the cells on the application of an electric field which is temporary for ease of transfection of the DNA plasmid in cells [78]. Another approach could involve the

encapsulation of the vaccine formulation into nanoparticles, liposomes or virus-like particles to be taken up by dendritic cells for entry into the cytosol of the cells [79]. Plasmid DNA can be trapped on the surface of the polymers, like polylactico-glycolides, cationic polymers, or chitosan, and can be administered parenterally or directly to mucosal surfaces (orally or via the respiratory tract), where they are taken up by dendritic cells [80,81] and induce systemic and mucosal immunity. Liposome vehicles are used to prevent DNA degradation during its transfection across the membrane of cells [82] and have been shown to induce cellular and humoral immunity activation [83,84]. More recently, microneedle patches have been considered the better formulation option for DNA vaccines with advantages such as improved immunogenicity, elimination of needles, no need for reconstitution, simplified supply chain, ease of use, reducing the need for healthcare providers, and greater acceptability compared to traditional hypodermic injections [85,86]. Thus, in the condition of an emergency, the manufacturing of vaccines based on DNA is easy, rapid, and straightforward, provided the viral genomic sequence is known.

3.3. DNA vaccine formulation improvements

To have increased efficacy from DNA vaccines the manufacturing platform can be optimized in many ways such as, (1) plasmid optimization to improve the expression of the gene of interest, (2) gene optimization by various methods like incorporating species-specific codon, (3) supplementing the vaccine with formulation adjuvants like alum, encapsulation in liposomes, microemulsions, etc., (4) plasmid adjuvants such as cytokines and toll-receptor ligands, (5) use of novel delivery methods which can improve the transfection ability of the DNA into cells like electroporation, needle-free delivery system, etc. In regards to the route of administration, the most popular route is intramuscular, where myocytes are predominantly targeted [87]. However, targeting skin dendritic cells is more beneficial and this could be achieved via microneedles, jet injection and electroporation [88–90]. Oral and mucosal routes are also being explored to increase the efficacy of the DNA vaccines, which are currently being conducted by Symvivo and Mediphage Bioceuticals, respectively [91].

3.4. DNA vaccines for COVID-19

A number of DNA vaccine candidates in phase I, II, III clinical trials for COVID-19 are under evaluation as shown in Table 2.

The S protein of the SARS-CoV-2 virus is responsible for entry into host cells via the ACE-2 receptor, so it is a favorable and obvious target utilized for the preparation of most vaccine candidates [101]. In the development of the DNA vaccines, the IgE signal sequence is commonly incorporated into the plasmid vector followed by insertion of the chemically synthesized S gene region. In the case of ZyCoV-D, pVAX-1 vector is used with a sequence encoding S protein from the Wuhan Hu-1 isolate (Genebank Accession No. MN908947.3). Zydus Cadila submitted its results of an adaptive phase I/II dose-escalation clinical trial in over 1,000 healthy volunteers to the Indian regulatory authorities at the end of December 2020 [95]. The

Table 1. Features of DNA-based vaccines.

Feature	Comment
Make-up Production	Easy construction and manipulation of plasmid DNA Can be easily produced on large scale, less time consuming, and consistent product obtained.
Safety	No harmful effects associated with those of live attenuated or killed (dead) vaccines; use of toxic treatment is not required
Shelf- life	Long and thermostable and no requirement for cold chain storage for transportation
Immunogenic potential	Both antibody and T cell immune responses have been shown.

Table 2. DNA vaccines under different clinical stage of development against SARS-CoV-2.

Name of company/ developer/ researcher	Development Stage	Description of product	Delivery method*	Clinical Trial Identification No.	Start Date/ Completion Date/Study location	Antigen encoded in the vaccine	Ref
Zydus Cadila Healthcare Limited, India	EUA in India	ZyCoV-D (pVAX-1 plasmid DNA vaccine vector)	Intradermal – Needle free delivery using Pharmajet Tropis® device	CTR/2021/01/030416	20 January 2021/ September-22/India	Spike protein (S) with RBD responsible for binding with angiotensin converting enzyme (ACE-2) receptor.	[33,92–95]
Inovio Pharmaceuticals U.S.A	Phase II/III [33,44,96,97]	INO-4800 (pGX001 plasmid DNA vaccine vector)	Intradermal using Electroporation technique using CELLECTRA 2000 device	NCT04642638,	23 November 2020/ 31 March 2022/ Japan	ChiCTR2000040146 and NCT04447781	30 November 2020/ Sep-22/ U.S.A
Spike protein (S) with RBD responsible for binding with ACE-2 receptor AnGes Inc/Osaka University/ Takara Bio, Japan	Phase II/III	AG0301 & AG0302 (pVAX-1 plasmid vaccine vector + alum adjuvant)	Intramuscular delivery	NCT04655625 and NCT04527081	23 November 2020/ 31 March 2022/ Japan	The spike glycoprotein containing the RBD, heptad repeat 1 (HR1), heptad repeat 2 (HR2), the transmembrane domain, and the cytosolic domain.	[33,98]
GeneOne Life Science, Korea	Phase I/II	GLS-5310	Intradermal delivery	NCT04673149	23 December 2020/ 31 December 2022/ Korea	Spike (S) protein and a second antigenic target (ORF3a, protein 3a) of SARS-CoV-2	[33,98]
Genexine Consortium, Korea	Phase I/II	GX-19 N	Intramuscular delivery	NCT04715997 and NCT04445389	30 December 2020/ 30 March 2022/ Korea	S-protein antigen including the Nucleocapsid protein (NP) antigen	[33,52]
Takis/Rottapharm Biotech, Italy	Phase I/II	COVID-eVax	Intramuscular or intramuscular + electroporation using Cliniporator and EPSPGun	EUCTR2020-003734-20 and NCT04788459	25 February 2021/ Jun-2022/ Italy	Monomeric form of SARS-CoV-2 S protein RBD	[33,99]
OncoSec Medical Incorporated/Providence Health and Services U.S.A	Phase I	CORVax12 (TAVO™ (plasmid IL-12) with a DNA-encodable version of the SARS-CoV-2 spike or 'S' glycoprotein)	Intradermal route using Cliniporator	NCT04627675	30 December 2020/May-2022/ U.S.A	SARS-CoV-2 spike or 'S' glycoprotein	[33,100]
Symvivo Corporation, Australia	Phase I	baCTRL-Spike	Oral route	NCT04334980	2 November 2020/ 28 February 2022/ Australia	Spike protein from SARS-CoV-2	[33]
University of Sydney, Bionet Co., Ltd Technovalia, Australia	Phase I	COVIGEN	Subcutaneous or intramuscular delivery using Pharmajet Stratis needle free delivery system	NCT04742842	15 February 2021/ 31 December 2022/ Australia	Not revealed	[33]
Entos Pharmaceuticals/ Cytiva, Canada	Phase I	Covigenix VAX-001 – DNA vaccines + proteo-lipid vehicle (PLV) formulation	Intramuscular route using Fusogenix delivery system	NCT04591184	7 April 2021/ Aug-2022/ Canada	Spike protein from SARS-CoV-2	[33]
Chula Vaccine Research Center, Thailand	Pre-clinical	DNA plasmid	Electroporation	Not available	Not Available	Not revealed	[33]
Ege University, Turkey	Pre-clinical	DNA plasmid	-	Not available	Not Available	Not revealed	[33]
Globe Biotech Limited, Bangladesh	Pre-clinical	Plasmid vaccine	-	Not available	Not Available	Not revealed	[33]
Immunoomic Therapeutics/ EpiVax/ PharmaJet, U.S.A	Pre-clinical	Plasmid vaccine	Needle-free intradermal delivery using PharmaJet Tropis® device	Not available	Not Available	Not revealed	[33]

(Continued)

Table 2. (Continued).

Name of company/ developer/ researcher	Development Stage	Description of product	Delivery method*	Clinical Trial Identification No.	Start Date/ Completion Date/Study location	Antigen encoded in the vaccine	Ref
Mediphage Biocellulars/ University of Waterloo/ Lambton College, Canada	Pre-clinical	msDNA-VLP	Ministering DNA encoding Virus like particle derived from SARS-COV-2 genome for intranasal delivery.	Not Available	Not Available	Not revealed	[33]
National Research Center, Egypt	Pre-clinical	plasmid vaccine S, S1, S2, RBD & N	-	Not Available	Not Available	Spike protein with RBD domain and N protein	[33]
National Institute of Chemistry, Slovenia	Pre-clinical	pcDNA3.1 (+) Plasmid DNA vector, nanostructured RBD	-	Not Available	Not Available	Nanostructured RBD domain	[33]
Karolinska Institute/Cobra Biologics (OPENCORONA Project), Sweden	Pre-clinical	DNA plasmid	Electroporation	Not Available	Not Available	Not revealed	[33]
Scancell/ University of Nottingham/ Nottingham Trent University, U.K.	Pre-clinical	SN14 (plasmid vaccine RBD&N)	-	Not Available	Not Available	RBD domain and N protein	[33]
Statens Serum Institute, Denmark	Pre-clinical	CoVAXIX plasmid vaccine	-	Not Available	Not Available	Not revealed	[33]
University of Cambridge/ DIOSynVax/ PharmaJet U.K.	Pre-clinical	DIOS-CoVax2 synthetic gene inserts	Intradermal delivery using PharmaJet Tropis delivery system	Not Available	Not Available	Not revealed	[33]
Center of Genomics and Bioinformatics of Academy of Science of Republic of Uzbekistan	Pre-clinical	The 3 regions of SARS-Cov-2 Spike- protein: NTD, RBD and HR1-HR2 inserted into the plasmid of PcDNA3.1 (+)	-	Not Available	Not Available	NTD, RBD and HR1-HR2 regions of Spike protein	[33]

*All these vaccines required two doses for providing sufficient immune protection

vaccine was shown to be safe and effective and Zydus Cadila was the first organization to initiate a large-scale phase III clinical trial for DNA -based anti-SARS-CoV-2 vaccine in India in the month of January 2021 [94]. The study was conducted on over 28,000 volunteers across more than 50 clinical sites pan India. The results of the study indicated that it is a safe and effective vaccine especially against the new mutant strain the delta variant. In addition, the trial was successfully carried out on 1000 children in age group of 12–18 years and the plasmid DNA vaccine was found to be safe. From the interim analysis in symptomatic RT-PCR positive cases primary efficacy of about 66.6% was found of ZyCoV-D. Administration of third dose of the vaccine showed 100% efficacy for moderate disease. The ZyCoV-D vaccine will be administered using the PharmaJet device known as Tropis®. This needle-free injection system can deliver vaccine intramuscularly or subcutaneously employing a narrow, high-velocity fluid jet, which penetrates the skin [92,102,103]. Earlier it was devised for a three-dose regimen but recently they conducted another study where two doses were administered each dose of 3 mg and the immunogenicity results were found to be equivalent to the three-dose regimen. Thus, ZyCoV-D has become the first plasmid DNA vaccine to get DCGI approval for Emergency Use Authorization [93].

With regards to the immunogenicity potential of ZyCoV-D, it was demonstrated well from the preclinical studies carried out in mice, guinea pig and rabbit models by intradermal route at dose of 25, 100 and 500 microgram respectively [92]. The vaccine was able to induce production of neutralizing antibodies (NAB) as well as Th-1 response in animal models which was confirmed by the elevated levels of interferon- γ . The phase I clinical studies of ZyCoV-D were done between July 2020 to October 2020 on 48 healthy adults aged between 18 to 55 years in a single center, open-label, non-randomized manner [93]. As mentioned earlier it was a three-dose regimen administered at a gap of 28 days and each subject was followed up up-to 28 days post vaccination for monitoring the safety and immunogenicity. No adverse effects were reported. The study regime consists of four treatment arms where volunteers in first and third arm received the dose through 1 mg and 2 mg needle-based injection system whereas second and fourth treatment arm received dose through 1 mg and 2 mg needle free injection system (Tropis). In the study the dose administered was 0.5 ml of ZyCoV-D vaccine which contained 5 mg of DNA plasmid with spike protein gene region insert suspended in phosphate buffer saline. The seroconversion rate in volunteers was based on IgG titers found at day 84 and around 36.36%, 33.33%, 100%, and 80% proportion of the subjects were found to be seroconverted in all four treatment arms successively [93].

In the case of INO-4800 (Inovio Pharmaceuticals, US), the pGX001 vector is used with sequence encoding the S protein with an N -terminal IgE along with the eukaryotic cytomegalovirus (CMV) promoter and bovine growth hormone polyadenylation signal. The resulting plasmid was designated as pGX9501, designed to encode the SARS-CoV-2 S protein. The research was funded by the Coalition for Epidemic Preparedness (CEPI)/Gates Foundation and US Department of Defense. This vaccine is given intradermally through its

proprietary device, CELLECTRA® [96,104], to stimulate local dendritic cells and induce immune responses [105]. Using this technique, short electrical pulses are given on the site of interest, which leads to a more efficacious immune response due to enhanced cell membrane permeability and better absorption of the antigen [96,97]. They have created two plasmids viz. pGX9501 and pGX9503 encoding SARS-CoV-2 IgE-spike protein for better transfection efficacy and immunogenicity which is further characterized *in vitro* by RT-PCR and western blotting [44]. Further, it was characterized in mice that demonstrated both B cell and T cell mediated immune response. In preclinical testing in guinea pigs neutralizing antibodies and T-cell responses were generated [44]. Anti-SARSCoV-2 binding antibodies were detected in lung washes of INO-4800-immunized mice and guinea pigs [96]. The outcomes of the phase I trial results were published in The Lancet's preprint server in December 2020 and noted that INO-4800 induced good safety and tolerability and elicited immune responses in all 38 vaccinated individuals [97]. On 23 April 2021, Inovio announced that given the universal eligibility for authorized COVID-19 vaccines in the US, the Department of Defense Joint Program Executive Office for Chemical, Biological, Radiological, and Nuclear Defense had discontinued funding for the candidate's phase III trial (NCT04642638) [106].

The third DNA vaccine in Phase III clinical trial is AG0301/AG0302 developed by AnGes, Takaro Bio, and Osaka University, and utilizes pVAX-1 as plasmid vector along with alum as an adjuvant and injected intramuscularly [107]. A phase I/II trial of the AG0301 vaccine is planned to recruit 30 healthy individuals in a non-randomized open-label non controlled study to determine its safety and immunogenicity (NCT04463472). The start date was in June 2020 with primary completion date in September 2020 and study end date 2021. The AG0302 vaccine is also recruiting 30 health individuals with 2 mg dose at either 2 or 4 week intervals, and the completion data is expected to be by September 2021 (NCT04527081) [98,108].

4. Concluding remarks

In order to eradicate the COVID-19 pandemic, there is an urgent need of an efficient vaccine against it. DNA vaccines are part of the cutting edge vaccination strategy that doesn't require carrier and the viral protein encoding gene are transfected to *in vivo* cell processing to induce adaptive immunity. DNA vaccination does have a strong safety and efficacy profile in comparison to many other vaccine platforms because DNA plasmids need not replicate or elicit vector-directed immune responses in hosts. Once the DNA is inside host cells fragment are transcribed into an RNA capable of inducing protein formation. Using the viral Spike protein from SARS-CoV-2 which serves as the virus's entrance key, to enter host cells is an obvious target for neutralizing antibody formation. Conventional vaccines could be replaced with DNA vaccines due to their potential to induce humoral and cellular immune responses. The fundamental idea behind DNA vaccines is to use a DNA plasmid that encodes for a protein from the virus. Plasmid DNA is cheap, durable, and clean, making the non-

viral platform a viable alternative for gene delivery. Amongst the vaccines in clinical trials for COVID-19, there are a number of vaccines that are DNA-based. Patient with mild to moderate symptoms of SARS-CoV-2 does not result in long-lasting humoral immunity, implying that an effective COVID-19 vaccine would induce a more significant immune response than a typical infection. Also, it is an ideal platform to deal with COVID-19 where the virus is continuously mutating as the vaccine is based on plug and play technology and thus make it adaptable to deal with it. Thus, due to good clinical safety profile, low manufacturing and transportation costs, and ability to induce strong immunity, plasmid DNA-based vaccines show promise as an effective vaccine strategy against COVID-19.

5. Expert opinion – Future prospects

The plasmid DNA vaccine platform is thought to have many complementary properties that make it a suitable choice for COVID-19. The DNA plasmid manufacturing process enables modular product development and can avoid the complexities of traditional vaccine productions, cell cultures, purification and scale-ups [44]. DNA vaccines are attractive as they are usually non-frozen and are stable for a number of years at 2° C–8°C and room temperatures and about 1 year at 37°C, whilst preserving effectiveness at temperatures up to 60°C [109]. The potential of these vaccine candidates to elicit humoral and cellular immune responses makes them an appealing candidates in vaccine formulations. With continuous vaccine formulation advancements in DNA Vaccine; it is thoroughly evaluated at developmental as well as during clinical development. Many such vaccines have been approved for veterinary use. This can be a breakthrough path for the strong commercial viability of DNA vaccine for human use, defining impactful research in this area. Using this platform, a number of vaccines have quickly be developed against SARS-CoV-2 virus and translated into human clinical trials with promising outcomes. While the slow vaccination rate in industrialized economies points to the need for simpler vaccination methods, DNA-based vaccines may alleviate these limitations of conventional vaccination procedures, especially in resource-limited settings. However, DNA-based vaccines for SARS-CoV-2 are currently unavailable, owing to a lack of existing manufacturing and regulatory infrastructure required for rapid production. Continued and increased investment in emerging vaccine delivery systems, such as DNA vaccines, is required to ensure that the technologies and facilities meet potential pandemic needs. On 20 August 2021, Drug Controller General of India approved ZyCoV-D from Zydus Cadila with EUA for people 12 years and above and it is the first DNA vaccine ever approved for mankind [110].

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Author contributions

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 - **It highlights the studies conducted on animal models and the results obtained of ZyCoV-D DNA vaccine. Focus on pre-clinical aspect of development.**