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REVIEW

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Development of synthetic antigen vaccines for COVID-19

Maria da Conceição Viana Invenção[a*], Alanne Rayssa da Silva Melo[a*], Larissa Silva de Macêdo[a], Thaís Souto Paula da Costa Neves[a], Cristiane Moutinho Lagos de Melo[b], Marcelo Nazário Cordeiro[a], Marcus Vinicius de Aragão Batista[c], and Antonio Carlos de Freitas[a]

[a]Laboratory of Molecular Studies and Experimental Therapy – LEMTE, Department of Genetics, Federal University of Pernambuco, Recife, Pernambuco, Brazil; [b]Laboratory of Immunological and Antitumor Analysis, Department of Antibiotics, Bioscience Center, Federal University of Pernambuco, Recife, Pernambuco, Brazil; [c]Laboratory of Molecular Genetics and Biotechnology, Department of Biology, Federal University of Sergipe, São Cristóvão, Sergipe, Brazil

ABSTRACT
The current pandemic called COVID-19 caused by the SARS-CoV-2 virus brought the need for the search for fast alternatives to both control and fight the SARS-CoV-2 infection. Therefore, a race for a vaccine against COVID-19 took place, and some vaccines have been approved for emergency use in several countries in a record time. Ongoing prophylactic research has sought faster, safer, and precise alternatives by redirecting knowledge of other vaccines, and/or the development of new strategies using available tools, mainly in the areas of genomics and bioinformatics. The current review highlights the development of synthetic antigen vaccines, focusing on the usage of bioinformatics tools for the selection and construction of antigens on the different vaccine constructions under development, as well as strategies to optimize vaccines for COVID-19.

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The current pandemic called COVID-19 caused by the *SARS-CoV-2* virus is responsible for over 200 million cases and 4 million deaths.[1] It has also brought the need for new political, economic, and social perspectives which maximize the search for fast alternatives to both control and fight the SARS-CoV-2 infection. Therefore, a race for a vaccine against COVID-19 took place, and in less than a year, some of the studies have reached phase 3 of vaccine trials as well as some others have been approved for emergency use in several countries.[2,3] Ongoing prophylactic research has sought faster, safer, and precise alternatives that can be reached by redirecting knowledge of other vaccines that already exist for other diseases, and/or the development of new strategies using available tools, mainly in the areas of genomics and bioinformatics.[4] The current review highlights the development of synthetic antigen vaccines, focusing on the usage of bioinformatics tools for the selection and construction of antigens on the different vaccine constructions under development, as well as strategies to optimize vaccines for COVID-19.

Vaccine development landscape in the context of COVID-19

Vaccines are excellent tools in controlling infectious diseases and preventing humanitarian epidemics crisis by inducing the establishment of an immune response capable of quickly controlling and eliminating pathogens. This long-term protection is usually characterized by antibody persistence and cell-mediated immune response.[5] As a result, vaccines are the main prophylactic alternative to prevent the spread of COVID-19.[6] There are currently 185 candidates being evaluated during the pre-trial vaccine and 102 with eight different technology platforms under clinical evaluation[7] (Table 1). So far, 17 vaccines have been approved for use in humans in several countries.

Many laboratories have invested in more modern vaccine strategies besides older vaccine platforms such as the attenuated or inactive virus, especially during the COVID 19 pandemic. A survey carried out in silico by Defrancesco2 showed that several vaccine platforms are being tested, such as protein subunit vaccines, virus-like particle vaccines, DNA- and RNA-based vaccines, viral vector-based vaccines, among other strategies.

Nucleic acid vaccines are new and versatile strategies that use recombinant DNA technology for immunization or immunotherapy. They consist of viral vector-based vaccines, in which a virus unrelated to the pathology, live or inactive, carries the genetic material of the target antigen, along with DNA- and RNA-based vaccine platforms, in which the gene sequence (of one or more genes) encoding the protein of the pathogen of interest will be delivered as a vaccine. Another alternative used in these nucleic acid approaches is the use of epitope coding sequences whose immunogenicity is rigorously selected *in silico*, in the so-called synthetic antigen vaccine. In this review, we will focus on these synthetic antigen vaccines, which are an interesting strategy since they can combine one or more antigens from the same pathogen or even from different variants in the same vaccine.[27,28] In this review, we will focus on DNA and mRNA vaccine platforms, especially multiepitope ones that use synthetic antigens.

CONTACT Antonio Carlos de Freitas acf_ufpe@yahoo.com.br Laboratory of Molecular Studies and Experimental Therapy – Lemte, Department of Genetics, Center for Biological Sciences, Federal University of Pernambuco, Av. Moraes Rego, Cidade Universitária, Recife, Pernambuco, Brazil.
*These authors contributed equally to this work.

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Table 1. Main vaccine candidates that are in phases 2/3 of clinical trials or have been approved for emergency use to date.

Platform	Description	Advantages	Disadvantages	References
Inactivated	Viral pathogens inactivated by chemical agents or radiation	Easy to prepare Safer in relation to attenuated vaccines.	Variable Efficacy Need for large-scale cultivation of highly pathogenic organisms under biosafety level 3 (BSL3) Requirement of strong protocols in quality control	
Vaccine Candidates:	Sinovac (CoronaVac) Sinovac Life Sciences and Butantan Institute. China/Brazil Type of vaccine: inactivated virus inoculated in African green monkey kidney cells (Vero cells). Antigen: SARS-CoV-2 (CN02 strain) Number of doses: 2; Dosing schedule: 0, 14 days; Administration Route: IM Phase: III; Age range (years): 18 or older Description: Good tolerance to low, medium, and high dose groups. No serious adverse event related to the vaccine was reported. Both the seroconversion and the level of GMT for elderly volunteers were comparable to adult age groups between 18 and 59 years old. The World Health Organization gave emergency authorization to the vaccine on June 2021. Currently, this vaccine is approved in 26 countries. Efficacy: 50,65% in Brazil trial and 91,25% in Turkey trial			8
	Inactivated novel coronavirus vaccine Wuhan Institute of Biological Products/Sinopharm. China Type of vaccine: inactivated virus cultivated in a qualified Vero cell line. Antigen: SARS-CoV-2 (WIV04 strain, National Genomic Data Center of the Chinese Academy of Science accession No. SAMC133237, and GenBank accession number MN996528) Number of doses: 2; Dosing schedule: 0, 21 days; Administration Route: IM Phase: III; Age range (years): 18 or older Partial Results: The results in phases I and II showed that the inactivated vaccine was demonstrated immunogenicity and well-tolerated in all dose groups under different injection procedures with no vaccine-related serious adverse events. Efficacy and adverse event data in phase III studies have been released. Currently, China is the only country that has approved the use of this vaccine for general use. Efficacy: 72,8%			9
	Inactivated novel coronavirus vaccine (BBIBP-CorV) Beijing Institute of Biological Products/Sinopharm. China Type of vaccine: inactivated virus cultivated in a qualified Vero cell line. Antigen: strain 19nCoV-CDC-Tan-HB02 (HB02) Number of doses: 2; Dosing schedule: 0, 21 days; Administration Route: IM Phase: III; Age range (years):18 to 85 Partial results: In the phase I/II studies, the inactive vaccine BBIBP-CorV administered as a two doses immunization was safe and well-tolerated, allowing people generate antibodies against the coronavirus. A robust humoral response was observed in 100% of vaccine receptors. In December of 2020, Sinopharm announced vaccine approval. The company has yet to publish the detailed results of its Phase III trial. The vaccine is currently approved for emergency use in more than forty countries, of which three have released definitive registration: Bahrain, China, and the United Arab Emirates. Efficacy: 78.1%			10
Non-replicant recombining viral vector	Unrelated virus, designed to encode the target gene of the pathogen. Viral vectors can be replicating or non-replicating	Induces high cell and humoral immune responses	Possible preexisting immunity against vector Virulence reversion risk Limitations to increase production	

(*Continued*)

Table 1. (Continued).

Platform	Description	Advantages	Disadvantages	References
Vaccine Candidates:	AZD1222 (ChAdOx1-S, Vaxzevria, or Covishield in India) University of Oxford/AstraZeneca. The United Kingdom Type of vaccine: Simian adenoviral vaccine vector. Antigen: Spike protein; Number of doses: 1; Administration Route: IM Phase: III; Age range (years): 18 to 55 Partial Results: This vaccine showed in phase I/II studies an acceptable safety profile and increased antibody responses with homologous reinforcement. These results allowed the evaluation on a large scale of this vaccine candidate in a phase III program. The vaccine has a register of approval in Brazil and a license for emergency use in over 70 countries. countries. Efficacy: 76% in a U.S. study			11–13
	Ad5-nCoV (or Convidecia) CanSino Biological Inc./Beijing Institute of Biotechnology. China Type of vaccine: Adenovirus Type 5 vector. Antigen: Spike protein Number of doses: 1; Administration Route: IM Phase: III; Age range (years): 18 or older Partial Results: The researchers published promising results from a Phase I safety trial. The phase II trial was started before the full analysis of the data from the phase I study was available. The COVID-19 vaccine with Ad5 vector in 5×10^{10} viral particles showed to be secure in phase II essays and induced significant immune responses in most receptors after a single immunization dose. In this study, most reactions reported post-vaccination were mild or moderate. Starting in August 2020, CanSino began running Phase 3 trials in some countries. In February 2021, China announced the approval of the CanSino vaccine for general use, and four other countries have approval for emergency use for this vaccine. Efficacy: 65,28%			14,15
	Gam-COVID-Vac (or Sputnik V) Gamaleya Research Institute Janssen. Russia Type of vaccine: Recombinant adenovirus type 26 (rAd26) (component I) + recombinant adenovirus type 5 (rAd5) (component II). Antigen: SARS-CoV-2 full-length spike glycoprotein coding gene Number of doses: 2; Dosing schedule: 0 (component I), 21 (component II) days; Administration Route: IM Phase: III; Age range (years): 18 or older Partial Results: The results published of phase I/II non-randomized studies of a heterologous prime-boost COVID-19 vaccine based on rAd26 and rAd5 vectors are safe and immunogenic in healthy adults. All reported adverse events were mostly mild. The results of the phase III trial show that the vaccine-induced robust humoral (n = 342) and cellular (n = 44) immune responses in all age groups. The vaccine is currently approved for emergency use in more than 60 countries. Efficacy: 91.6%			16,17
	Ad26.COV2.S (or JNJ-78436735) Janssen Pharmaceutical Companies of Johnson & Johnson. USA Type of vaccine: Ad26.COV2.S (a non-replicating adenovirus 26 based vector). Antigen: The stabilized pre-fusion spike protein of SARS-CoV-2 Number of doses: 2; Dosing schedule: 0, 56 days; Administration Route: IM Phase: III; Age range (years): 18 or older Partial results: Phase I/II tests showed satisfactory results in the safety profile and immunogenicity after only a single dose. The results of phase III tests were also satisfactory. This vaccine is currently approved for emergency use in more than 40 countries. Efficacy: 72% in The United States, 68% in Latin America, 64% in South Africa			18
Subunit vaccines	Antigen components of the target protein produced in the laboratory	High-scale production Safety	Low immunogenicity and may require the use of adjuvants or repeated doses High cost	

(*Continued*)

Table 1. (Continued).

Platform	Description	Advantages	Disadvantages	References
Vaccine Candidates:	SARS-CoV-2 rs/NVX-CoV2373 Novavax. USA Type of Vaccine/antigen: Full-length recombinant SARS CoV-2 Spike glycoprotein nanoparticle vaccine adjuvanted with Matrix M™ Number of doses: 2; Dosing schedule: 0, 21 days; Administration Route: IM Phase: III; Age range (years): 18 to 84 Partial Results: Phase I/II essays showed that the NVX-CoV2373 showed acceptable safety results and induced high immune responses. The Matrix-M1 adjuvant induced responses of CD4 + T cells that were influenced toward a Th1 phenotype. The general reactogenicity was practically absent or mild, and the second vaccinations were neither suspended nor delayed due to reactogenicity. Novavax's vaccine is one of several being tested in an Oxford study that gauges how well alternating doses can boost immunity. The vaccine is in phase III clinical trial in some countries. This vaccine is not yet approved and the results are in progress. Efficacy: 96% against original coronavirus; 86% against B.1.1.7 and 49% against B.1.351			19
DNA vaccines	DNA encoding the target antigen	Rapid large-scale vaccine construction and production Good cost-benefit, reproducible, noninfectious	It naturally has low immunogenicity It may require certain approaches for administration as electroporation devices and the use of adjuvants	
Vaccine Candidates:	INO-4800 Inovio Pharmaceuticals/ International Vaccine Institute Type of vaccine: DNA plasmid + EP (CELLECTRA® 2000 device). Antigen: plasmid pGX9501 expressing a sequence of the SARS-CoV-2 full-length spike glycoprotein Number of doses: 2; Dosing schedule: 0, 28 days; Administration Route: ID Phase: II–III (combined phases); Age range (years): 18 to 64 Partial Results: The initial data from a Phase I study did not reveal any serious adverse effects and measured an immune response in all 38 volunteers. INO-4800 was well tolerated and safety data further suggest that the vaccine can be safely boosted since there was no increase in the frequency of side effects after the second dose. Phases II and III are currently being carried out in some countries. Efficacy: data not available AG0301-COVID19 Osaka University/ AnGes, Inc./ Takara Bio. Japan Type of vaccine: DNA plasmid vaccine + Adjuvant; Antigen: spike protein Number of doses: 2; Dosing schedule: 0, 14 days; Administration Route: IM Phase: II–III (combined phases); Age range (years): 20 to 65 Partial Results: study not reported Efficacy: data not available			20
mRNA vaccines	The mRNA encoding the target antigen. It is usually complexed with lipids or polymer-based nanoparticles	It is easy, fast, scalable, and economical to produce Once in the cytoplasm of the cell, the vaccine is ready for translation and does not need to reach the nucleus	It naturally has low immunogenicity and presents high instability It may require specific storage conditions at very low temperatures	
Vaccine Candidates:	mRNA-1273 Moderna/NIAID. USA Type of vaccine: LNP-encapsulated mRNA. Antigen: Lipid-nanoparticle (LNP)–encapsulated mRNA vaccine expressing the prefusion-stabilized spike glycoprotein Number of doses: 2; Dosing schedule: 0, 28 days; Administration Route: IM Phase: III; Age range (years): 18 or older Partial Results: In the initial phase I/II trials, the mRNA-1273 vaccine induced anti-SARS-CoV-2 immune responses in all participants, and no trial-limiting safety concerns were identified. In addition, in a small study involving older adults, the adverse events associated with the mRNA-1273 vaccine were mostly mild or moderate. In the phase III trial, the mRNA-1273 vaccine showed 94.1% efficacy in preventing Covid-19 illness, including severe disease. Aside from transient local and systemic reactions, no safety concerns were identified. The vaccine is currently approved for use in Switzerland and emergency use in more than twenty countries. Efficacy: More than 90%			21,22

(*Continued*)

Table 1. (Continued).

Platform	Description	Advantages	Disadvantages	References
	BNT162b1/BNT162b2 (or Comirnaty, and tonizameran) BioNTech/Fosun Pharma/Pfizer. Germany/USA Type of vaccine: 3 LNP-mRNAs. Antigen: Lipid nanoparticle–formulated with SARS-CoV-2 full-length spike protein Number of doses: 2; Dosing schedule: 0, 28 days; Administration Route: IM Phase: III; Age range (years): 16 or older Partial Results: Phase I/II essays show two versions of an mRNA vaccine. The version BNT162b2 was associated with a lower incidence and severity of systemic reactions than that of BNT162b1, particularly in the elderly. In younger and older adults, both vaccine candidates produced SARS-CoV-2 GMT depending on similar doses, which were similar or superior to the geometric mean titer of a sample panel with convalescent SARS-CoV-2 serum samples. The data presented in the phase III trial showed that a two-dose regimen of BNT162b2 conferred 95% protection against Covid-19 in subjects 16 years of age or older. The safety profile of BNT162b2 was characterized as mild-to-moderate, and the incidence of serious adverse events was low, with similarity in the vaccine and placebo groups. The vaccine is currently approved for use in Bahrain, Brazil, New Zealand, Saudi Arabia, and Switzerland. The emergency use of the vaccine covers more than 50 countries. Efficacy: 91,3%			23–28
	CVnCoV Curevac. Germany Type of vaccine: mRNA. Antigen: SARS-CoV-2 spike protein Number of doses: 2; Dosing schedule: 0, 28 days; Administration Route: IM Phase: III; Age range (years): 18 or older Partial Results: study not reported Efficacy: data not available			

IM = intramuscular; EP = electroporation; ID = intradermic; GMT = Geometric Means Titer from detected antibodies.

Vaccination with non-viral delivered nucleic acid-based approaches has the potential of combining the advantages of live-attenuated vaccine platforms and subunit vaccines, however with no need for cultivation of highly pathogenic organisms on a large scale under biosafety level 3 (BSL3). Furthermore, the inactivation process of viral vaccines can modify the structure of epitopes present in inactivated virus vaccines, which does not occur with nucleic acid approaches. Moreover, because they have no viral particles in their constitution, they do not offer viral reactivation risks, thus providing an excellent option for vulnerable populations, including pregnant women, the elderly, infants, and immunosuppressed people.[29] Table 1 provides a brief description of the different vaccine platforms used against COVID-19 with their advantages and disadvantages.

Another advantage of the next-generation approaches is the much faster and more versatile production of the immunogen. This production makes these platforms ideal for the current chaotic pandemic situation, in which it is necessary to produce billions of doses simultaneously. Another aspect is that, although nucleic acid vaccines have limited coded gene information capacity compared to inactive or attenuated virus vaccines, such synthetic antigens are predicted to be more immunogenic and, because of their reduced size, there is the possibility of combining epitopes from different viral strains in the same vaccine, in addition to working with several vaccine targets simultaneously.

About the flexibility of synthetic antigen vaccines, once the manufacturing process is established, a similar process can be applied to produce a different vaccine by simply replacing the viral antigen coding region with a new insert. Such flexibility makes this vaccine platform ideal for controlling the current pandemic since there is a great possibility of the emergence of new viral variants resistant to the current vaccines in the near future, a situation that requires rapid adaptation of the vaccine. During the construction of synthetic gene, it is possible to evaluate the epitopes conservancy in front of the new coronavirus lineages from the United Kingdom (B.1.1.7),[30] South Africa (B. 1.351),[30] Brazil (B.1.1.248 – P.1 and P. 2),[30,31] India (B.1.617 – B.1.617.1, B.1.617.2 and B.1.617.3),[30,32–34] USA (B.1.427 and B.1.429)[30,35] and Nigeria (B.1.525),[36] as well as its variants. The immunoinformatics tools that work with this analysis will be more detailed in the topic Epitope Conservation analysis.

mRNA vaccines were the first group of platforms approved for emergency use against COVID-19, also representing the platform with the highest levels of effectiveness among all vaccine platforms to date. Although multiepitope vaccines have not registered clinical trials to date, they are still in the immunoinformatics approach phase.[37–39]

Most candidate vaccines developed to control *SARS-CoV-2* infection have the structural antigen S (total length or specific subunits) as their main target. The S glycoproteins are the main responsible for interaction and viral entrance into host cells and based on research on *SARS-CoV* and *MERS-CoV*, a strong neutralizing effect was associated to trigger specific cell T responses and neutralizing antibodies, which makes this protein an excellent vaccine target.[40,41] Other targets can also be incorporated into multiepitope vaccines, like viral proteins

such as E protein, which forms the viral envelope and can be found in higher concentrations during replication of the virus. It can also interact with some cellular proteins and, after the virion construction process, it can break the cell membrane and release the pathogen to the extracellular environment[42,43] which may contribute to the presentation of this antigen to immune system cells. The M protein, in turn, is a membrane protein that is also associated with viral assembly and its specific phosphorylation sites can interact with the host.[43,44] While the protein N remains associated with the genetic material of *SARS-CoV-2* being related to the viral transcriptional and translational apparatus.[43] In addition, Mu et al.[45] reported that it can also act in immune system evasion.

Although little explored in studies involving vaccines against COVID-19, accessory proteins can be potential targets for future vaccine constructions based on their importance in the viral construction and how it deals with the immune response from infection. ORF1ab is a polyprotein that is part of the virus replication apparatus. To become functional after entry into the cell, it is cleaved into 11 non-structural proteins that have different functions, being nsp1 known for the possible ability to evade the immune system.[43,46] While ORF3a is an ion carrier protein that may be related to the development of the inflammatory process of COVID-19 due to the promotion of cytokine storm besides virulence and viral replication.[47–50] ORF6 was considered the protein that showed the highest immunosuppression of primary interferon and its signaling.[43] ORF7a, on the other hand, is a protein that acts together with nsp1 and nsp3c in a probable interference in the innate immune response.[43,51] Furthermore, mutations in this region should receive greater attention considering that this protein can act as a virulence factor.[43] The ORF8, in turn, is a protein that is either related to the pathogenicity or the coronavirus replication apparatus, acting in the interferon pathway of the host. It may also affect the recognition of cytotoxic T lymphocytes by interfering with presentation via MHC and thus evading the immune system,[43,52–54] which allows them to explore its use for humoral immune response activation.

The nucleic acid vaccines can stimulate different arms of the immune response through cross-presentation pathways. The intracellular antigens produced by these vaccines are processed through the endogenous pathway and, therefore, are capable of generating a specific cellular response while still generating antibodies. Besides, synthetic antigen vaccines allow the directioning of immune response by including in the vaccine construct epitopes recognized by B lymphocytes, and MHC-I (cytotoxic response) or MHC-II ligands (helper response). After translation in the cytoplasm, these antigens are generated by proteolysis within the proteasome, followed by their entry into the endoplasmic reticulum via TAP transporter for cell surface presentation. Meanwhile, activation of the helper response occurs via the endocytic pathway, in which somatic cells transfected at the injection site produce the vaccine peptides and these, in turn, can be engulfed by DCs or internalized as apoptotic bodies. Furthermore, such peptides released into the extracellular environment can be directly recognized by B cells or even be presented to these cells via a helper response. More details on all activation pathways generated by nucleic acid approaches, including cellular and humoral responses, can be found in Figure 1.

Given the importance of correct processing for the generation and presentation of vaccine epitopes, it is essential to include spacer sequences (also known as linkers) between epitopes in the vaccine construct to provide proteasomal cleavage and TAP binding sites.[55] In addition, other linker sequences perform various other functions such as addressing and activating specific routes within cell compartments, more details can be found in Table 2. Meanwhile, the schematic representation of a synthetic multi-epitope vaccine construct containing linker sequences can be seen in Figure 2. Another important in synthetic antigen vaccines is the stability of the antigen after intracellular processing. This analysis is performed using immunoinformatics approaches to each epitope of the vaccine construct. More details of this analysis will be discussed later in the topic of molecular docking analysis and molecular dynamics simulation.

Development of nucleic acid approaches using immunoinformatics tools

One of the approaches used in the production of genetic vaccines is the usage of Immunoinformatics tools.[67] *In silico* analysis is becoming more important each day, especially because of the pandemic, the lack of financial resources, and the need to construct a vaccine in a short amount of time. Thus, the search for free computational tools became a viable alternative, capable of minimizing the possible limitations that the traditional methods, both in vitro and in vivo, of vaccine construction demand, such as the need for experiments that are not only time-consuming but need a good laboratory infrastructure, which is very expensive.

Advancements in bioinformatics contributed to the development of new tools for the analysis of protein compounds with drug potential and the assistance in vaccine construction.[67] It was noted that after the first *SARS-CoV-2* genetic sequence was deposited in GenBank,[68] many studies were able to use these computational tools during the pandemics.[4,69–75] Therefore, it is possible to believe that immunologic bioinformatics tools, also named immunoinformatics approaches, tend to grow even more after the pandemic.

In silico analysis encompasses a wide range of production steps for a gene vaccine against COVID-19, such as the prediction of epitopes, immunogenicity and conservation analysis, populational coverage evaluation, molecular docking, and molecular dynamics simulation of the epitope-MHC complex.[76] These analyses allow the selection of epitopes that potentially are more effective,[77] which is less time-consuming when compared to in vitro screening.

It is possible to build a synthetic multiepitope gene that will be further validated in vitro and in vivo in order to be used in the vaccine trials (Figure 3). This synthetic gene, when transcribed and translated by cells, will act as a synthetic antigen, which will hopefully be recognized by the immune system, activate the T and B lymphocytes, and produce antibodies.[78] Following, there is a list of steps and tools used in the construction of a synthetic antigen.

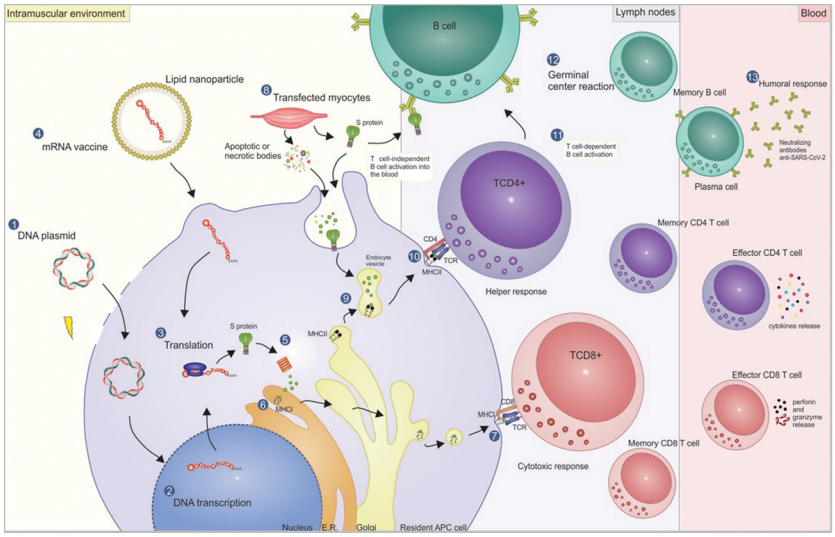

Figure 1. Mechanism of action of DNA and mRNA vaccines and the pathways for activating the cellular and humoral response. DNA vaccines are commonly delivered by electroporation through transient pores formed in the membrane (1). Thus, the DNA reaches the cell cytoplasm and then the nucleus, where it will be transcribed (2). Then the mRNA goes to the cytoplasm, where it is translated in the vaccine peptide (3). Another strategy is the direct delivery of the mRNA (mRNA vaccine) encapsulated in lipid nanoparticles in the cell cytoplasm (4). After the endosome escape, the mRNA is translated in the cytoplasm, followed by the vaccine antigen processing in the proteasomes (5), where they are cleaved into smaller peptides. Next, the peptides are transported by the TAP transporter (not shown) into the endoplasmic reticulum, where they are linked to the MHC-I (6) for TCD8 lymphocyte presentation at the cell surface (7), activating the cytotoxic response and generating effective and memory cells. While the cytotoxic response is triggered through the processing of intracellular antigens, the helper response, as a general rule, is triggered through the exogenous pathway, in which transfected somatic cells – such as myocytes at the injection site – produce the vaccine peptide (8). The peptides can be released outside the cell and be directly engulfed by DCs, or they can be internalized by the apoptotic or necrotic bodies, provoked by an inflammatory environment caused by the electroporation. Thus, the fusion of endocytic vesicles – containing the peptides processed by the lysosomal pathway – with vesicles containing MHC-II molecules of DCs (9), allows the presentation of epitopes to the TCD4 lymphocytes at the cell surface (10), with the activation of helper response and generation of memory cells. The TCD4+ lymphocytes, in turn, play a fundamental role in the activation (11) and maturation of B cell affinity inside the germinal centers (12) for the activation of the humoral response (T cell-dependent B cell activation) generating plasmatic cells that can produce high-affinity neutralizing antibodies, as well as memory cells. Another possible activation pathway for humoral response, but with the induction of a weaker immune response, is the direct linkage to the vaccine antigen with B cell receptors (BCRs) (T-cell independent B cell activation).

Table 2. Usage of linker sequences in different studies with the aim to ensure the correct processing/directing of peptides in multiepitope vaccines.

Linker Sequence	Description	Reference
Ubiquitin	The introduction of the coding sequence of the ubiquitin gene at one extremity of the vaccine construction aims to favor the peptide degradation by proteasomes during the epitope-specific CTL response	56–58
GPGPG	The introduction of this spacer between MHC-II binding epitopes in multiepitope vaccine construction promotes the disruption of junctional epitopes in these vaccines, restoring immunogenicity against the target epitopes during helper response	55,59–61
EAAAK	It consists of a helical linker to control the distance and reduce the interference between the domains of functional proteins with other protein regions in the vaccine construction. Thus, it is ideally incorporated into N and C-terminal of B cell conformational epitopes	59,60,62
ALL and SSL	These linkers are expected to direct the cleavage to the C-terminus of the preceding peptide and to the N-terminus of the next peptide	63
RKSYL and RKSY	Similar to the previous sequence, these motifs are expected to direct the cleavage to the C-terminus of the proceeding peptide, but enable a more flexible cleavage at the N-terminus of the next peptide with multiple potential cleavage sites, optimizing binding to TAP transporter	63
KFLRQY; ADRIW; ADKQW; ADRQW; ADNQY; AKRW; ADNIW.	The initial amino acids of each of these flanking sequences aim to optimize the processing and release of epitopes by the proteasome, and, after cleavage, the following amino acids provide binding sites to TAP transporter	57
ARY	This sequence is a high-affinity motif for TAP recognition based on the preferences of human TAP for flanking of epitopes in the polyepitope construct	64
R/K-R/K	The introduction of a dibasic motif flanking MHC-II binding epitopes in a polyepitope construct enhances its processing, since these motifs represent cleavage sites for lysosomal cathepsins B and L, thus optimizing helper response activation	56,65
RKRSHAGYQTI; YQTI	This sequence represents the C-terminal tyrosine-based motif of LAMP-1 (lysosome-associated membrane protein-1) glycoprotein and its function is to direct the immunogen from the secretory pathway to lysosomes for degradation, where the peptide fragments bind to MHC class II molecules. Thus, this strategy allows the redirecting of gene vaccines activation route for the activation of the helper response as well	56,66

Epitope prediction

The GenBank (https://www.ncbi.nlm.nih.gov/genbank/), at the National Center for Biotechnology Information (NCBI), is a database of genetic sequences known worldwide, where nucleotide sequences for a wide range of organisms can be found. In addition, NCBI has a database for amino acid sequences, the Protein Database (https://www.ncbi.nlm.nih.gov/protein). The availability of amino acid sequences

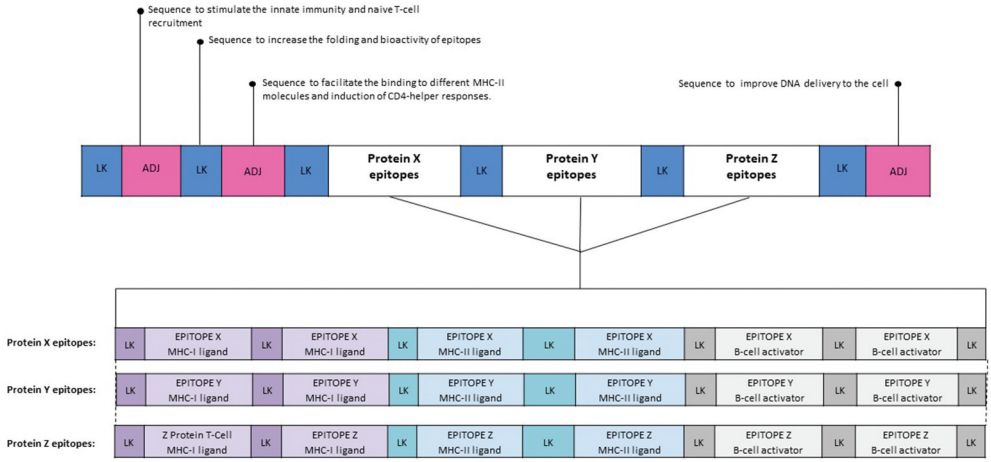

Figure 2. Structure of a hypothetical synthetic multiepitope vaccine construct containing adjuvant and linkers sequences. In this example, the construct contains sequences that act as adjuvants, which are capable of increasing the immunogenicity of nucleic acid vaccines. Moreover, linker sequences were added between each epitope in order to provide proteasomal and lysosomal processing sites, and TAP transporter binding sites. Concerning the epitopes, in this construction MHC-I, MHC-II ligands, and linear B cell epitopes were added in order to induce both cellular and humoral responses. The epitopes shown in purple are intended for binding to MHC-I molecules and must have between 8 and 11 amino acids. In light blue, the MHC-II ligands are found, these must feature more than 11 amino acids. Meanwhile, the epitopes for B cell activation are shown in gray and contain larger-sized epitopes, up to about 16 aa. LK: Linker, ADJ: Adjuvant.

Table 3. In silico methods to predict T cells epitopes.

Method	Description	Reference
Artificial Neural Network (ANN)	Corresponds to a system similar to the brain neural connection, where each cell receives a signal and sends it to another cell. The union between these cells works as a network In an ANN, each cell would be a knot that contains a kind of analysis. One or more entry information are inserted and pass through this knot, resulting, at the end of the network, in different exit information Example: An ANN to predict epitopes creates layers with weights that correspond to characteristics related to the binding affinity between the peptide and the MHC. Thus, by identifying the presence of a certain characteristic, the software goes to the next knot in the network to verify the status of that peptide in relation to another characteristic, and so forth, forming something similar to a status matrix with n characteristics	80,85
NetMHCpan 4.0	Uses an ANN method to predict epitopes using peptide sequences as entry information, and the exit information is generated from the binding affinity data and elution of linkers with mass spectrometer. This method structure is pan because it analyzes just one model, HLA data (Human MHC), and the peptide length	80
Stabilized Matrix Method (SMM)	It is a method that does specificity modeling of sequences of biological processes that can be quantified. When it comes to epitope prediction, it can be used to predict information regarding the peptide capacity to bind to MHC, TAP transport, and proteasome cleavage The entry data corresponds to amino acid or nucleotide sequences, where the coding is done binarily (0 or 1). To each nucleotide sequence, the weight of each residue that can occur in each position of the sequence will be multiplied. The result of this product is the value of prediction y. To measure the efficacy of the process, an experimental average y value will be generated	82–84,86
Support Vectors Machine (SVM)	Through machine learning and statistic learning theory, a model capable of recognizing linear and nonlinear data patterns is created. The data is classified by Kernel functions, linear, radial basis, string, and others For epitope prediction, the SVM is used in the differentiation among peptides that are T cell epitopes from those that are not epitopes	79
NetCTL	It is a prediction method by ANN that uses information about binding affinity, TAP efficacy, and peptide cleavage via proteasomes To measure the binding affinity of each peptide to the MHC-I, values are attributed to each peptide that is inside an interval that has extreme values 0 (low affinity) and 1 (high affinity) To predict cleavage through the proteasome pathway for residues that are used in the NetChop 2.0 C-term 2.0, NetChop C-term, and NetChop 20S-3.0 The TAP transport efficacy is measured through SMM	79,82,83,87–89
NetCTLpan	Epitope prediction in different vertebrate species (pan-specific), amongst which is the human species The NetCTLpan differentiation is the possibility of adjusting different parameters, such as choosing the species; selecting species-specific alleles, and for human studies, it is possible to choose the size of the peptide between 8 and 11-mer; allele selection that is more commonly found in the population; determining the minimal score limit for the prediction and the percentage to consider the prediction as positive (peptides are considered epitopes; defining the proteasome cleavage weights and TAP efficacy, and higher these weights are, higher the possibility of finding epitopes Prediction residues can be seen in two formats, using a graphic that shows the peptides in green as epitopes and in red as non-epitopes, and through a table that shows in columns the MHC prediction values, TAP efficacy, proteasome cleavage score, and the general/combined prediction, and ranking in crescent order of the prediction percentages of a set of peptides with a length of 9 amino acids	79,90
NetChop	Allows the choice of prediction methods named NetChop C-term 3.0 and NetChop 20S-3.0 and allows the alteration of limit score that might interfere with specificity and sensitivity The prediction results can be seen in a similar way to the NetCTLpan, differing only by the table visualization because it presents information related to amino acid residues	79
Consensus	Gather different epitope prediction methods in a single open approach, with the aim of obtaining the best performance of the peptide selection process to those considered epitopes	91

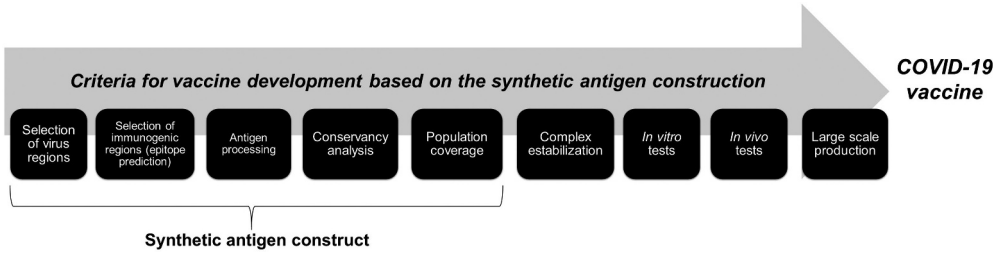

Figure 3. Summary showing, step by step, the criteria for the development of a COVID-19 vaccine through the construction of synthetic antigens.

for each protein of the new coronavirus enables the prediction of epitopes. This step is fundamental to the construction of a synthetic antigen that can be used in nucleic acid approaches against COVID-19 because it corresponds to the selection of peptides from virus proteins that could bind to MHC (major histocompatibility complex) molecules capable of inducing T (CD8+ and CD4+) and B cells activation.

The predictions can be carried out through different computational methods, such as Artificial Neural Networks, NetMHCpan, Stabilized Matrix Method (SMM), Matrix Vector Support (SVM), NetCL/NetCLpan/NetCHOP, Consensus,[79–85] among others (Table 3). Those methods are used in different databases and online servers, such as the Immune Epitope Database and Analysis Resource (IEDB) (https://www.iedb.org/), Virus Pathogen Resource (ViPR) (https://www.viprbrc.org/), NetCTLpan – 1.1,[90] NetMHCpan – 4.0,[80] NetMHCstab – 1.0,[92] NetMHCstabpan – 1.0,[93] NetCTL 1.2,[94] ProPred-I and ProPred,[95,96] RANKPEP,[97] among others.

Some of these tools and servers are already used in COVID-19 research, such as Abdelmageed et al.,[71] Rahman et al.,[75] and Dong et al.,[59] who have used the IEDB tools to select T cell epitopes, Ahmed et al.[70] used ViPR to predict T and B cell epitopes, Bhattacharya et al.[73] used the ProPred-I and ProPred to predict MHC-I and MHC-II linker epitopes, Grifoni et al.[98] did the epitope prediction of MHC-II using the NetMHCpan EL – 4.0 server, and Enayatkhani et al.[74] predicted MHC-I and MHC-II epitopes using the RANKPEP server in order to design a multiepitope vaccine against COVID-19.

These immunoinformatics tools available in the databases and servers demand that the type of human MHCs (HLAs) of interest is informed, so it can provide the epitopes for T CD8 + and CD4 + . For vaccines against COVID-19,[99] a list of HLAs with high affinity to *SARS-CoV-2* peptides was made available, displaying the worldwide amplitude that can be used in prediction tools. Some of the alleles that present a strong binding with these peptides were HLA-A*02:11, HLA-A*02:22, HLA-A*02:02, HLA-A*02:03, HLA-A*02:06, HLA-B*15:03, HLA-B*15:17, HLA-B*35:10, HLA-B*15:25, HLA-B*15:39, HLA-C*03:02, HLA-DRB1*01:01, HLA-DRB1*10:01, HLA-DRB1*01:04, HLA-DRB1*11:02, HLA-DRB1*13:01. All these alleles were capable of binding to more than 100 peptides. Besides these, other HLAs ligands to *SARS-CoV-2* can be found in the consortium formed during the pandemics, named COVID-19 HLA & Immunogenetics (http://www.hlacovid19.org/), which has a specific database for those who work with COVID-19. Another database containing HLAs of different populations worldwide is the Allele Frequency Net Database,[100] which was used by Moura et al.[76] to identify epitopes in the S protein of *SARS-CoV-2*.

According to the processing of peptides by the cell proteasome, the efficiency of its displacement by the TAP channel, and the binding capacity to HLAs molecules, it is possible to detect potential epitopes.[82] The NetChop-3.1 server[89] detects the peptide from the proteasomal cleavage sites, while the MHC I processing tool (Proteasome, TAP)[84] was used in the in silico design for the COVID-19 vaccine from S, M, and E proteins done by Rahman et al.,[75] which generates a ranking based on the potential of each T cell epitope.

The peptides that have a higher potential to be considered a T cell epitope must go through an immunogenicity analysis since not all peptides are immunogenic.[101] This analysis generally consists of an evaluation of the peptide capacity of inducing lymphocyte activation. It can be done using a tool available in the IEDB named Class I Immunogenicity,[102] as suggested by Kardani et al.,[103] or the C-ImmSim server,[104] as used by Dong et al.[59] for the construction of in silico multiepitope vaccine against COVID-19. It can also be done through the NETMHCpan – 4.0 server,[80] which was used by Moura et al.[76]

The general method for the prediction of B cells is based on the residual value and the informed quantity of amino acids around the residue. The amino acid amplitude capable of defining a peptide that has the antigenic potential varies between 5 and 7 amino acids. Rahman et al.[75] performed this analysis in their coronavirus studies using the ABCPred servers[105] and BepiPred-2.0.[106] The same methods are also available in the IEDB database, the Antibody Epitope Prediction (http://tools.iedb.org/bcell/), which was used by Bhattacharya et al.[73] and Grifoni et al.[98] The prediction tool available in the Virus Pathogen Resource (ViPR) (https://www.viprbrc.org/) was used in the *SARS-CoV-2* study done by Ahmed et al.[70]

From the predicted epitopes it is possible to identify their antigenic potential. In studies related to COVID-19, such as the ones done by Baruah and Bose,[72] Bhattacharya et al.,[73] Dong et al.,[59] Enayatkhani et al.[74] and Rahman et al.,[75] the antigenicity analysis was done through the VaxiJen server.[107]

Epitope clusters

It is possible to have sequence similarities among the predicted epitopes, thus allowing for clusters to be created. Clusters are groups that unite the epitopes that were predicted over the

same regions. This step avoids information redundancy regarding the same epitope. The Epitope Cluster Analysis[108] can be used in the design for the vaccine against COVID-19, focusing on cluster identification, which is available at the IEDB. This tool gathers epitopes that have over 80% similarity and defines the epitopes represented in each cluster. EpiMatrix and ClustiMer are also servers capable of identifying epitope clusters that can be used in vaccine constructions, as observed in the study of Scholzen et al.[109]

Epitope conservation analysis

Among the virus protein variants, the predicted epitopes can be conserved or not. Thus, in order to have a vaccine that prolonged immunity even when faced with different variants, it is important to verify the level of conservation of these epitopes and select those that have higher conservation levels.[103] The Epitope Conservancy Analysis tool,[110] available at the IEDB, can be used to identify the more conserved epitopes of T and B cells to be added in the multiepitope construction against *SARS-CoV-2*. This tool calculates a value referring to the level of conservation from a certain level of identity (obtained by the analysis of epitope clusters) and defines a ranking from the generated values.

Populational coverage analysis

Considering the importance of a vaccine capable of covering most of the population for containing the pandemic, it is vital to perform an analysis of the populational coverage. This analysis will verify the populations around the world and check for common alleles capable of interacting with the epitopes. Kardani et al.[103] mentioned different tools for in silico vaccine design against different pathogen microorganisms, amongst which is *SARS-CoV-2*, reporting the use of Population Coverage tool,[111] available at the IEDB. Abdelmageed et al.[71] and Rahman et al.[75] also used this tool to analyze the population coverage of predicted epitopes. This tool calculates the coverage fractions of HLAs for the populations.

The best results found in this phase can define whether more than one vaccine will need to be designed. Kibria et al.[112] demonstrated the importance of this analysis when they realized that it would be needed to design two vaccines at the end of the study because one of the epitopes predicted presented low coverage for the South African population (3.15%) when compared to another predicted epitope (40.9%). Therefore, it was necessary to design a vaccine exclusive for the South African population and another for the rest of the populations worldwide.

Molecular docking analysis and molecular dynamics simulation

The epitopes that presented higher populational coverage values can be used in a molecular docking analysis. The docking is performed to calculate the best pose and the binding energy between the predicted epitopes and MHC molecules. ClusPro,[113] PatchDock,[114] HADDOCK 2.4,[115] AutoDock 4.0 (http://autodock.scripps.edu/), CABS-dock[116] and ZDOCK 3.0.2[117] are some of the online servers used in many studies about COVID-19.[59,71–76]

For this kind of molecular docking, it is necessary to use 3D structures of the HLAs available at the Protein Data Bank (PDB) (https://www.rcsb.org/). Intending to aid COVID-19 studies, the PDB has a section exclusively for *SARS-CoV-2* structures. Bhattacharya et al.[73] used in their study for the design of a vaccine against the new coronavirus, the file with the docked complex so it can be visualized in PyMOL software (https://pymol.org/2/).

With the complexes formed with the peptides bound to HLA molecules, it is possible to perform a molecular dynamics (MD) simulation. This analysis assesses the stability of the peptide-HLA complex through a certain amount of time under specific temperature, pressure, ion presence, and water molecule conditions, simulating the conditions of the biological process related to the peptide-HLA binding complex. For that, the complex needs to remain stable during enough time for lymphocyte activation.[118]

NAMD (https://www.ks.uiuc.edu/Research/namd/) is one of the programs that performs molecular dynamics simulation, and the Visual Molecular Dynamics (VMD) program can be used to visualize its results (https://www.ks.uiuc.edu/Research/vmd/). Baruah and Bose[72] used these programs to perform an MD simulation to assess the stability of the complex peptide-MHC of T and B cells of glycoproteins on the surface of the new coronavirus. Dong et al.[59] used the server GROMACS (http://www.gromacs.org/) for MD simulations in their multiepitope vaccine constructions against COVID-19.

Reverse translation and synthetic antigen production

After filtering the epitopes that present higher stability in MD simulations, the amino acid sequences can be back translated into nucleotides, so a synthetic gene can be constructed. The Reverse Translate program[119] allows the back translation of amino acid sequences into nucleotides. These sequences, when put together, form a bigger sequence composed of nucleotides capable of synthesizing all selected epitopes. Therefore, it is possible to insert it into a plasmid vector, for example, configuring a gene vaccine. When it enters the organism, the body recognizes it as a synthetic antigen and activates the immune system, providing the necessary response to protect the person who was vaccinated.[78]

This construction step of the candidate vaccine structure against *SARS-CoV-2* was possible to be observed in the study of Enayatkhani et al.[74] who constructed the secondary structure of the vaccine using the server PSIPRED (http://bioinf.cs.ucl.ac.uk/web_servers/psipred_server/psipred_overview/) and in silico cloned it using the SnapGene software (https://www.snapgene.com/). Dong et al.[59] opted to use the JCat tool[120] to design their multiepitope vaccine against COVID-19.

The use of different computational tools for the prediction and analysis of epitopes allows that only virtually the best epitopes are selected, with the best results of immunogenicity, conservation, populational coverage, binding energy, and stability. Therefore, these filters can make vector-based approaches faster and more efficient.[121]

DNA and RNA based vaccines are essentially poorly immunogenic,[122] thus, the administration of adjuvants is essential to overcome this limitation.[123] An important class of adjuvants are Toll-like receptors (TLR) ligands. When stimulated, the TLR rapidly identify these molecules as "dangerous" and trigger the production of pro-inflammatory cytokines, as well as the activation of innate immune response, and the increase of antigen presentation to lymphocytes by dendritic cells (DCs). Examples of TLRs agonist are the TLR-9 agonist composed of CpG motifs, which are capable of inducing a strong cytotoxic response[124] and the TLR-3 agonist molecule polyriboinosinic polyribocytidylic acid [Poly(I:C)], which is a double-stranded RNA analogue capable of inducing cell signaling through multiple inflammatory pathways.[125,126] Another promising class of immunomodulator are cytokines, since these proteins play a critical role in immune cell signaling. Several studies have included plasmids encoding cytokines in their assays,[127] such as the use of IL-2 and IL-12 in vaccines for influenza,[128] *SARS-CoV*,[129] and HIV[130–132] which demonstrated the significant increase of immunogenicity. Finally, it is essential to ensure the efficiency of the vaccine transfection, so the most promising delivery systems for nucleic acid approaches include electroporation (EP) for DNA-based vaccines and lipid nanoparticles (LNPs) for mRNA vaccines, resulting in increased uptake of the vaccine plasmid and consequently increasing its efficiency.[133–135]

Conclusion

The COVID-19 pandemic brought to light that viral diseases have the potential of decimating millions of people in a short amount of time, something that happened before until efficient vaccines were developed that allowed the control of these diseases. Such vaccines were developed by classic platforms that contributed to major advances in public health, such as the eradication of smallpox. However, certain limitations are associated with these platforms, which make them less susceptible to the rapid response that a pandemic requires. We are currently facing an unprecedented effort at accelerated speed during vaccine development, in which numerous research groups worldwide have been working simultaneously, along with governmental and private efforts to try to curb the infection.

The enormous advances in molecular engineering and biotechnology in recent decades have enabled the development of increasingly efficient bioactive molecules, such as the latest generation vaccines. Such vaccine platforms have numerous advantages, such as greater safety; better immune response directioning; the possibility of coverage against multiple viral subtypes; the fast development, production, and ease of storage, which justifies the growing effort to establish these vaccine strategies. Additionally, the databases and the bioinformatics tools currently available allow the prediction of the most promising epitopes to use in essays in vivo, also allowing rapid replacement of these epitopes in other vaccine constructs in response to pathogen mutations, thus preventing epidemics with emerging viral subtypes.

The current pandemic context is surrounded by challenges. One of them is the development in record time of a vaccine for a new virus in which it is still spreading at alarming rates and constantly mutating, in which there is a need for the production and distribution of billions of doses. In addition, the immunopathogenesis of COVID-19 is not fully understood, and previous studies from vaccines against the following viruses (*SARS-CoV* and *MERS-CoV*) in some animal models raised safety concerns regarding Th2 mediated immunopathology.[136]

Another challenge is the reconsideration of current approaches to regulatory assessment and the licensing process of new vaccine platforms by government agencies in order to ensure the safety and efficacy of these new vaccines, which is a time-consuming factor. However, time is a crucial element in the current context, since the *SARS-CoV-2* virus reached an average worldwide infection rate of 828 thousand people a day and 14,7 thousand deaths during the peak of the pandemic (to date). History showed us that these crises also generate unique opportunities for the development of new technologies, and it is possible that the learning generated with *SARS-CoV-2* will revolutionize vaccine development technology for human usage, which is already proving to be highly effective and safe, and therefore, this can open the field to many possibilities that are not restricted to prophylactic but also therapeutic purposes.

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ORCID

Maria da Conceição Viana Invenção http://orcid.org/0000-0001-9360-1275
Alanne Rayssa da Silva Melo http://orcid.org/0000-0002-1786-3369
Larissa Silva de Macêdo http://orcid.org/0000-0003-1660-4901
Thaís Souto Paula da Costa Neves http://orcid.org/0000-0003-3573-9916
Cristiane Moutinho Lagos de Melo http://orcid.org/0000-0002-8831-0163
Marcelo Nazário Cordeiro http://orcid.org/0000-0003-4912-3530
Marcus Vinicius de Aragão Batista http://orcid.org/0000-0003-4745-8919
Antonio Carlos de Freitas http://orcid.org/0000-0002-4957-9549

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