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Innovative vaccine platforms against infectious diseases: Under the scope of the COVID-19 pandemic

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ABSTRACT

While classic vaccines have proved greatly efficacious in eliminating serious infectious diseases, innovative vaccine platforms open a new pathway to overcome dangerous pandemics via the development of safe and effective formulations. Such platforms play a key role either as antigen delivery systems or as immune-stimulators that induce both innate and adaptive immune responses. Liposomes or lipid nanoparticles, virus-like particles, nanoemulsions, polymeric or inorganic nanoparticles, as well as viral vectors, all belong to the nanoscale and are the main categories of innovative vaccines that are currently on the market or in clinical and preclinical phases. In this paper, we review the above formulations used in vaccinology and we discuss their connection with the development of safe and effective prophylactic vaccines against SARS-CoV-2.

1. Introduction

The COVID-19 outbreak, caused by the virus SARS-CoV-2, proved that a single nano-sized virus can cause intense unrest worldwide, with serious consequences for modern societies (Bedford et al., 2019; Rauch et al., 2018). Lockdowns and personal prophylactic measures played a key role in controlling the transmissibility of the virus. However, more direct solutions for the elimination of a pandemic are necessary. Effective vaccines remain on top priority for handling those situations.

Vaccination was first performed by Edward Jenner (1796) (Stern and Markel, 2005) and vaccines were advanced by Louis Pasteur (Baxby, 1999). Nowadays, we can classify vaccines in three main generations. The first generation contains the live-attenuated or inactivated vaccines of pathogens. Several efficacious vaccines of this type have been produced against smallpox (WHO, 2004), tuberculosis (Covián et al., 2019), poliomyelitis (Kew et al., 2005) and the seasonal or the pandemic flu (Herold and Sander, 2020). Indeed, smallpox was eradicated in the 1980s and poliomyelitis is nearly eradicated today (Minor, 2015). Subunit (second-generation) vaccines contain only specific immunogenic domains of the pathogen. Domains that are usually used are the membrane, capsid proteins or toxins (Torii et al., 2017) of germs and viruses (Morein and Simons, 1985). Sometimes, the pathogenic proteins have the ability to self-assemble into particulate systems, known as virus-like particles (VLPs) (Noad and Roy, 2003). Subunit vaccines seem to have a better safety profile than live-attenuated or inactivated vaccines. However, they are less immunogenic, resulting in weaker and sometimes insufficient response. Thus, the majority of these vaccines require an immunomodulator, i.e., an adjuvant, to induce an immune response (Cimica and Galarza, 2017). Some of the most widely used vaccines nowadays belong to this category, such as human papillomavirus and hepatitis B vaccines (Qian et al., 2020; Yadav et al., 2019).

Third-generation vaccines do not contain antigenic proteins of pathogens, but part of their genetic material. Such vaccines have recently been approved for human use (EMA, 2020a; Kim et al., 2020) and the expectation is that they will bring a revolution in the field of vaccinology. The genetic material is encapsulated into nanoparticles and transferred inside the target host cells. When released, the genetic material, DNA or RNA, is expressed by the host, as in the case of an infection. Hence, an accurate architecture of the expressed antigenic protein is produced. The cost of production for nucleic acid vaccines is one of their main advantages, as the manufacturing process is simpler and has higher repeatability in comparison with previous generation vaccines (Lee et al., 2018; Tejeda-Mansir et al., 2019). On the other hand, when DNA is the cargo, it must pass into the nucleus to produce the intermediate mRNA molecule that will be then translated into the immunogenic protein. mRNA vaccines need only to pass the plasma membrane, which is easier than passing the nuclei membrane. The mRNA molecules have a dual role in the stimulation of the immune

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system. First, they are the templates for the formation of the desired antigenic protein and secondly, they can act as adjuvants that are recognized by endosomal and cytosolic innate immune receptors, such as Toll-like receptors 3,7 and 8 (Rauch et al., 2018). Despite their important advantages, nucleic acid vaccines have not been evaluated for a long period and a certain degree of doubt about their mechanism of action still exists. For example, the exogenous plasmid DNA that is inserted into the nucleus might remain there for a longer time period than intended, leading to worries for genomic integration and mutagenesis (Rauch et al., 2018). As many DNA-based vaccines utilize stable plasmids for the transfer of the desirable genes and not a platform or a vector, here we emphasize on mRNA vaccines.

At this point, it is important to note that many of the aforementioned innovative technologies follow the physicochemical principles of nanotechnology. In the last decades, nanomedicine has boosted progress and some impressive results have been achieved in its short-term history. In vaccinology, nanoparticles (NPs) have a dual role, as their action includes both antigen delivery and adjuvanticity. Functional platforms, consisting of stimuli-responsive biomaterials that can be administrated by alternative routes result in decrease of the cost, time and effort needed for the design and development of effective vaccines. Nanovaccinology allowed the development of a smart-vaccine against COVID-19 in less than a year.

2. Overview of the immune system

The two arms of human immunity are equally important for the protection against infections and cellular malfunctions, producing an enhanced humoral and cellular cascade.

2.1. Innate immunity

When a pathogen or antigen is presented into a tissue or in body fluids the innate response is immediately activated. The Complement, granulocytes, macrophages, and dendritic cells promote the release of a plethora of active molecules, including chemokines, cytokines, interferons, and complement components. Firstly, complement activation leads to the opsonization or lysis of the antigen membrane via three routes: the classical, the alternative and the mannan-binding lectin pathway (Zipfel and Skerka, 2009). Furthermore, neutrophils are granulocytes that normally flow in the blood and can recognize signals coming from infected cells and macrophages. After recognition, neutrophils gather to the site of the infection and phagocytose the antigens, while simultaneously producing chemokines and other chemoattractants, leading to a further activation of the immune response. Eosinophils and basophils are other granulocytes in the bloodstream but they exist in much lower concentrations, compared with neutrophils. These two types of leucocytes have an important role in hypersensitivity responses and anti-parasite immunity (Falcone et al., 2001; Jiao et al., 2016; Voehringer, 2009).

Tissues also have their own immune cells. Immature dendritic cells and macrophages are located in peripheric tissues and in the presence of a pathogen, they have the ability of phagocytosis. The main difference between these two cell types of the innate system is that dendritic cells do not only phagocytose the pathogens but are also capable of antigen presentation, in contrast with macrophages. The presentation occurs via binding of the antigen with major histocompatibility complex class I or II (MHC I or II). MHC class II is associated with the presentation of extracellular peptides derived from allergens, bacteria, protozoa, or dead host cells, while MHC class I is used for the presentation of intracellular proteins, such as viral proteins expressed by infected cells. MHC I can be expressed by almost all nucleated cells while, MHC II is a privilege presentation complex of only professional antigen-presenting cells (Delamarre and Mellman, 2011; Mellman, 2013). Phagocytic cells interact with the pathogens via recognition of the pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) by their pathogen recognition receptors (PRRs).

Lastly, natural killer cells (NK) are lymphocytes that detect infected host cells, allogenic cells, or cancer cells and cytolyze them. NK sense inflammatory signals such as cytokines, antibodies, viral signals, or host cell stress-signals (Hammer et al., 2018). Although NK belong to the innate immune system, they have the ability to produce memory. Antigen-specific or non-antigen- specific NK memory cells are induced after activation by viruses or haptens. Hence, certain markers that are present in the surface of NK memory cells provide long-lasting protection against stimuli (Cerwenka and Lanier, 2016).

2.2. Adaptive immunity

There are two main arms of adaptive immune cells, T-lymphocytes and B-lymphocytes. As in innate immune response, the adaptive system also produces both humoral and cellular responses. After the activation of the innate response, the antigen-presenting cells, mostly the dendritic cells, migrate from the peripheral tissues to the lymph nodes. In the lymph nodes, they present antigens of the pathogen they have phagocytosed, via the help of MHC class I or II.

T-cells mature in the thymus and are classified in $CD4^+$ and $CD8^+$ T-cells. $CD8^+$ T-cells, also called cytotoxic T-cells, recognize antigens presented by MHC I. After recognition, the $CD8^+$ T-cell evoke lysis of the infected cell membrane, concluding in the cell's death. On the other hand, $CD4^+$ T-cells or T-helper cells (T_H) are activated by antigens in MHC II and produce cytokines to interact with other cells. Naive $CD4^+$ T-cells differentiate in five subcategories: T_H1, T_H2, T_H17, T_{FH}, and T_{REG}. The differentiation is associated with the presence of certain cytokines. Briefly, T_H1 induce the activation of innate cellular immunity (macrophages), while T_H2 is associated with the production of antibodies via the activation of B-lymphocytes. T_H17, which apart from inducing innate immunity, also seem to play a key role in autoimmune diseases (Leung et al., 2010). Follicular T-helper cells (T_{FH}) are located in the germinal centers inside the tonsils and physiologically, they are responsible for the differentiation and the proliferation of B-cell clones (Crotty, 2011). In conclusion, regulatory T-cells (T_{REG}) are the immunosuppressive cells of the immune system. They can be further subclassified, and depending on their special active biomolecules, e.g., FOXP3, they down-regulate the proliferation of certain types of T-cells and they protect organs (e.g., liver) or immune cells from immune mediated injury by suppressing the immune response. The equilibrium between the necessity to encounter the "enemy" and preserve a functional and effective immunity is therefore based on the work of T_{REG} cells (Karkhah et al., 2018).

The other main class of adaptive immunity cells are B-cells that are responsible for the production of specialized antibodies - immunoglobulin A (IgA), IgE, IgM, IgG, and IgD – that can inactivate the pathogens and simultaneously render them more recognizable by other immune cells. B-cells mature in the bone marrow and are afterward located in the lobules of the lymph nodes. There, they form certain structures, named B-cell follicles. When activated by APCs, B cells start to proliferate and form the germinal centers (Schudel et al., 2019). In germinal centers, B-cells undergo a series of mutations in the domain of IgG genes. Only B-cells with high affinity to the present antigen develop and mature to plasma cells, which then secrete antibodies of high quality and quantity (Kräutler et al., 2017).

Both classes of adaptive lymphocytes produce immunity memory, as they are able to provide long-lasting protection against the same or even very similar antigens (an action called cross-protection) (Netea et al., 2019). Indeed, memory B-cells seem to have a much broader repertoire, leading to more efficient and faster antigen neutralization in the case of infection through a closely related antigenic epitope. T-cell-dependent and T-cell-independent memory B-cells are the two major types of long-lasting B-cells (Kurosaki et al., 2015). In contrast with memory B-cells, which induce prophylaxis through humoral memory, T-cells can also induce memory. Central memory T-cells (T_{CM}) trafficking through

lymphoid tissues, whereas effector memory T-cells (T_{EM}) can accumulate other tissues. Thus, T_{CM} and T_{EM} can provide the functionalities of mature T-cells in the case of reinfection (Jameson and Masopust, 2018).

3. Classification of vaccine platforms

Many innovative vaccines take advantage of the use of particulate antigen delivery systems and nano-adjuvants. Such "smart" platforms provide protection of the antigen from biological degradation, targeted delivery to the desirable cells after administration and higher immunogenicity. Below, we classify and analyze the most commonly used vaccine platforms. Table 1 presents the vaccines that utilize nanoscale vaccine platforms.

3.1. Virus-like particles (VLPs)

Virus-like particles (VLPs) are the most common vaccine nanoplatforms for both prophylactic and therapeutic purposes (Smith et al., 2013). The first nanovaccine, which was authorized in 1986 for hepatitis B prophylaxis, is classified in VLP-based vaccines (Zhao et al., 2013). Until today, authorized VLP vaccines against hepatitis B virus (HBV), hepatitis E virus (HEV), and human papillomavirus (HPV) are globally used and many others are in clinical trials (Qian et al., 2020). According to ClinicalTrials.gov, prophylactic vaccines against chikungunya, encephalitis virus and norovirus are currently under clinical evaluation.

Most VLPs have a size of 20–100 nm and consist of pathogen surface proteins, without the presence of genetic material (Fig. 1A) (Smith et al., 2013). This is the main difference between VLPs and viruses, which leads to the advantage that there is no danger of pathogen proliferation. In other words, VLPs combine the immunogenicity of the viruses, based on highly organized supramolecular structures, without their pathogenicity, leading to safe and effective vaccines (Mohsen et al., 2017).

The proteins used must have the ability to self-assemble into functional and immunogenic nanostructures, mainly with the use of an expression system, prokaryotic or eukaryotic cell line. Yeast, bacteria, insect, mammalian and plant cells have been utilized for this purpose (Balke and Zeltins, 2020; EMA, 2020b; Li et al., 2015; Qian et al., 2020; Shouval et al., 2015; Yusibov et al., 2002). The above is achieved by the

Table 1
Authorized nano-scaled vaccines.

Name	Targeted Disease/ Pathogen	Antigen	Adjuvant or Vector	Route of Administration	Ref.
VLP-based vaccines					
Engerix-B (GSK)	HBV	SHBs	Aluminum hydroxide	IM	(EMA, 2020d)
Recombivax HB (Merck & Co.)	HBV	SHBs	Aluminum salt	IM	(Zhao et al., 2011)
Sci-B-Vac (SciGen)	HBV	SHBs, MHBs, LHBs	Aluminum hydroxide	IM	(Shouval et al., 2015)
Heplisav-B (Dynavax)	HBV	SHBs	CpG1018	IM	(FDA, 2020a)
Fendrix (GSK)	HBV	SHBs	AS04	IM	(EMA, 2017)
Hepavax-Gene (Crucell Berna Biotech)	HBV	SHBs, MHBs	Aluminum hydroxide	IM	(Rebedea et al., 2006)
Cervarix (GSK)	HPV	HPV 16/18	AS04	IM	(EMA, 2020b)
Gardasil (Merck & Co.)	HPV	HPV 6/11/16/18	Aluminum salt	IM	(EMA, 2020e)
Gardasil-9 (Merck & Co.)	HPV	HPV 6/11/16/18/31/33/45/52/58	Aluminum salt	IM	(EMA, 2020f)
Cecolin (Xiamen Innovax Biotech Co.)	HPV	HPV 16/18	Aluminum hydroxide	IM	(Zou et al., 2020)
Cecolin-9 (Xiamen Innovax Biotech Co.)	HPV	HPV 6/11/16/18/31/33/45/52/58	Aluminum hydroxide	IM	(Qian et al., 2020)
Hecolin (Xiamen Innovax Biotech Co.)	HEV	P239	Aluminum hydroxide	IM	(Yin et al., 2020)
LNP-based vaccines					
Inflexal (Crucell Berna Biotech Ltd.)	Influenza	HA, NA	Virosomes	IM	(Herzog et al., 2009)
Epaxal (Crucell Berna Biotech Ltd.)	HAV	RG-SB strain	Virosomes	IM	(Bovier, 2008)
Viral vector-based vaccines					
Dengvaxia (Sanofi Pasteur Inc.)	Dengue virus	CYD genome 1–4	rYFV	SC	(EMA, 2020a)
Imojev (Sanofi Pasteur Inc.)	JEV	JE SA 14–14-2	YFV17D	SC	(FDA, 2013)
Ervebo (Merck Sharp & Dohme B. V.)	Ebola virus	ZEBOV-GP	rVSV	IM	(Wolf et al., 2021)
Zabdeno (Janssen-Cilag International N.V.)	Ebola virus	ZEBOV-GP	Ad26	IM	(Tomori and Kolawole, 2021)
Mvabea (Janssen-Cilag International N.V.)	Ebola virus	ZEBOV-GP, SEV-GP, TFEV-NP, MMV-GP	MVA-BN	IM	(Tomori and Kolawole, 2021)
Nano-adjuvant-based vaccines					
Fluad (Seqirus Inc.)	Influenza	3 inactivated influenza strains	MF59	IM	(FDA, 2020b)
Fluad Tetra (Seqirus Inc.)	Influenza	4 inactivated influenza strains	MF59	IM	(EMA, 2020g)
Mosquirix (GSK)	Malaria caused by P. falciparum & HB	RTS,S	$AS01_E$	IM	(EMA, 2019b)
Shingrix (GSK)	HZ & PHN	varicella zoster virus glycoprotein E	$AS01_B$	IM	(EMA, 2020h)

Abbreviations: HBV, hepatitis B virus; HPV, human papilloma virus; HEV, hepatitis E virus; HAV, hepatitis A virus; rYFV, recombinant yellow fever virus; rVSV, recombinant vesicular stomatitis virus; JEV, Japanese encephalitis virus; Ad26, adenovirus 26; MVA-BN, modified vaccinia arnaka – Bavarian Nordic; HB, hepatitis B; HZ, herpes zoster; PHN, post-herpetic neuralgia; IM, intramuscular; SC, subcutaneous

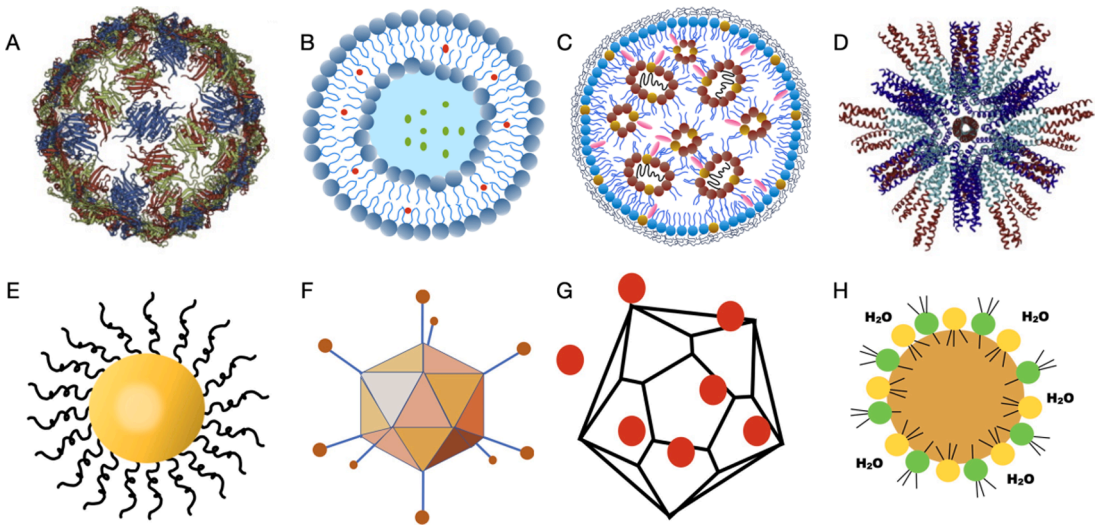

Fig. 1. Novel nano-scaled vaccine platforms. A, VLP; B, Liposome; C, LNP specialized to encapsulate nucleic acid; VLP; D, SAPN; E, Inorganic NP; F, Viral vector; G, ISCOM-Matrix adjuvant platform; H, MF59 o/w nanoemulsion. A and D adapted from Karch and Burkhard, 2016 with permission from Elsevier.

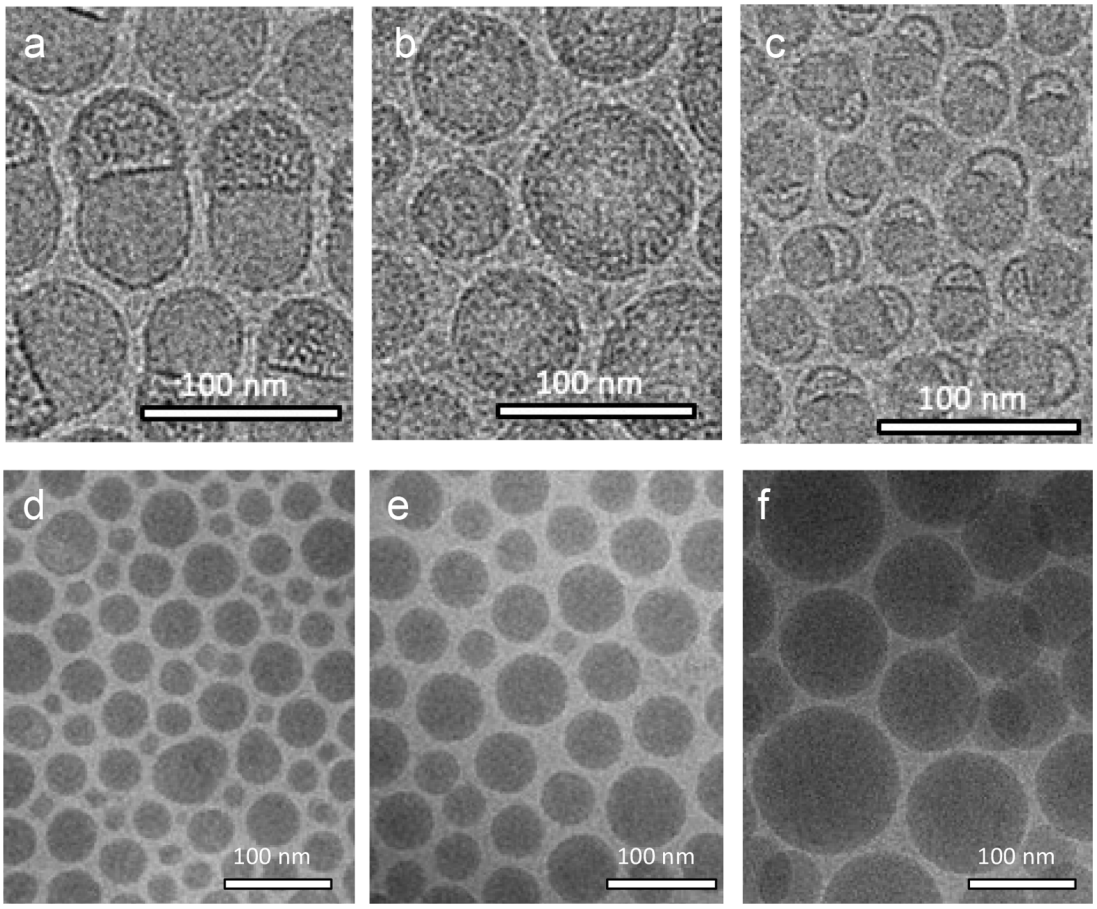

Fig. 2. Cryo-TEM images of mRNA-LNPs. Unique LNP morphology is presented depending on the qualitative and quantitative composition of the systems. The images are showing "bleb" structures (a & c), multivesicular particles (b) and spherical structures (d,e & f) with different electron-dense cores. Fig. 2a-c are adapted from Brader et al. (2021) and Fig. 2d-f are adapted from Yanez Arteta et al., 2018.

right conformational orientation of the antigenic epitopes in the VLP surface and thus, the production of high titers of specialized neutralizing antibodies (nAbs). The structure is stabilized with many intra- or intermolecular covalent or hydrophobic interactions. Amino acids, such as cysteine and lysine have an important role in this activity due to their physicochemical properties (Berthier et al., 2020; Li et al., 2016). Moreover, equally important is the switching of hydrophobic and hydrophilic domains for the creation of stable formulations (Berthier et al.,

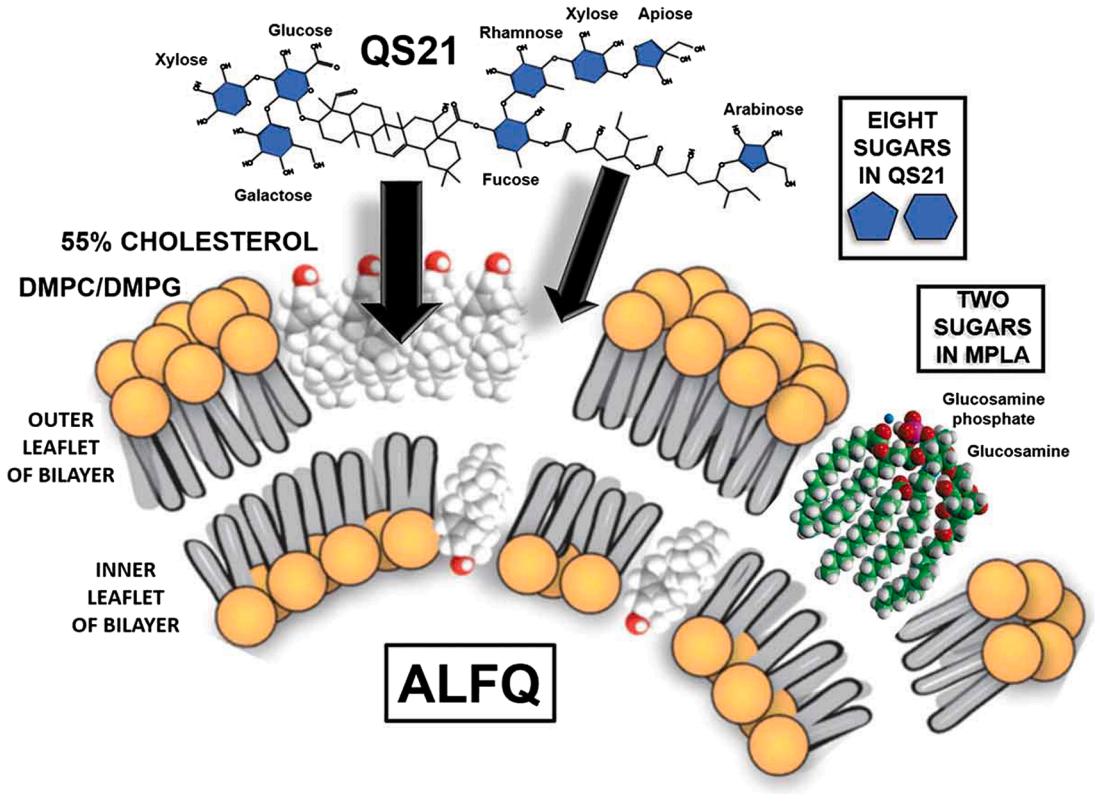

Fig. 3. Theoretical formation of a 'sugar lawn' of 10 sugars on the surface of ALFQ by interaction of QS21 with ALF liposomes. Adapted from Alving et al., 2020.

2020). As in the case of virus capsids, the antigenic proteins of VLPs self-assemble into highly symmetrical and strict architectures, usually icosahedral and octahedral, with statistically preferable repeatability (Gilbert et al., 2005; Lu et al., 2020). Even in cases of complex hybrid systems that consist of more than one protein, the final formations do not present high lot-to-lot deviation (Zhang et al., 2020), maybe because the information of the functional conformation is encrypted in the monomer structure. However, significantly important variations may be observed if the expression system changes. For example, prokaryotic cell lines do not have the ability of post-translational processing that may result in a difference in glycosylation and the quadruple structure of the VLPs (Mohsen et al., 2017). Such differences might lead to alteration of the immunogenic response of the vaccine receiver.

Concerning immunogenicity, VLPs have proved to activate both the innate and the adaptive response. Complement activation via the classical pathway occurs after the vaccination, resulting in opsonization of the VLPs (Gomes et al., 2017). In this way, the repetitive epitopes of VLPs are recognized by the Toll-like receptors and become more easily visible from the components of the cellular immune system, especially dendritic cells (Tagliamonte et al., 2017). VLPs result in the stimulation of $CD4^+$ T-helper cells - T_H1 and T_H2. Furthermore, because of their virus-like behavior, DCs cross-present the epitopes in MHC I, concluding in the activation of $CD8^+$ T-cells and a more intense immune response (Bachmann and Jennings, 2010). Small particulate antigens (<200 nm), such as VLPs, have the ability to enter the lymphatic system without the need of APCs (Manolova et al., 2008). This is extremely important as the cell-free antigenic VLPs can directly interact with the follicular B-cells in the secondary lymphoid organs (Bachmann and Jennings, 2010). The cross-linking interaction is much stronger than the DC one, and results in a more effective activation of the immune response with a much lower quantity of antigens (Hong et al., 2018).

Currently, scientists design standardized VLP platforms. An interesting idea was presented by Garg et al. They managed to synthesize a multivalent VLP-based prophylactic vaccine against four arthropod-borne viruses – chikungunya, Japanese encephalitis, yellow fever and Zika virus. The VLPs are secreted by 293T stable cell lines and generate a high amount of nAbs for all viruses in mice experiments. Such an approach is preferable for both manufactures and populations sensitive to these viruses, as it is a more economic technology than the production of live attenuated virus (LAV) vaccines and can protect against four viruses, minimizing vaccine administrations (Garg et al., 2020). Another appealing procedure is the formation of chimeric VLPs. Chimeric VLPs can be produced either by genetic fusion or by chemical conjugation. SpyCather-SpyTag methodology is an innovative decoration of VLPs via the spontaneous isopeptide bond formation (Brune and Howarth, 2018). Recently, a vaccine of this type was synthesized, utilizing as a VLP platform the core-capsid protein of AP205 phage. The platform contained antigens of *P. falciparum* (VAR2CSA epitope) and HPV (L2 RG1 epitope) for protection against malaria and HPV infection and its *in vitro* results were encouraging for the production of combinational vaccines via the use of a single VLP-scaffold (Janitzek et al., 2019).

3.2. Lipid nanoparticles (LNPs)

Lipid nanoparticles (LNPs) that are utilized in vaccines can be distinguished in three main categories: liposomes, virosomes and LNPs specified for nucleic acid transfer (Fig. 1B and 1C). The word liposome comes from two Greek words: 'lipos' (λίπος) meaning fat and 'soma' (σώμα) meaning body. Liposomes are lipid bilayers and can be uni- or multi-lamellar. Except phospholipids, they may also contain cholesterol, other lipids, and polymers (Demetzos, 2016). They were discovered by Bangham in 1964 (Bangham and Horne, 1964) and in 1974 they were first mentioned as possible adjuvants in vaccine formulations by Allison and Georgiadis (Allison and Georgiadis, 1974). Nowadays, many liposome-based formulations have been approved. Among them, there are two vaccines, Inflexal® and Epaxal®, both from Crucell Berna

Table 2
Innovative nano-scaled COVID-19 vaccines according to the WHO (08/2021).

Vaccine Name	Platform	Developers	Number of Doses	Route of administration	Phase
AZD1222	VVnr ChAdOx1-S	AstraZeneca + University of Oxford	2	IM	4
mRNA-1273	RNA - LNP	Moderna + NIAID	2	IM	4
BNT162	RNA - LNP	Pfizer/BioNTech + Fosun Pharma	2	IM	4
Gam-COVID-Vac	VVnr (rAd26-S + rAd5-s)	Gamaleya Research Institute; Health Ministry of the Russian Federation	2	IM	3
Ad26.COV2.S	VVnr (rAd26)	Janssen Pharmaceuticals	1–2	IM	4
Recombinant novel coronavirus vaccine	VVnr (rAd5)	CanSino Biological Inc./Beijing Institute of Biotechnology	1	IM	4
CVnCoV	RNA - LNP	CureVac AG	2	IM	3
SARS-CoV-2 rS/Matrix M1	PS	Novavax	2	IM	3
ARCoV	RNA - LNP	AMS, Walvax Biotechnology and Suzhou Abogen Biosciences	2	IM	3
GRAd-COV2	VVnr	ReiThera + Leukocare + Univercells	1	IM	2/3
CoVLP	VLP	Medicago Inc.	2	IM	2/3
rVSV-SARS-CoV-2-S Vaccine	VVr (rVSV)	Israel Institute for Biological Research	1	IM	2/3
ARCT-021	RNA	Arcturus Therapeutics	ND	IM	2
DeINS1-2019-nCoV-RBD-OPT1	VVr (Intranasal flu-based RBD)	University of Hong Kong, Xiamen University and Beijing Wantai Biological Pharmacy	1	IN	2
AV-COVID-19	VVr + APC	Aivita Biomedical, Inc. + National Institute of Health Research and Development, Ministry of Health Republic of Indonesia	1	IM	2
MRT5500	RNA	Sanofi Paster and Translate Bio	2	IM	2
SARS-CoV-2 VLP Vaccine	VLP	The Scientific and Technological Research Council of Turkey	2	SC	2
RBD SARS-CoV-2 HBsAg VLP vaccine	VLP	Serum Institute of India + Accelagen Pty + SpyBiotech	2	IM	1/2
V591-001 –	VVr (TMV-o38)	Merck & Co. + Thernis + Sharp & Dohme + Institute Pasteur + University of Pittsburgh	1–2	IM	1/2
LV-SMENP-DC vaccine	VVnr + APC	Shenzhen Geno-Immune Medical Institute	1	SC & IV	1/2
hAd5-S-Fusion+N-ETSD	VVnr	ImmunityBio, Inc. & NantKwest, Inc.	1–2	SC or Oral	1/2
AdCLD-CoV19	VVnr	Cellid Co., Ltd.	1	IM	1/2
COVIVAC	VVnr	Institute of Vaccines and Medical Biologicals, Vietnam	2	IM	1/2
VBI-2902a	VLP	VBI Vaccines Inc.	2	IM	1/2
DS-5670a	RNA	Daiichi Sankyo Co., Ltd.	2	IM	1/2
EXG-5003	RNA	Elixirgen Therapeutics, Inc	1	ID	1/2
Modified MVA	VVnr	German Center for Infection Research	2	IM	1/2
LV-SMENP-DC	VVnr + APC	Shenzhen Geno-Immune Medical Institute	1	SC & IV	1/2
VXA-Cov2-1	VVnr	Vaxart	2	Oral	1
MVA-SARS-2-S	VVnr	University of Munich	2	IM	1
LNP-nCoVsaRNA	RNA -LNP	Imperial College London	2	IM	1
ChulaCov19	RNA - LNP	Chulalongkorn University	2	IM	1
COH04S1	VVnr	City of Hope Medical Center + National Cancer Institute	1–2	IM	1
AdCOVID	VVnr	Altimmune, Inc.	1–2	IN	1
BBV154	VVnr	Bharat Biotech International Limited	1	IN	1
PTX-COVID19-B	RNA	Providence Therapeutics	2	IM	1
CoV2 SAM	RNA - LNP	GlaxoSmithKline	2	IM	1
ChAd68	VVnr	Gritsstone Oncology	2–3	IM	1
SC-Ad6-1	VVnr	Tetherex Pharmaceuticals Corporation	1–2	IM	1
ABNCoV2 cVLP +/- MF59	VLP	Radboud University	2	IM	1
HDT-301	RNA - LNP	SENAI CIMATEC	2	IM	1
HDT-301	RNA - LNP	SENAI CIMATEC	2	IM	1
mRNA COVID-19 vaccine	RNA	Shanghai East Hospital and Stemirna Therapeutics	2	IM	1
LNP-nCOV saRNA-02	RNA - LNP	MRC/UVRI + LSHTM Uganda Research Unit	2	IM	1
PIVS-Spike	VVnr	CyanVac LLC	1	IN	1

Abbreviations: VVnr, viral vector non-replicating; VVr, viral vector replicating; LNP, lipid nanoparticle; APC, antigen presenting cell; IM, intramuscularly; IN, intranasally; SC, subcutaneously; ID, intradermal

Biotech, Switzerland, for the protection against the influenza virus and the hepatitis A virus, respectively (Bulbake et al., 2017).

Under the right environmental pressure, phospholipids are organized into pseudo-spherical architectures, whose properties are highly connected with the biophysical behavior of their building blocks (Demetzos, 2016). Hence, liposome size, lamellarity, surface charge, and bilayer fluidity vary depending on the physicochemical characteristics of the monomers and their combination (Watson et al., 2012). The bilayer has an amphiphilic character, as the polar heads of phospholipids are oriented toward the water molecules and the hydrophobic chains are placed in the internal area of the membrane (Demetzos and Pippa, 2014). The hydrophobic interactions of the hydrocarbon chains in aqueous medium are the driving force for the liposomal structure.

Due to their conformation, they can transfer both small hydrophobic molecules (incorporated in the membrane) and hydrophilic ones (encapsulated in the aqueous core) (Metselaar and Storm, 2005), as presented in Fig. 1B. Furthermore, as phospholipids are the basic components of the cell membrane, liposomes have biomimicking properties and are well-tolerated non-toxic platforms (Yang et al., 2019). They are biodegradable and usually, they do not bioaccumulate after administration. Finally, they can transfer more than one antigen and, with the right surface functionalization, they can slowly release their cargo, leading to controlled release platforms (Riaz et al., 2018).

Several studies showed that when antigenic proteins or peptides are conjugated onto the lipid membrane, the activation of defensive mechanisms is more intensive than when they are encapsulated in the aqueous area (Blom et al., 2017; Serre et al., 1998). According to the vaccine glossary, liposomes with conjugated antigenic epitopes on their surface are called virosomes. More specifically, virosomes are produced by appropriate handling (e.g., ultracentrifuge) of the virus of interest,

Table 3
Comparisson of the COVID-19 mRNA-LNP vaccine formulations Abbreviations: LNP, lipid nanoparticle; N/P, ratio of the nitrogen of the ionizable cationic lipid / phosphate group of the nucleotides.

	Pfizer/BioNTech	Moderna
LNP components	ALC-0315 ALC-0159 DSPC Cholesterol	SM-102 PEG-2000 DMG DSPC Cholesterol
% molar ratio of cationic lipid: helper lipid: cholesterol: PEGylated lipid	46.3:9.4:42.7:1.6	50:10:38.5:1.5
N/P molar ratio	6	6
Storage conditions	6 months at −90 °C to −60 °C 1 month at 2 °C to 8 °C	7 months at −25 °C to −15 °C 1 month at 2 °C to 8 °C

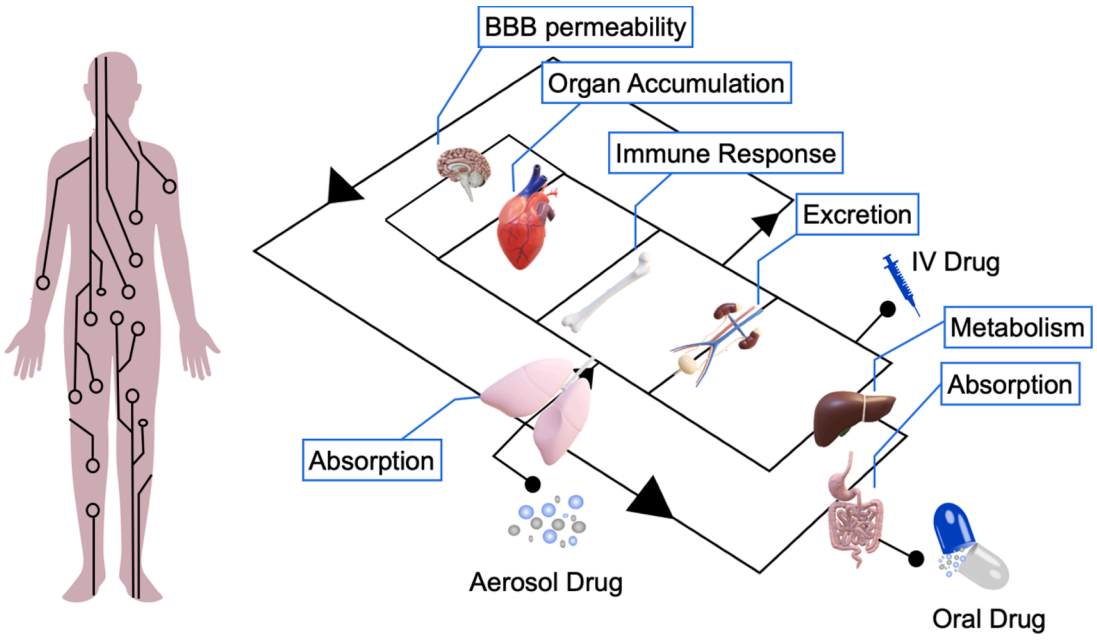

Fig. 4. Multi-organs-on-chip illustration. Organs-on-chip are microfluidic devices that could significantly decrease the number of human subjects needed for the evaluation of novel vaccines in preclinical and clinical phases.

isolation of the membrane components and mixing with other pharmacologically inactive molecules, e.g., lecithin or phospholipids (Mischler and Metcalfe, 2002). In this way, virosomes have better biomimicking properties. Inflexal® and Epaxal® belong to this category.

Finally, cationic lipids are preferable when the cargo is a hydrophilic, anionic molecule, i.e., siRNA and mRNA, due to the development of electrostatic interactions or hydrogen bonds between the molecules. LNPs that contain cationic lipids have the ability to disrupt the endosomal and/or phagosomal membrane because of its low-durability to proton influx (Henriksen-Lacey et al., 2011; T. Li et al., 2018). However, the positive charge is responsible for toxicity effects, mainly lysis of the negative charged cell membranes (Lv et al., 2006; Semple et al., 2001). Such observations mobilized scientists to develop the category of ionizable cationic lipids (apparent pKa < 6.5). Ionizable cationic lipids are charged during the manufacturing process (pH = 4) while they are uncharged in higher pH values. The main differences of these LNPs in comparison with liposomes is that the ionizable cationic lipids are distributed mainly in the core, developing internal lipid hydrophobic areas while interacting with the nucleic acid polyanionic macromolecules. According to recent literature, siRNA-LNPs may importantly vary in morphology, depending on the lipid molar ratio, the mixing process and the N/P ratio (ionizable cationic lipid amine to nucleotide phosphate ratio) (Akinc et al., 2019; Kulkarni et al., 2020, 2019; Viger-Gravel et al., 2018). As mRNA is approximately 100-fold larger than siRNA, different morphologies might be produced when entrapping mRNA molecules (Fig. 1C). According to the literature, these platforms encapsulate mRNA into aqueous cavities inside the hydrophobic core of LNPs. PEGylated and "helper" lipids mostly cover the core, while the ionizable cationic lipids form a highly symmetrical, reverse-hexagonal, hydrophobic structure (Sebastiani et al., 2021; Yanez Arteta et al., 2018). Interestingly, Arteta et al. proved that the empty LNPs did not present the internal reverse-hexagonal morphology that was found for the mRNA loaded LNPs. Although the core is characterized as hydrophobic, aqueous cavities and channels seem to exist at approximately 24%, based on small-angle X-ray scattering (SAXS) and small-angle neutron scattering (SANS) experiments (Yanez Arteta et al., 2018). In contrast with Arteta's group results, which demonstrate that the shell of their mRNA-LNPs is a lipid monolayer, other groups reported a lipidic bilayer shell of their mRNA-LNPs or other sophisticated architectures as the formation of internal liposomal-like blebs (Brader et al., 2021). According to the above, it can be concluded that the exact external and the most important internal morphology of the RNA-LNPs is depended on the physicochemical characteristics of the LNP lipids, as well as the manufacturing processes. Fig. 2 presents some of the mRNA-LNP morphologies that have been observed by cryo-TEM. Although the above indicate a high degree of system morphology freedom and uncertainty, they could also provide the ability to design different RNA-LNPs that exhibit unique entrapment and release properties.

All types of LNPs have proved to have enhanced immunomodulatory properties and to activate both $CD4^+$ and $CD8^+$ T-cell pathways, as well as B-cell responses (Bulbake et al., 2017). By tailoring their morphology and physicochemical characteristics, different innate and adaptive

immune responses may be possible. Larger LNPs (≥400 nm) induce T_H1 type immune response while the smaller ones (100 nm) induce T_H2 type response (Badiee et al., 2012). These may result in a significant difference in the immune response as T_H1 is mostly involved with the cell-mediated immunity and phagocyte response, while T_H2 is connected with humoral immunity (Romagnani, 1999). In conclusion, membrane fluidity affects the immunogenicity of the LNPs. More rigid liposomes, composed of saturated lipids and low concertation of cholesterol have proved more immunogenic than liposomes with lower transition temperatures and higher cholesterol ratio (Kaur et al., 2014; Watson et al., 2012).

3.3. Polymeric nanoparticles (PNPs)

Several polymeric NPs have been used in nanovaccinology either to entrap/conjugate the antigens or to act as adjuvants (Fig. 1D). Polymeric materials can cooperate well with many other biomaterials such as liposomes or inorganic NPs to create sophisticated nanostructures with the ability of "smart response" when administrated *in vivo*.

One of the most well-studied polymeric biomaterial utilized in vaccines is poly(lactide-co-glycolic acid) (PLGA). PLGA is a biodegradable and biocompatible material that has been approved as a vesicle by both the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for human use. Its excellent safety profile, ease of surface modification and size distribution allow the formation of unique systems with different properties (Allahyari and Mohit, 2016). PLGA formations have been studied for their ability of cargo prolonged release, a beneficial property for the enhanced activation of the immune system. Dhakal et al. have extensively researched the use of PLGA for the formation of effective vaccines (Binjawadagi et al., 2014; Dhakal et al., 2017; Hiremath et al., 2016). They have created innovative platforms against the respiratory syndrome virus, H1N1 and H1N2 influenza viruses. The results after intranasal administration in pigs show that both cytotoxic T-cells and T-helper cells can induce the immunity and memory mechanisms against the above pathogens. Another effort for the development of a mucosal vaccine was presented by Tallabaka et al., who developed a PLGA-based immunostimulant, covalently conjugated with a C5a receptor agonist, EP67. The modified PLGA NPs present an enhanced T-cell long-lasting mediated protection in mice population (Tallapaka et al., 2019). Finally, some studies have evaluated the value of PLGA NPs for the dual role to deliver both the antigen and the adjuvant. A PLGA nanoparticulate formulation was synthesized to encapsulate the *Anjelica sinensis* polysaccharide as an adjuvant and ovalbumin as a model antigen. BALB/c mice, vaccinated subcutaneously with those systems, presented improved lymphocyte proliferation and enhanced T_H1 and T_H2 response, resulting in promising cellular and humoral immunity (Gu et al., 2019).

Apart from synthetic linear or grafted polymeric formulations, another group of polymeric nanoparticles has gained attention in the last years. Self-assembled protein (or peptide) NPs (SAPNs) are excellent vaccine platforms due to their biocompatibility and their special morphological characteristics. SAPN architecture was inspired by the viral capsids (Doll et al., 2015). Peptide monomers assemble into oligomers, which then form NPs, usually with an icosahedral conformation (Raman et al., 2006), as presented in Fig. 1G. The main difference with VLPs is that in the case of SAPNs, not the antigens but other peptides, which interact with the antigens, have the ability to self-assemble. Thus, SAPNs can be utilized as scaffolds of antigenic epitopes that cannot self-organize in particulate systems on their own. Hence, SAPN development is a rational bottom-up technique that is based on our knowledge on structural biology and biophysics to create sophisticated engineered proteins, capable to self-assemble via hydrophobic interactions (Karch and Burkhard, 2016).

Many antigens have been incorporated into SAPNs, such as human immunodeficiency virus 1 (HIV-1) (Karch et al., 2019; Wahome et al., 2012), *Plasmodium falciparum* (Burkhard and Lanar, 2015; Kaba et al., 2018, 2012; Seth et al., 2017) and bronchitis virus (J. Li et al., 2018) antigenic epitopes. Some experts support that the nature of these systems give them the privilege of highly antigenic conformations that do not require an extra adjuvant. Thus, simplest systems with more predictable behavior could be produced. On the other hand, the history of VLP structural formations does not confirm such hypothesis. Already licensed VLP vaccines provide higher titers of antibodies when combined with adjuvants, despite the repetitive presentation of the antigenic epitopes, as mentioned above. Based on these data, scientists have already started to study the integration of their SAPNs with the right adjuvants to provide a safe and effective stimulation of the immune system. One of these approaches proposes that the action of presentation of the HIV-1 V1V2 loop on the surface of SAPNs can be increased by the addition of extensively studied liposomal adjuvant conformations (Karch et al., 2019).

3.4. Inorganic nanoparticles (INPs)

Gold and silica NPs are the most famous representatives of this category. Until today, no inorganic nanoparticulate-based vaccine has been approved for therapeutic or prophylactic reasons, although experiments show beneficial results. Their "clean" and stable morphology combined with their capability of high antigen payloads are some of their basic advantages (Fig. 1E). However, their major drawback is their toxicity issues. As they are non-biodegradable materials, they may bioaccumulate in target organs and trigger unwanted immune responses and inflammatory cascades. Thus, enhanced toxicity studies remain extremely important for the understanding of their absorption-distribution-metabolism-excretion (ADME) profile after *in vivo* administration.

Gold NP (GNP) size ranges between 2 and 100 nm and can be synthesized in various shapes, like spheres (Gregory et al., 2012), rods (Tazaki et al., 2018), cubes (Niikura et al., 2013), nanocages, stars, prisms (Kumar et al., 2015) and nanoclusters (Wang et al., 2016). All these different morphologies have been utilized for the preparation and evaluation of many prophylactic vaccines against viruses, bacteria, and parasites. The physicochemical characteristics of GNPs allow the easy conjugation with both the antigen and adjuvant by simple, even one-step procedures (Tao et al., 2017). Quach et al. associated the immunostimulation of GNPs with their size and concentration, concluding that larger chimeric particles (80 nm) showed a better efficacious and toxicological profile for vaccination against the dengue virus than the smaller ones (20 and 40 nm), after subcutaneous administration in BALB/C mice. Both $CD4^+$ and $CD8^+$ T-cell activation were induced, while promising nAbs titers were produced (Quach et al., 2018). Moreover, another interesting example is the synthesis of an AuNP-M2e-sCPG formulation as a universal vaccine against Influenza A serotypes, as M2e is a highly conserved N-terminal extracellular portion of the M2-ion channel protein (Tao et al., 2014). Furthermore, the addition of CpG adjuvant could additionally enhance the immunogenicity of the vaccine. Indeed, this formulation has proved to be effective after intranasal administration to mice, by inducing high titers of IgG antibodies and memory B-cells, even in elderly mice (Bimler et al., 2019).

Silica nanoparticles (SiNPs) can form either core-like, non-porous spherical structures (Thalhauser et al., 2020) or mesoporous morphologies (Ferreira Soares et al., 2020). Concerning solid SiNPs, the antigenic protein can be either adsorbed on the surface of the particle or conjugated by covalent bonds, while in the mesoporous SiNPs, the antigen is encapsulated into the porous and can be stabilized by electrostatic or hydrophobic forces (Huang et al., 2020). Additionally, positive charge appears to further improve the cellular uptake of the SiNPs by APCs (Amin and Boateng, 2020). As unfunctionalized SiNPs are negatively charged, due to the silanol groups on their surface (Huang et al., 2020), additional positively charged moieties could be added (Amin and Boateng, 2020). A promising study by Bai et al. showed that hollow mesoporous silica nanoparticles loaded with VLPs for the prophylaxis

against the foot-and-mouth disease virus (FMDV) presented better immunostimulating results in comparison with the use of VLPs immunomodified with Freund's complete adjuvant after IM administration to guinea pigs. High antibody titers as well as INF-γ and proliferation of T-cells were induced (Bai et al., 2019). Moreover, Huang et al. assume that mesoporous SiNPs of 200–400 nm have the best size and pore diameter for the activation of the innate cellular immune response (Huang et al., 2020). Mahony et al. reported that amino-functionalized mesoporous SiNPs of 90 nm diameter presented better humoral and cellular immune response against ovalbumin in comparison with a higher quantity of ovalbumin subunits adjuvanted with QuilA, a famous immunomodulator saponin mixture, after IM administration to mice. The preclinical trial showed that the functionalized mesoporous SiNP formulation, additionally to its promising adjuvant and delivery platform properties, did not lead to any morphological changes of high-risk organs and tissues (kidneys and spleen) (Mahony et al., 2013).

3.5. Viral vectors

Although viral vectors do not belong to nanoformulations, they are biological platforms at the nanoscale (Fig. 1F). The first effort to create such a vaccine was by Moss and colleagues in 1984 for the protection from HBV infection, using the vaccinia virus (Moss et al., 1984). Today, after the authorization of five viral vector-based vaccines and the subscription of many such formulations in clinical trials, their use remains a taboo. Causes for the aloofness are safety issues, as recombinant viruses, attenuated or not, promote the immunity against another pathogen via the infection of the host cells. Many modern technologies and different viral species were tested to verify the safety and efficacy of these formulations.

Genetic engineering processes modify live viral vectors, either replicating (usually attenuated) or non-replicating, to encode heterogenous antigens. This technique is mainly achieved by the insertion of the desirable antigen genes and the deletion of the possibly harmful ones. Certain pathogen-associated molecular patterns (PAMPs), present in the surface of the viral platforms, result in a more effective recognition by the cells of the immune response than the use of a subunit antigen. Thus, the addition of an adjuvant is usually unnecessary, decreasing the complexity and the cost of the vaccine (Ewer et al., 2016). In many cases, the antigen delivered by those platforms is not expressed in the virus, but it is expressed after the infection of the host cell by the translational and post-translational mechanism of the second one, via the virus replication cycle. The major advantage of the above is the correct protein conformation and glycosylation that reassures an effective presentation motif and a potent immune response (Rauch et al., 2018). After the absorption of the virus into the host cell and the expression of the immunogenic protein, the protein can be presented by the MHC I and activate the $CD8^+$ T-cell pathway (Ewer et al., 2016).

Despite all these unique properties of viral vectors, some serious concerns remain about their mechanism of action and subsequently, their safety. Synthesis of antibodies against the vector is the main drawback of these platforms. As a result, in the case of human viruses, a high human seroprevalence for certain strains results in quick recognition and inactivation of the virus before the promotion of the immunity against the desirable antigen. This problem is clear in the case of adenoviruses (Ads) (Coughlan, 2020). They are classified in two main categories, human Ads (HAds) and non-human primate Ads. Ad5 is a HAd that has been extensively studied as a viral vector with encouraging results for the prevention of many pathogens due to the ease of its genetic modification (Humphreys and Sebastian, 2018; Rauch et al., 2018). Ad5 is a common virus and large human populations, already appearing to be Ad5-seropositive, decreasing the efficacy and the predictability of this platform (Buchbinder et al., 2008). Hence, rarer human Ads, such as Ad35 (Crank et al., 2016) and chimpanzee Ad (ChAd) are preferable (Osman et al., 2017; Tiono et al., 2018). In some cases, different viral vectors are utilized for the first and the booster dose, to reassure the activation of the immune response (Crank et al., 2016).

The history of viral evolution has shown that their behavior is sometimes unpredictable. Certain EU regulations are active at the moment for the evaluation of viral vehicles to reassure the safety of both the vaccines and the environment (Baldo et al., 2013). The recent example of an authorized by the FDA and the EMA vaccine against dengue, utilizing the yellow fever virus as a vector (EMA, 2020c), reminds us that these innovative vaccine platforms have not been studied for a long period and thus, extensive trials are necessary. In 2015, Dengvaxia® was licensed in the Philippines for protection of 9 to45-year-old people against dengue. Two years later, the vaccination program was terminated due to suspicions that Dengvaxia® caused increased danger for aggressive infection by the dengue virus (Halstead, 2018). Indeed, post-hoc clinical trials and sample re-analysis by Sanofi verified the concerns. Dengvaxia® proved to increase the risk of severe dengue and dengue hospitalization in seronegative populations, mostly for children, and was related to some deaths (Sridhar et al., 2018). This effect on seronegative populations is a result of the dengue virus nature and is not correlated with the viral vector. After these events, Dengvaxia® was licensed by the FDA and the EMA in 2018 only for people 9 through 45 years of age who live in areas where the disease is epidemic and have already been infected once with the virus (EMA, 2018). Unlike Dengvaxia®, Imojev® (Sanofi Pasteur), the first viral vector vaccine seems to have great efficacy and safety results. It is a modified yellow fever virus (YFV17D) that encodes two envelope proteins of the Japanese encephalitis SA 14–14-2 strain. Imojev® is currently licensed in a plethora of countries with high epidemic risks (Kim et al., 2020; Ma et al., 2020; WHO, 2013). Interestingly, the live attenuated JE SA 14–14-2 vaccine is now evaluated for the ability of cross-protection against other similar mosquito-borne flaviviruses (Wang et al., 2020). Such a result could be extremely positive, as many of the flaviviruses co-exist in epidemic dangerous areas.

Finally, the three authorized vaccines for protection against the Embola virus belong to this vaccine category, as well. Ervebo™ (rVSVΔG-ZEBOV-GP), the first authorized Embola vaccine, and the only one fully approved by both the EMA (2019a), is based on the modification of the attenuated vesicular stomatitis virus so that it expresses the surface glycoprotein of Zaire Embola virus. Ervebo™ was used, under a compassionate use protocol, during the Kivu Embola epidemic with promising results as a one dose intramuscular protective vaccine. Ervebo can be administrated to adults $\geq$ 18 years (Andrea et al., 2015; Choi et al., 2021; Ollmann Saphire, 2020; Wolf et al., 2021). On the other hand, the hepes vaccines are administrated as a first (Zabdeno) and a booster (Mvabea) dose and are approved for use under 'exceptional circumstances' in individuals $\geq$ 1 years of age by the EMA (2020). To avoid the phenomenon of viral vector seropositivity, Zabdeno consists of the replication-incompetent, recombinant Ad26.ZEBOV, while Mvabea contains the non-replicating, recombinant Vaccinia Ankara Bavarian Nordic virus (MVA-BN-Filo). Ad26.ZEBOV expresses the glycoprotein of the Zaire Ebola virus (ZEBOV) Mayinga strain and the booster vaccine, MVA-BN-Filo, encodes 4 proteins of different strains of the Ebola virus [Zaire Ebola virus Mayinga strain glycoprotein (GP); Ebola virus Sudan Gulu strain GP; Ebola virus Taï Forest strain nucleoprotein and the Marburg virus Musoke strain GP] (Tomori and Kolawole, 2021).

3.6. Vaccine adjuvants

The word "adjuvant" comes from the Latin "adjuvare", which means "to help". According to the EMA: "A vaccine adjuvant is a component that potentiates the immune responses to an antigen and/or modulates it towards the desired immune responses" (EMA, 2005). Depending on the type of the pathogen, different categories of adjuvants can be used to provide the best result (Petrovsky and Aguilar, 2004). Adjuvants alone do not have an immunogenic ability, but when co-administrated with an antigen, they activate the innate mechanisms of the immune system and

improve the efficacy of the vaccine. The activation is triggered by the recognition of adjuvant domains on the cellular PRRs. According to the active EMA guidelines, even if an adjuvant does not present serious adverse effects, its use must also be beneficial and improve the safety and efficacy profile to be approved (EMA, 2005). Below, we review some of the most common and promising innovative adjuvants at the nanoscale.

3.6.1. Immune-stimulating complexes (ISCOMs) and ISCOM-matrix

Immune-stimulating complexes (ISCOMs) were first described by Morein and colleagues in 1984 and since then, an evolution in the adjuvant technology arrived (Morein et al., 1984). They are spherical cage-like structures of an approximately 40 nm diameter and they consist of cholesterol, phospholipids, specific saponins from *Quijalla saponaria* and incorporated antigens (Reimer et al., 2012). Later, it was noted that the integration of the ISCOM formulation with the antigen is not necessary for the immunomodulatory character of the adjuvant and empty ISCOM particles were developed. These particles are now called ISCOM-matrix **(**Fig. 1G**)** and their main representative is Matrix-M™. Matrix-M™ consists of two different types of NPs, Matrix-A and Matrix-C (7:3). These NPs differ in the saponin fraction (Fraction-A and Fraction-C respectively) (Hu et al., 2005; Magnusson et al., 2018; Skene et al., 2008). A negative surface charge is present due to the glucuronic acid of the *S. saponaria* saponins. This charge is useful for electrostatic interactions with positively charged antigens and also provides physicochemical stability in the system's conformation (Pearse and Drane, 2005).

As early as 1997, it was proposed that ISCOMs induce both T_H1 and T_H2-mediated immune responses (Magnusson et al., 2018), while in 2012, a study took place for the evaluation of the mechanism of action of Matrix-M™ in murine. The results showed an increase of leucocytes and DCs in lymph nodes in a murine model (Reimer et al., 2012). The ISCOMATRIX formulation promotes high levels of both humoral (high titers of specialized antibodies) and cellular (Ag-specific $CD8^+$ T-cells) immunity, while a plethora of chemokines and cytokines contribute to generate a potent, robust and long-lasting immune response (Morelli and Maraskovsky, 2017).

3.6.2. Oil-in-water nanoemulsions

Although conventional o/w emulsions have been effectively used in many prophylactic vaccines, nanoemulsions **(**Fig. 1H**)** show a more efficient and safer profile. According to the literature, higher immunogenicity and milder topic reactions are observed after the use of o/w nanoemulsions in comparison with larger-sized emulsion droplets (Shah et al., 2014). In this review, we analyze the MF59 and AS03 o/w nanoemulsions.

The MF59 adjuvant has been utilized in seasonal and pandemic influenza vaccines. The first MF59 adjuvanted vaccine was licensed by the FDA in 1997 under the trade name Fluad® (Seqirus Inc.) as an inactivated influenza vaccine for elderly populations. Till today, four more MF59 adjuvanted vaccines have been authorized (Focetria®, Celtura®, Aflunov® and Fluad Tetra®). MF59 contains squalene (4.3% w/w) and the surfactants Tween 80 (0.5% w/w) and Span 85 (0.5% w/w). The size of the droplets is approximately 160 nm (O'Hagan, 2007). The nanoemulsion is biocompatible, biodegradable, and non-toxic (Kommareddy et al., 2017). As to the MF59 mechanism of action, it mainly promotes a T_H2 response. The potency of MF59 is caused by the development of a local immunostimulating environment. A plethora of chemokines and cytokines, such as the CCL2 monocyte chemoattractant, lead to the production of high antibody titers (O'Hagan et al., 2012; Villarreal and Casale, 2020). As MF59 does not activate the T_H1-mediated mechanism, studies should be proposed for the incorporation of the safe MF59 with a T_H1 inducer, such as the CpG oligodeoxynucleotide (O'Hagan, 2007; S. Wang et al., 2020).

Adjuvant System 03 (AS03), such as MF59, is a squalene-based emulsion and additionally contains α-tocopherol (Vitamin E) that provides enhanced immunogenicity (Del Giudice et al., 2018; Morel et al., 2011). AS03 has been utilized in vaccines against avian influenza (H5N1) and the H1N1 influenza pandemic (2009). During the pandemic, two AS03-containing vaccines were authorized, one in Europe (Pandermix) and one in Canada (Arepanrix). One year after, Pandermix was associated with narcolepsy syndrome in the adolescent population and until 2015, more than 1300 cases had been reported in the EMA EudraVigilance database. Although the mechanism of this adverse action might be correlated with the production of an antibody for an influenza nucleoprotein and not with the existence of AS03, more research is necessary on the subject (Ahmed et al., 2018; Johansen et al., 2018). It is also of exceptional importance to research whether the same result would appear after immunization of a non-adjuvanted vaccine or with the use of another similar adjuvant, as AS03 may not be the major causative of narcolepsy but the driving force.

3.6.3. Liposomal adjuvant systems

After the evaluation of the saponin QS-21 and the 3-*O*-desacyl-4′-monophosphoryl lipid A (MPL) immunostimulating possibilities and dangers, the GlaxoSmithKline (GSK) and US Army developed liposomal platforms - AS01 ($AS01_B$ and $AS01_E$) - to incorporate the above constituents **(**Fig. 3**)**. Two vaccines containing $AS01_B$ have been approved, Shingrix and Mosquirix. According to the EMA risk-management plan, updated in 2019, one of the adverse effects that have been observed and associated with the administration of Mosquirix is febrile convulsion in subjects 5–17 months and it shall further be investigated (EMA, 2019b, 2015).

The liposomal formulation of these materials decreases the hemolytic danger caused by QS-21, as high doses of QS-21 can cause lysis of the plasma membranes of erythrocytes, due to the amphiphilic behavior of the molecule (Lacaille-Dubois, 2019). This action is correlated with the interaction of QS-21 with the plasma cholesterol, leading to the formation of pores and defects of the membrane bilayer. Furthermore, the chemical instability of QS-21 should also be taken into consideration, as the pure mixture of the saponins is thermo- and pH-sensitive, leading to hydrolytic diacylation (Ragupathi et al., 2011). Hence, the production of an adjuvant system with a liposomal structure that contains cholesterol, orientates QS-21 to interact with the cholesterol of the liposomes rather than the cell membrane cholesterol. AS01, as well as a similar system developed by the US Army, called Army Liposome Formulation containing QS-21 and MPL (ALFQ), can develop sophisticated architectures that protect from hemodialysis and orientate the eight sugars of QS-21 in a conformation visible for interaction with the lectin receptors, present on the surface of innate immune system cells (Alving et al., 2020)

4. COVID-19 vaccines at the nanoscale

After one and a half years since the first cases of COVID-19, more than 216 million cases have been identified and more than 4,5 million people have lost their life due to SARS-CoV-2. According to numbers, the above approaches the population of New Zeeland. Recently, four COVID-19 vaccines have been authorized by the FDA and the EMA (emergency use authorization in the US and conditional market authorization in the EU) and more are used in other territory regions, such as in China and Russia. All of these vaccines have successfully contributed in the fight against the pandemic, as approximately 4 billion doses have already been administrated. According to the World Health Organization (WHO), as of May 2021, 100 vaccine candidates are under clinical evaluation (phases 1–3) or authorized (phase 4) worldwide. Almost half of them (42) contain platforms at the nanoscale, while all the authorized COVID-19 vaccines are based on nano-sized vectors. 20 are based on the viral vector (replicating or non-replicating) technology, 16 are RNA vaccines, 5 utilize the VLP formations and 1 belongs to recombinant protein nanosystems, adjuvanted with a nanoplatform. In this section, we analyze COVID-19 nanoscale vaccines that are authorized or in the

final stage of their clinical evaluation. Table 2 presents information about all the COVID-19 nanoscale vaccines.

4.1. Viral vector COVID-19 vaccines

Three non-replicating viral vector (VVnr) vaccines have been approved by the UK Medicines and Healthcare Regulatory Agency, the EMA, the FDA, and the regulatory authorities of Russia by the names AZD1222 (authorized by EMA), Sputnik V (under rolling review in EU and US), and Janssen COVID-19 vaccine. The first one, which was developed by the University of Oxford and AstraZeneca, utilizes the replication-deficient chimpanzee adenovirus ChAdOx1 to carry the structural surface glycoprotein antigen (S-protein) gene (Voysey et al., 2021). The second one was developed at the Gamaleya Research Institute of Russia and consists of two different human recombinant adenoviruses – rAd5 and rAd26. Both vectors carry the full-length S-glycoprotein gene of SARS-CoV-2. Although it is not always clarified in the literature, the term 'combined vaccine' means that the subjects' first dose of the vaccine is with the one vector and the second dose contains the other vector. Hence, we are talking about two different formulations that are used each time (Logunov et al., 2020). Why such an administration design is necessary has the same answer as the question of why a different dosage schedule gave better results for the ChAdOx1 nCov-19 vaccine: seropositivity to the vector, as mentioned above. To minimize this phenomenon, Sputnik V takes advantage of the two types of Ads, while ChAdOx1 nCov-19 highlights that when the first booster dose is lower than the second one, higher efficiency is achieved. Specifically, administration of low dose/standard dose provided 90% efficiency while administration of stand dose/ standard dose provided approximately 65% efficiency in group populations 18–55 years old. Interestingly, a phase 1/2 clinical trial (NCT04684446), sponsored by AstraZeneca, evaluates whether AZD1222 vaccine can be given in combination with the rAd26 strain of Sputnik V vaccine, as well as the dose timing of these two vaccine doses.

In contrast with the above two vaccines, the COVID-19 vaccine that was developed by Janssen Pharmaceutical (Johnson & Johnson), is authorized as an one-dose vaccine. In fact, it is the only one-shot authorized COVID-19 vaccine. According to the clinical trials result, the vaccine showed 66.3% efficiency two weeks after the vaccination, while further effectiveness could be achieved after a second dose. Janssen's vaccine utilizes the human Ad26 virus as a vector to transfer the immunogenic information to activate the immunity. The CanSino Biological vaccine, based on the rAd5 virus, also provides protection against SARS-CoV-2 after a single immunization and is currently under phase 3 evaluation (Zhu et al., 2020).

All these four vaccines are administrated intramuscularly (IM). Two pharmaceutical companies, Vaxart and ImmunoBio, have developed oral vaccines against SARS-CoV-2, currently in phase 1 and 1/2 trials, respectively. Both vaccines utilize Ad5 as a vector but the Vaxart formulation is enriched with a TLR agonist (dsRNA) (Jin et al., 2010; Poteet et al., 2016).

According to the WHO, 4 vaccines based on replicating viral vectors are in clinical evaluation worldwide. The mechanism of action for these vectors is the same with Imojev® and Dengvaxia® formulations, meaning that the chimeric vectors have the potential to replicate but they can barely cause illness due to the vector.

Although viral vector vaccines have proved to be highly effective and safe, concerns about some very rare side effects have arisen, specifically unusual blood clots with low blood platelets. According to EMA data, AZD1222 (Vaxzevria) can cause such clots in 1/100,000 of vaccinated people (EMA, 2021). The FDA has reported some similar cases after immunization with Janssen's vaccine (Shay et al., 2021). These clots seem to be correlated with the production of platelet factor 4 (PF4) antibodies and the pathogenicity is known as the VIPIT (vaccine induced prothrombotic immune thrombocytopenia) syndrome (Greinacher et al., 2021; Scully et al., 2021; Thaler et al., 2021). Although very rare, VIPIT is a serious adverse effect, capable to cause high level of vaccine hesitancy. Thus, some European countries chose to use Vaxzevria only in older populations, where the danger of severe disease and death after infection with SARS-CoV-2 is higher, while other vaccines are preferred for younger people.

4.2. RNA-based COVID-19 vaccines

As mentioned above, RNA vaccines are promising vaccines because they combine high effectiveness and safety with cost-effective and rapid manufacturing processes. Moreover, the RNA modification to provide protection against SARS-CoV-2 variants is easily feasible. Evidence of that is the authorization of the two first COVID-19 vaccines by the EMA and the FDA. Pfizer/BioNTech's and Moderna's technologies are based on the encapsulation of mRNA molecules into LNPs (Table 3). Both are administrated IM and two doses are necessary to provide a high level of protection against SARS-CoV-2. The first one is currently the only COVID-19 vaccine that has received a standard marketing authorization, while the rest vaccines have received a conditional marketing authorization.

Pfizer/BioNTech's vaccine (BNT162b2 – 30 mg per dose) encodes a membrane-anchored SARS-CoV-2 full-length spike, stabilized in the prefusion conformation (Walsh et al., 2020). As it is mentioned in the Summary of Product Characteristics (SPC) of the vaccine, the other excipients included are an ionizable cationic lipid (ALC-0315), a surfactant PEGylated molecule (ALC-0159), cholesterol and a helper phospholipid (distearoylphosphatidylcholine DSPC). The mentioned biomaterials form the LNPs that encapsulate the mRNA molecules into internal aqueous cavities. The result of a phase 2/3 trial showed 95.0% vaccine efficacy and limited, mostly mild adverse events. Some of the serious but rare adverse events are shoulder pain, right axillary lymphadenopathy, paroxysmal ventricular arrhythmia, and right leg paresthesia. HIV, HBV and HCV-positive patients, as well as older populations or populations with other chronic diseases were included in the trials, but people with other immunodeficiencies were excluded. Evaluation processes will continue for two years after the second administration, so that more reliable results will be obtained (Polack et al., 2020). Although this vaccine seems to have a beneficial profile, it must be stored at −80 °C (Mishra and Tripathi, 2021). These storage conditions hinder vaccination in low-income countries.

Moderna's vaccine, on the other hand, is stable inside a typical refrigerator (2–8 °C) for one month and for seven months at −25 °C to −15 °C. The mRNA (mRNA-1273) encodes the stabilized prefusion SARS-CoV-2 spike protein. Some of the mRNA modifications are similar to ones in Pfizer's mRNA (5′cap, 3′UTR and poly(A) tail) and result in higher stability of the molecule (Corbett et al., 2020). Although the manufacturing processes are not fully transparent at the moment, due to active patents, we hypothesize that except for mRNA stability differences, the differentiation in the storage temperature between the two vaccines is mainly owed to the materials of the LNPs. Moderna's LNP formula contains SM-102 as an ionizable cationic lipid, DSPC, cholesterol and dimyristoyl glycerol - polyethyleneglycol 2000 (DMG-PEG2000). Both platforms use the same system for the development of the LNPs (ionizable lipid:DSPC:cholesterol:PEGylated surfactant). However, the morphology of the two ionizable lipids, ALC-0315 and SM-102, is significantly different (e.g., in the number of hydrophobic tails). Moreover, the molar ratios between the utilized biomaterials are currently unknown. All of the above result in two LNP platforms of unique physicochemical profiles.

Finally, the CVnCoV vaccine was designed by CureVac AG and is currently under rolling review by EMA. CVnCoV is a lipid nanoparticulated platform that encapsulates an unmodified mRNA and leads to a more physical activation of the immune system. According to CureVac declarations, the vaccine is stable and effective for at least three months in a regular refrigerator (5 °C). While this vaccine is almost in the market, GSK and CureVac AG agreed to develop a next-generation

vaccine that will be effective in multiple variants and mutations of SARS-CoV-2, confirming the adaptability of this vaccine technology against the variants. Four other LNP-mRNA platforms are in early-stage evaluation.

4.3. Nano-adjuvant COVID-19 vaccines

Novavax is the only pharmaceutical industry, at the moment, that developed not only an antigenic nanosystem but also a nano-adjuvant vaccine (NVX-CoV2373 – 0.5 mL). NVX-CoV2373 contains a recombinant nanoparticulate platform (rSARS-CoV-2, ~27.2 nm) and a nano-adjuvant and is almost ready to be authorized by the medicine agencies. The platform is formed by the self-assembly of the trimeric, full-length, wild type S-glycoprotein SARS-CoV-2. The protein monomers are expressed, assemble into nano-micelles and are purified by baculovirus *Spodoptera fruigiperda* (Sf9) insect cells (Tian et al., 2020). The vaccine is adjuvanted with Matrix-M1 that has been extensively described above. The vaccination process requires 2 IM injections, 21 days apart. rSARS-CoV-2 and Matrix-M1 are separately stored at 2–8 °C and mixed right before the administration (Keech et al., 2020). Hopefully, NVX-CoV2373 seems to be efficacious against both the UK and South Africa variants, according to Novavax. However, scientific reports and results are necessary to confirm this claim.

5. Future perspectives

The programming of each immune cell that receives certain information from its environment, as well as the interaction with other cells of the immune system is the reason for the production of an effective response. The ability of the immune system to receive and respond immediately to a plethora of different messages is remarkable, noting that immune cells are “blind” to recognize information that does not activate its receptors. We should not correlate its function with a binary self-not-self algorithm. Interestingly, researchers correlate our immunity mechanism of action with artificial intelligence terms, such as crowd wisdom and machine learning (Cohen and Efroni, 2019). We would like to bring these observations a step further and connect them with quantum mechanics. Principles such as the impossibility to differentiate the input and output, the hardware (chemistry) from the software (bio-information) and the immediate communication and interaction of a population (crowd wisdom) are basic phenomena observed in the science of quantum computation (Davies, 2004). Indeed, several important decisions of the immune system are based on events that happen at the nanoscale, where quantum effects are proven to exist (McFadden and Al-Khalili, 2018).

In regard to the above, it is beneficial to repurpose formulations at the nanoscale as vaccine platforms. The results of their use in vaccine technology can be placed into three basic axes: a) low cost, b) efficiency, and c) safety. Firstly, nanotechnology can be useful for the development of innovative platforms that can be administrated via alternative routes, including intranasal, (Marasini et al., 2017; Nakahashi-Ouchida et al., 2018) intradermal (Al-Zahrani et al., 2012; Caucheteux et al., 2016) or mucosal (Faruck et al., 2020; Johnson et al., 2020; Zhang et al., 2018) administrations. Although these sophisticated formulations might seem expensive, the final immunization per person cost can be decreased due to the minimization of doses, the ability to combine more than one antigenic epitope, the development of multifunctional chimeric vaccines (cross-protection) and the lower storage cost.

Secondly, due to the high surface-to-volume ratio of nanoscale systems, enhanced immunogenicity of both the innate and adaptive immunity can be induced. Furthermore, the enrichment of such formulations with the right adjuvant that will activate certain pathways of the immune response can target specific key cells. HIV-1 (Brinkkemper and Sliepen, 2019), Zika (Shanmugam et al., 2019) and Ebola (Yang et al., 2017) are some of the viruses on which nanovaccines have shown promising results. Not surprisingly, many of the currently authorized vaccines against SARS-CoV-2 are based on nanotechnology. These innovative vaccines provide extremely high level of efficiency, more than 65% and in some cases more than 90%, including difficult subpopulations, such as older people with comorbidities.

In vivo trials, as well as human trials are necessary today for the development of immunogenic and safe vaccines. Because of ethical issues, scientists are trying to find effective methods to decrease the number of subjects that are necessary in vaccine and drug development. We hypothesize that systems biology and 3D bioprinting will have a promising contribution to accomplishing the above purpose. Over the last years, it has been evident that a disease is not attributed to a single factor but instead, several complex processes are taking place at the same time. Systems biology uses big datasets of the ‘omics’ - transcriptomics, metabolomics and proteomics - and predicts the behavior of a biologic system, for instance, a cell, tissue, organ, or even a living organism to certain stimuli (Schneider and Klabunde, 2013). Developing *in silico* models that could forecast the systemic effect of the administration of an active substance could minimize the number of *in vivo* trials. Assessment of critical process parameters and optimization of quality by design (QbD) processes could be the first fields of pharmaceutical manufacture that will take advantage of systems biology (Richelle et al., 2020).

3D bioprinting is another innovative idea for minimizing *in vivo* preclinical and clinical trials. 3D bioprinting utilizes smart thermo-responsive biomaterials, mainly polymers, that are biodegradable, to synthesize highly hierarchical structures of medicinal implants, tissues, or organs (Lee and Cho, 2016). Except for the designing of complex scaffolds or extracellular matrixes (ECM), modern bioprinters can also deposit cells. Organs-on-a-chip is a promising idea based on the microfluidic technology. Microfluidic organ-based platforms could be manufactured by high-technology bioprinters with the prospect of mimicking the functionality of real organs. Thus, scientists can study the effects of administrating a certain formulation (e.g., a vaccine) in a human-like chip of only some micrometers diameter (Sun et al., 2020). Recently, chips that connect more than ten compartments/organs have been produced, as presented in Fig. 4, and their ability to correctly predict the real pharmacokinetic and/or pharmacodynamic *in vivo* behavior in different stimuli is in evaluation stage (Berthier et al., 2020; Novak et al., 2020).

According to all the above, we propose that universal medicine agencies, such as the EMA and the FDA, should recognize the benefits of utilizing innovative technologies that are taking place at the nanoscale for the development of high quality, efficient, safe, and economically affordable vaccines and adopt the right legislative framework. In this way, not only high, but also low-income countries can have access to effective and safe vaccines the most important human privilege: human health. Standard, well-characterized, safe vaccine platforms are the key parameters to quickly develop vaccines against epidemic viruses.

6. Conclusion

The present review outlines the innovative vaccine platforms and their role in the design and production of safe and effective COVID-19 vaccines. These platforms can transfer both antigens and adjuvants.

Each class of these platforms provides certain advantages and disadvantages, the knowledge of whom will lead to the most effective selection for each pathogen. Three main vector types can be classified by the type of active substance they deliver - protein subunits, nucleic acids, or immunostimulant molecules. Each platform is responsible for a unique activation of the human immune system, due to a different antigen presentation. The presentation is highly connected with the platform’s structure and stability. Thus, the physicochemical and morphological characteristics of each vaccine platform affect the final functionality of the system and finally the vaccine effectiveness.

The incorporation of biomaterials that belong to different categories, such as lipids and polymers could result in the development of “smart”

delivery nanoplatforms i.e., LNPs that interact with their environment and release the desired antigenic information to the target immune cells.

On the other hand, viral vector vaccines have the ability to incorporate the antigenic information into their genetic code and via a non-pathogenic infection of the host cells, mimic the natural infection route of the virus.

Subunit formulations self-assemble into nanosystems with a highly organized and responsive morphology. The surface of these systems presents certain areas of the antigenic proteins, the epitopes, in a way that the activation of the immune process is more intense and effective.

Finally, even when the immunogenicity of the formulation is low, the addition of an adjuvant system with immunostimulant factors can lead to an effective immune response. Although a nanosystem that contains the adjuvant is not always necessary, in some cases the platform can significantly reduce the toxicity of the adjuvant and/or increase the immunostimulant properties.

The COVID-19 pandemic has up till now resulted in the loss of a great number of lives, which overcomes the population of a small country i.e., Croatia. Such an aggressive virus and a threatening health issue resulted in a worldwide coordinated effort to fight against the virus spread and boosted the development of innovative vaccine platforms. Although these nanoscale vaccines thrived under an emergency situation, their results are highly promising for the design of future vaccines against other pandemics or even existing viruses for which effective vaccines have not been found yet.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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