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Review Article

From bench to bedside: Unveiling the background and benefits of nanovaccines tested in clinics

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ABSTRACT

Despite the growing body of literature on nanovaccines, focused analyses of platforms tested in and undergoing clinical investigation remain limited. This review addresses this gap by critically examining recent advancements and highlighting nanovaccine technologies that have undergone human clinical trials. Using an extensive search on clinicaltrials.org, we explored the diverse applications of nanovaccines, including leading SARS-CoV-2 candidates and platforms targeting other infectious diseases and cancers. We also highlighted foundational research that has enabled clinical investigation of nanovaccines over the past decade, highlighting their potential to address a range of medical conditions. While many technologies have been developed to combat SARS-CoV-2, several key innovations targeted a broader spectrum of diseases. This review details these technologies, focusing on their materials and mechanisms of action in inducing immune protection, while also exploring how nanomedicine facilitates nanovaccine development and introduces novel adjuvant concepts. Finally, we provided a retrospective analysis of the development journey of these platforms, offering insights into the intellectual and

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technological efforts behind their clinical translation. By bridging the gap between research and application, this review aims to give readers a comprehensive understanding of how nanovaccines progress from the laboratory to clinical practice.

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1. Introduction

The past 3 decades have witnessed an exponential growth of the nanomedicine field, providing new tools for improving disease management and diagnosis [1]. The ability to manipulate and engineer carriers, probes and sensors at the nanometer scale has paved the way for the development of complex and multicomponent devices with unparalleled properties, primarily focused on revolutionizing targeted medicine and enhancing the safety of toxic therapeutics [2]. This effort has also revolutionized the delivery of various molecules that were previously challenging to administer or possessed unfavorable pharmacokinetics profiles, including highly hydrophobic and hydrophilic drugs [3]. At the forefront of these advancements lies the pioneering role of nanomedicine in delivering biologics (i.e., proteins and nucleic acids), representing one of the cornerstone of vaccine development, widely recognized as the most effective strategy for preventing infectious diseases [4]. In this scenario, nanotechnology was developed to generate vaccines targeting infections, chronic and degenerative conditions such as cancer, nervous system disorders and autoimmune diseases. Traditional vaccines prime the immune system by exposing it to an attenuated or inactive form of a pathogen, enabling the body to develop a robust immune response against the actual disease-causing agent [5]. However, this "modus operandi" have some limitations, such as difficulty in achieving adequate vaccine efficacy [5], difficult storing, preservation, dosage control and poor targeted delivery [6]. Nanomedicine offered the potential to overcome these limitations by improving vaccine stability and controlled release of adjuvants and antigens. Also, nanoparticles can resemble or mimic the structure of viruses or bacteria, which can support the immune system activation. They can also be designed to target specific cells or tissues, which can improve their overall efficacy [7,8]. Furthermore, they can be precisely formulated or enriched with different antigens and adjuvant molecules that are fundamental to build an appropriate immune memory and response [9,10]. Controlled release mechanisms allow for sustained antigen delivery, potentially reducing the number of doses required, while personalized vaccines can be tailored to individual genetic profiles. These advances in material development allowed combining antigenic properties with the ability of cell internalization and cytoplasmic delivery, avoiding endosomal degradation. This effort, boosted by the nefarious recent events related to the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemics mortality and the consequent need to control the disease spreading, opened new avenues of research to develop and optimize novel nano-based vaccines. Many works and reviews were dedicated to this topic [11–13] and to the pre-clinical and experimental efforts performed to generate nanovaccines. Most of these works focus on nanovaccines designed for cancer treatment [14], while giving little attention to the efforts generated to translate these technologies in clinics. In this work, we aim to analyze critically the status of the research, providing tangible examples of such technologies that satisfied the requirements (production yield, scalability, quality control, efficacy and safety) for human experimentation, therefore focusing exclusively on technologies investigated in clinical trials.

A significant portion of this work relies on a study conducted on clinicaltrials.org. A search for "nanocarriers and vaccine" yielded dozens of clinical trials on nanovaccines, while many others came to light with more in-depth research. Most of these investigations were established to validate vaccines against SARS-CoV-2, the virus that cause Coronavirus disease 2019 (COVID19), other notable studies focused on vaccines for different infections and for cancer, representing the latest therapeutic frontiers against these diseases. A retrospective analysis of the path performed to develop these platforms was also provided with insights about the efforts behind the development of clinical nanovaccines. The metrics of our literature search are shown in Fig. 1, which summarizes current clinical studies by their starting year, Phase, status and disease.

2. Diseases targeted by nanovaccine

In theory, effective non-toxic vaccines and nanovaccines could be developed to prevent every disease. However, some conditions are more readily targetable than others. Infectious diseases represent classic targets for vaccines because the pathogens expose non-self-antigens that can be isolated, characterized, and reproduced. For the sake of clarity, the different diseases studied in nanovaccine clinical trials are summarized below briefly, allowing the subsequent section to be categorized based on the various nanovaccine platforms, which are designed usually for multiple conditions.

Since late 2019, SARS-CoV-2 triggered a global pandemic, necessitating urgent scientific intervention. Nanotechnologies, including mRNA vaccines, played a key role in mitigating the virus's spread and fatalities. In the first half of 2023 (June excluded) [15], 680 million new infections and nearly 7 million deaths were reported. Ongoing research on viral mutations and booster strategies underscores the need for vigilance and new vaccination approaches. Respiratory Syncytial Virus (RSV) is a common respiratory pathogen causing mild to severe illness in infants, young

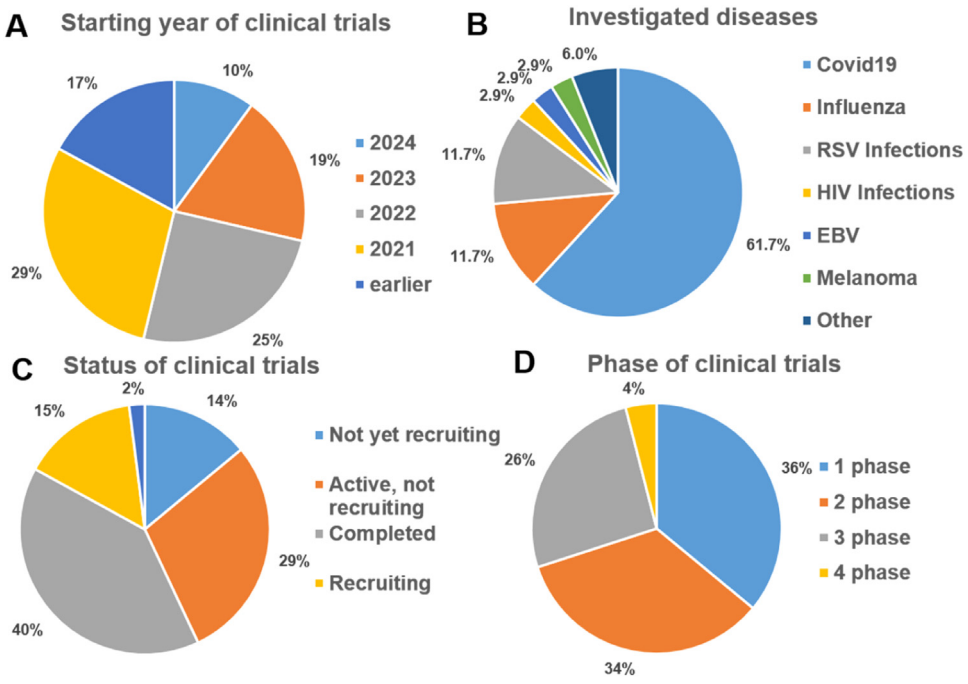


Fig. 1 – Statistics of the research in clinicaltrial.gov: The results were obtained by typing "NANOVACCINE" in the engine search of the website and they were characterized by: (A) year, (B) disease, (C) clinical trial status, and (D) clinical trial Phase.

children and immunocompromised adults [16,17]. It can lead to bronchiolitis and pneumonia, particularly in vulnerable populations [16]. There are no specific treatments [18], making vaccine development critical. Ebola is endemic to West and Central Africa with ~20 outbreaks in 40 years. Current vaccines (rVSV-ZEBOV and Ad.26ZEBOV+MVA-BN-Filo) are effective but require stringent storage conditions (−70 °C refrigeration) [19]. Protein nanovaccines, which are easier to store, are under development [20] . Dengue Fever caused by the Dengue flavivirus affects 400 million people annually in Southeast Asia, the Pacific, Latin America and Africa. Severe cases can lead to hemorrhagic fever and shock syndrome and vaccination might result paramount for reducing the death rate of the disease [21]. The only vaccine, Dengvaxia, has low efficacy and adverse effects like antibody-dependent enhancement (ADE) [22]. Nanovaccines undergoing clinical trials are discussed later in the text (see Section 7, Table 1).

Influenza is a major global health concern, causing severe illness, hospitalizations and deaths annually [27]. Vulnerable groups include the elderly, children, pregnant women and individuals with comorbidities. Influenza also imposes significant economic burdens [28]. Effective vaccine development and distribution are vital for reducing its impact [27]. Human Papillomavirus (HPV) associated with nearly 100% of cervical cancers and significant proportions of anal, vaginal and penile cancers in Europe [29]. HPV vaccines are considered the first example of anticancer vaccines (Fig. 2), although earlier technologies for hepatitis B laid the groundwork [30,31]. Approved nanovaccines for HPV will be discussed later (see Section 5). Human Immunodeficiency Virus (HIV) is a global health threat with a high mutation rate [32] and latent biological reservoirs [33], complicating vaccine development. Despite advances

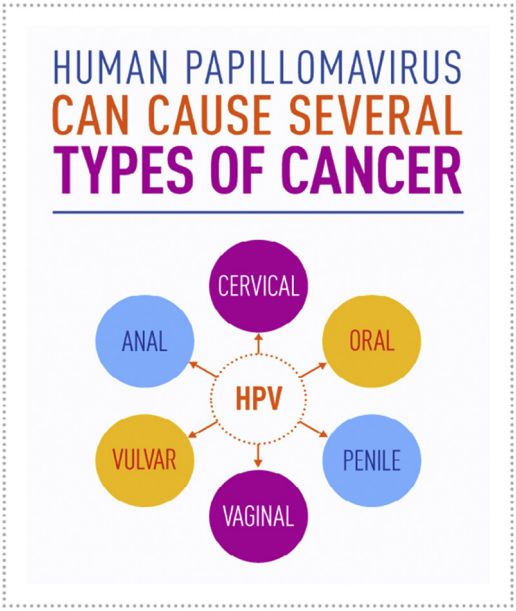


Fig. 2 – Kinds of cancer related to HPV. National Cancer Institute poster used to advertise HPV vaccination campaign. Picture taken from National Cancer Institute [31].

transforming HIV into a manageable chronic illness, eradication via vaccination remains challenging. Malaria caused by Plasmodium parasites, malaria claims over 600,000 lives annually, primarily in tropical and subtropical regions [34]. Disproportionately affecting resource-limited areas, malaria vaccines are still unavailable. Nanovaccines under clinical trials hold promise (see Section 5). Autoimmune

Table 1 – Pros and cons of different nanovaccine technologies.

Platform	Advantages	Disadvantages	Approved	Clinical trials
Liposomes	High safety, approved for children and HIV patients	Low efficiency and stability, refrigeration for shipping and long-term storage	HAV -Epaxal (J&J) Influenza -Inflexal-V (J&J) Herpes zoster -SHINGRIX (GSK)	Tuberculosis -Candidate TB vaccine (GSK) P-II
Lipid nanoparticles (LNP)	Increased stability and delivery efficiency; Can encapsulate mRNA, proteins, gene editing tools; Long-term storage	Rarely reported dangerous side effects (pericarditis, myocarditis, cardiomyopathy)	COVID-19 -Cominarty (Pfizer/BioNTech) -SpikeVax (Moderna/NIAID) -GEMCOVAC (Gennova)	COVID -Cominarty (Pfizer/BioNTech) P-III and P-IV in different therapeutic indications -SpikeVax (Moderna/NIAID) P-IV in combination influenza vaccination RSV -Lipid nanovaccine (Sanofi Pasteur) P-I/II Influenza -mRNA-1010 (Moderna) PIII -mRNA-1020/30 (Moderna) P-I/II - DCVC)P-I Cancer -ABO2102 (ABOGEN Bio) P-I
Virus-like particles (VLP)	Induce effective cellular and humoral immune response; Do not require special conditions to ship and storage; Long-lasting immune response	High costs; Difficult to scale-up manufacturing process; Reactogenicity	HBV -Recombivax-HB® (Merck) -Engerix-B® (GSK) -Heplisav-B (Dynavax) HEV -Hecolin (Wantai BioPharm) SARS-CoV2 -Covifenz® (Medicago/GSK)	HPV -Multivalent HPV VLP vaccine V503 (Merck Sharp & Dohme LLC) P-III -Quadrivalent HPV VLP vaccine V501 (Merck Sharp & Dohme LLC), P-III Chikungunya -PXVX0317 Chikungunya virus VLP vaccine (Bavarian Nordic) P-II Norovirus-induced gastroenteritis -Norovirus GI.1/GII.4 bivalent VLP vaccine (Takeda) P-II Zika Virus -VLP vaccine MV-ZIKA-RSP (Themis Bioscience GmbH) P-I Influenza -A (H1N1) 2009 Influenza VLP vaccine (Novavax) P-II COVID-19 -VBI-2902a and VBI-2905a -VLP vaccine candidates (VBI Vaccines Inc.) P-I -SARS-CoV-2 VLP vaccine (Ihsan GURSEL) P-II RSV -RSV F (NIAID) P-II, P- III HIV - UVAX-1197 and UVAX-1107 UVAX Bio LLC
Polysorbate-80-based protein nanovaccine with Matrix-M adjuvant	Effective, safe, stable	Labor-intensive production; High costs; Short-term effect toxicity of saponins	COVID-19 -NVX-CoV2373 (Novavax)	Influenza -NanoFlu (qNIV, Novavax) P-I/III Influenza and COVID-19 rS+qNIV (Novavax) P-II
PLGA nanoparticles	High stability, biocompatibility, and loading capacity	Difficult to scale-up manufacturing process	/	Melanoma -PLGA scaffold WDVAX in melanoma (Dana-Farber Cancer Institute) P-I Advanced and/or metastatic solid tumors -NY-ESO-1 (Radboud University Medical Center) P-I

(*continued on next page*)

Table 1 (*continued*)

Platform	Advantages	Disadvantages	Approved	Clinical trials
OMV nanovaccines	Provide innate and adaptive responses; Intrinsic adjuvant Properties; Cost effective	Batch-to-batch variation	Meningitis -VA-MENGOC-BC™ (Finlay Institute -Bexsero™ (GSK)	Meningitis -menPF-1 (Sponsor: University of Oxford, Phase I CT NCT01640652) COVID-19 -Avacc 10 (Intravacc B.V.) P-I
AuNP	Act as adjuvants; safe	High cost; Non-biodegradable	/	Dengue fever -DengueTcP™ (Emergex Vaccines Holding - Center for Primary Care and Public Health in Switzerland) P-I
Ferritin NP	Effective and safe; Easy to scale up the manufacturing process	High cost	/	EBV -EBV gp350-ferritin vaccine (NIAID) P-I Influenza -UFluA (Emergent BioSolutions) P-I - H1ssF-3928 (NIAID) P-I
Dexosomes (DEX)	Activate the innate and adaptive immunity	Time-consuming production; High costs.	/	Lung cancer -Tumor antigen-loaded DC-derived exosomes (Gustave Roussy) P-II

Diseases with complex conditions affect millions worldwide, causing chronic pain, fatigue, organ damage and disability. The lack of vaccines stems from the difficulty in targeting a singular cause due to the highly individualized nature of autoimmune diseases.

Cancer is a significant global health burden, with up to 20% of cases linked to viral agents (e.g., cervical and liver cancers) [23–25]. Vaccines targeting these agents can prevent certain cancers. Some cancers express specific antigens (e.g., cancer testis antigen-CTA [26]), which can be used for vaccine development. Colorectal cancer (CRC) is a focus for oral nanovaccine research (see Section 9). Epstein-Barr virus (EBV) is responsible for a diverse range of health issues, including infections, immune disorders and several types of cancer [35], several benefits can arise from a successful vaccine strategy to mitigate the risks posed by this virus. Currently, the most common preventive approach targets the glycoprotein 350/220 (gp350), which plays a crucial role in viral attachment to B cells through the complement receptor 2 [36]. EBV gp350-ferritin nanovaccine is investigated clinically. In Table 1 are listed pros and cons of major nanovaccine platforms described in the review.

3. Lipid-based nanovaccines

As occurred in the drug delivery field, a wide array of materials has been investigated and utilized in preclinical and clinical testing to develop nanovaccines. However, lipid-based structures have notably dominated the field, particularly in response to the SARS-CoV-2 outbreak. Therefore, we believe that a comprehensive introduction to the foundational work behind these technologies is essential for discussing the nanovaccine platforms that have progressed to clinical testing. While recent mRNA nanovaccines have garnered significant attention for their effectiveness in against COVID-19 pandemic, the concept of utilizing lipid nanovesicles as carriers to deliver biologics for immunization dates back nearly 5 decades, effectively opening the field of nanovaccine technology. Gregoriadisand Dr. Ryman pioneered the *in vivo* delivery of biologics encapsulated in liposomes, paving the way for this innovative approach [37]. Dr. Gregoriadis, in particular, investigated extensively the potential of this delivery platform to prime the immune system, demonstrating that liposomes loaded with diphtheria toxoid elicited a remarkable immune response due to their adjuvant properties. This strategy proved to be safer than repeated administration of the unadulterated toxin in vaccination regimens [38]. These studies also anticipated that cationic liposomes were more efficient in inducing an immune response than negatively charged carriers [37], and more generally that nanomedicine could be applied to vaccine technology. This effort was pivotal for the generation of virosomes that allowed for the development of Food and Drug Administration (FDA)-approved carriers against hepatitis A (Epaxal®) and influenza (Inflexal V®) developed at the Swiss Serum and Vaccine Institute [39]. Epaxal® was approved in 1994 and comprised a liposomal formulation enriched with viral particles. In particular, the hepatitis A virus (HAV)inactivated via formalin was absorbed on the surface of the liposomes. These particles were 150 nm in diameter, negatively charged and synthesized with a standard formulation of phosphatidylcholine and phosphatidylethanolamine. The phospholipid bilayer of these particles was also enriched with the fusion viral glycoprotein hemagglutinin (HA) and neuraminidase (NA) (Fig. 3A). Epaxal® showed high immunization power and safety compared to other vaccines that use aluminum salts as adjuvant. Its tolerability profile suggested its large use in children and travelers since it could provide a quick immune protection against the virus [40]. Inflexal V® is another virosome vaccine with similar characteristics to Epaxal®, approved in 1997 for the prevention of influenza. The vaccine comprised a mixture of three virosomal formulations, each targeting a different strain of the influenza virus. Inflexal V® had a high safety profile and it was administered to people

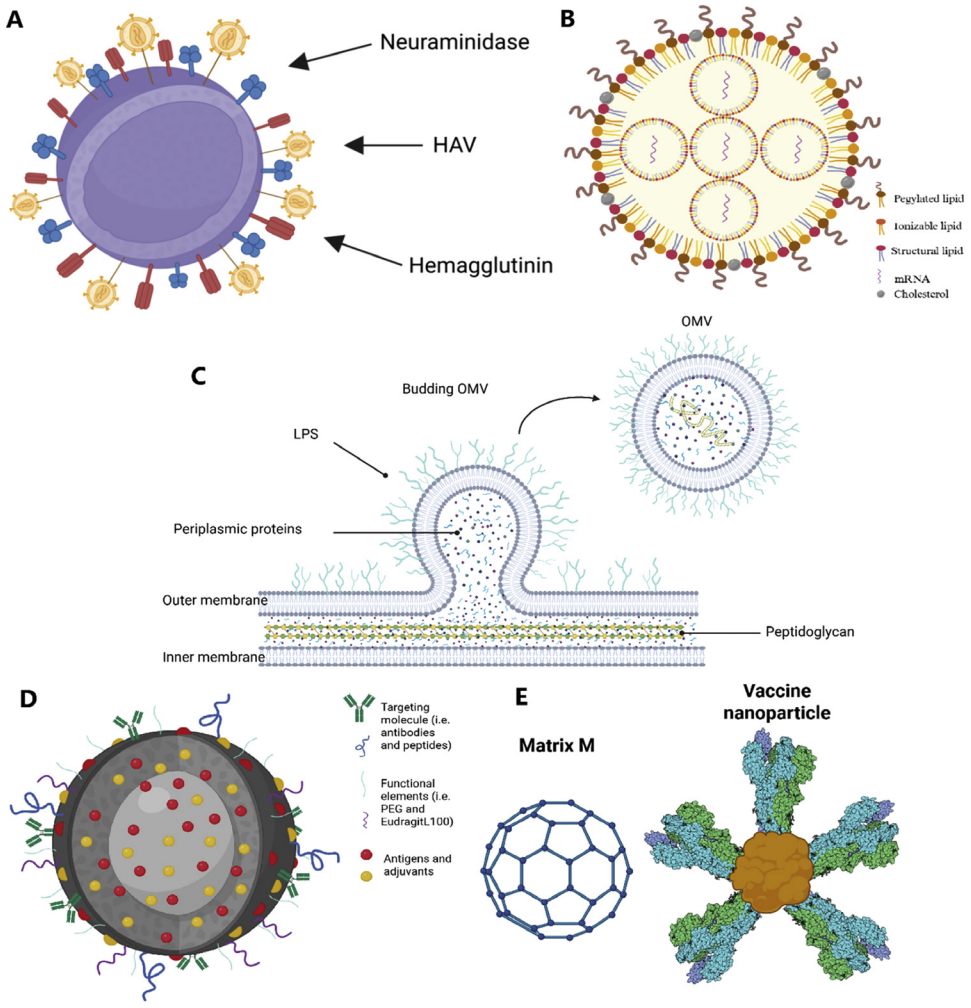


Fig. 3 – Schematic representation of different nanovaccines' structures. (A) Epaxal surface schematics according to Bovier PA [40]; (B) Schematics of LNP mRNA vaccine structure. Organization of the structural and functional elements and the payload within a single particle; (C) Outer membrane vesicle formation: vesiculation process and detachment of the outer membrane from the peptidoglycan layer and the inner membrane. OMV – outer membrane vesicle, LPS – lipopolysaccharide. Figure adapted from [142], copyright© 2022 Lieberman. (D) Versatility of PLGA-based vaccine surface modification. Established protocols can be used to determine the biological identity and surface properties of PLGA nanovaccine including targeting molecules, multiple antigen and adjuvants and functional polymers, generating hybrid systems; (E) Novavax protein nanovaccine. The vaccine formulation comprises Matrix-M nanoadjuvant represented with a truncated icosahedron structure and a nanotechnology comprising transgenic antigens mounted on a core of polysorbate 80. The Figure was drawn according to the Novavax website [226] with the support of free images.com. All figures were generated with Biorender.

of all ages, including those immunocompromised (such as people with HIV). The vaccine had been shown to induce a strong humoral response in these patients, as well as in healthy individuals. A more recent definition of virosomes is VLPs [41] that will be discussed in deep in the next sections.

3.1. *Novel mRNA vaccines*

The emergence of the COVID19 triggered an unprecedented global health crisis. The urgent need for effective countermeasures against this rapidly spreading pathogen spurred an international scientific collaboration of unprecedented scale. Among the most significant breakthroughs in this endeavor was the development of mRNA-based vaccines. Two mRNA vaccines, Comirnaty® [42] and SpikeVax® [43], were the first to receive authorization for emergency use and widespread public distribution. Comirnaty®, developed by Pfizer and BioNTech, was the result of a pre-existing collaboration between the two companies on a nano-based platform for influenza vaccination [44,45]. SpikeVax®, on the other hand, emerged from a cooperative effort between Moderna and the National Institute of Allergy and Infectious Diseases (NIAID) in the United States [46]. Both these vaccines are based on LNPs as delivery vehicles for their mRNA payloads. Compared to liposomes, the gold-standard of lipid-based delivery systems, LNP (80–100 nm) exhibit a unique spatial organization of their lipid components, better resembling micelles rather than the well-defined bilayer

structure of liposomes. This distinct architecture contributes to the enhanced stability and delivery efficiency of LNP. This carrier comprises a single layer of lipids interconnecting with the other lipid molecules (potentially ionizable) organized in the lumen of the LNP (Fig. 3B) [47,48]. LNP technology has evolved over several decades and is now recognized as a leading non-viral gene delivery system. Its versatility allows for encapsulating a variety of therapeutic molecules, including siRNA [49], gene editing tools [50] and proteins [51], into cells. In 2018, LNP made their clinical debut with the approval of Onpattro®, the first siRNA-based therapeutic, which employs LNP to deliver siRNA to the liver for inhibiting the expression of transthyretin in patients affected by transthyretin amyloidosis. Even though this nanotherapeutic is not a vaccine, its legacy is linked directly to the Pfizer/BioNtech and Moderna vaccines. Many scientists consider Onpattro® as the prototype of these mRNA vaccines. The structural and physicochemical properties of LNP made them ideal candidates for the development of mRNA vaccines. Their ability to encapsulate and protect mRNA molecules while facilitating cellular internalization and subsequent protein expression proved crucial in the rapid development of these vaccines. LNP also exhibit favorable biocompatibility and safety profiles, further supporting their suitability for therapeutic applications [52–54]. Comirnaty® and SpikeVax® marked a turning point in the COVID-19 pandemic. These mRNA vaccines, powered by LNP technology, demonstrated remarkable efficacy and safety, becoming instrumental in curbing the spread of the virus and saving countless lives [55], building a clinical background opened to a large number of applications that could revolutionizing modern medicine.

3.2. *Structure*

Comirnaty® and SpikeVax® are discussed together because despite the use of some proprietary functional lipids, the overall design and idea behind these vaccines is the same, and they have been developed at the same time to prevent infectious disease. They comprise five components and three of them are covered by intellectual properties.

3.2.1. *Structural elements*

The structural elements are represented by the cholesterol and 1,2-distearoyl-sn–glycero-3-phosphocholine (DSPC). DSPC was already successfully used in clinics as a component of Onpattro® [42,46]. Besides their structural role, neutral phospholipids such as DMPC (1,2-dimyristoyl-sn–glycero-3-phosphocholine) and DOPE (2-dioleoyl-sn–glycero-3-phosphoethanolamine) are extensively investigated in the field and have shown great promise as delivery materials in LNP-based vaccines, particularly for cancer immunotherapy [56]. DMPC facilitates the uptake of nanoparticles by cells due to its resemblance to cell membrane components and aids in targeting lymph nodes [57,58]. Likewise, DOPE, known for its ability to transition from bilayer to non-bilayer phases to enhance cargo release, has been utilized for generating cancer nanovaccines to co-deliver antigens and adjuvants, efficiently accumulate in lymph nodes, promote dendritic cell (DC) maturation, and favor $CD8^+$ T cell infiltration into tumor tissues [59].

The other two components, represented by PEGylated and ionizable lipids instead, are considered functional lipids and protected by intellectual property rights. Selecting the functional lipids for LNP formulation typically involves the screening of a library containing tens to thousands of lipids with the desired characteristics *in vitro* [60]. The PEGylated lipids in Comirnaty® (ALC-0159 [61]) and SpikeVax® (PEG2000-DMG [43]) constitute a small fraction of the LNP structure but play a crucial role in maintaining polydispersity, preventing particle aggregation in biological environments, and enabling long-term storage [62]. Both Comirnaty® and SpikeVax® technologies demonstrated a relatively low risk of anaphylactic shock [63,64] following administration, with the PEGylated components hypothesized to be the primary contributors to this phenomenon. Although >20% of patients who experienced anaphylaxis had a history of previous anaphylactic reactions, the link between PEG and this adverse effect has been reported as via complement activation-related pseudoallergy [64]. However, the benefits of the vaccination were deemed to far outweigh the risks of these side reactions (4.7 and 2.5 cases per million for Pfizer and Moderna, respectively) [65].

3.2.2. *Ionizable lipids*

ALC-0315 and SM-102, the ionizable lipids of Comirnaty® and SpikeVax®, respectively, possess a tertiary amine group in their structure that becomes protonated at acidic pH. This protonation imparts a positive charge to the lipids, lending them the ability to interact with the negatively charged lipids of the endosome. These interactions disrupt the integrity of the endosomal membrane, facilitating the escape of the mRNA payload into the cytoplasm. This mechanism, driven by the pH-sensitive ionizable lipids, underscores the smart technology aspect of these vaccines, enabling endosomal escape and subsequent mRNA translation, antigen presentation and immune activation [66]. Other theories regarding this phenomenon indicate that a buffering action of the amines of the lipids occurs in the lumen of the endosomes, eventually increasing the osmotic pressure of these vesicles, ultimately damaging these organelles [67]. It was estimated that only 1%–5% of the biologics can escape the endosomal compartment, and that this phenomenon can occur only in a limited period of time after particle internalization, when the sequestered carriers are entrapped in early endosomes maturing in late endosomes [68]. The group of Tasciotti investigating the events following nanoparticle endosomal escape reported a similar observation. They designed a technology comprising a silica core coated with a pH responsive polymer. These particles possessed both PEGylated and ionizable groups and they were efficient in delivering siRNA [67]. In endothelial cells, after endosomal escape, they were sequestered again in autophagic multilaminar bodies within 48–72 h [69], potentially blocking any further release. Ionizable lipids play a crucial role in stabilizing the negatively charged payload within the nanoformulation. Some studies suggest that these components can also enhance cell internalization by promoting particle interaction with the negative cell membranes [60] or by facilitating interactions with surrounding proteins that may mediate cell surface interactions. While this phenomenon still needs further

investigation after intramuscular injection, studies have demonstrated that following intravenous administration, a protein corona, including the apolipoprotein ApoE, can form, promoting nanoparticle hepatic clearance via the low-density lipoprotein receptor [70]. Onpattro® technology, for example, relies on this working mechanism. Finally, these ionizable lipids showed high biocompatibility and immunogenicity [71]. Their ability to facilitate efficient transfection hinges on their pKa value, which typically falls between 6.5 and 6.2 [72]. The immunogenicity of the ionizable lipids is attributed to their capacity to induce inflammation via cytokine secretion, and some studies suggest that these molecules can activate toll-like receptors [73].

3.2.3. *mRNA payload*

The fifth, intellectually protected, component of these vaccines is the mRNA payload (BNT162b2 [74] and mRNA-1273 [75,76] for Comirnaty® and SpikeVax®, respectively), both of which trigger the translation of the SARS-CoV-2spike protein. The choice of the mRNA molecules is very important not only for the proper translation of the antigens, but also for the working mechanism of the vaccine [77]. The payload of Comirnaty® and SpikeVax® contain modified uracil groups that increase its cellular stability and the adjuvant properties of the vaccine. The use of natural mRNA was indicated as one of the reason at the basis of the failure of CureVac vaccine, a mRNA-based LNP developed by GSK [77]. The science underpinning the development of mRNA for vaccination purposes is fascinating and insightful, but it falls outside the scope of this work, which aims to highlight the role of the carriers. Recent reviews on this topic can be found here [78,79].

3.3. *Clinical applications*

Multiple trials were dedicated to evaluate the efficacy and safety of mRNA vaccines and, as expected, in the last years, most of them were focus on evaluating their benefits in preventing COVID19. Current studies are focused on investigating the effects of Comirnaty® and SpikeVax® vaccines in fragile patients (i.e., NCT04780659, NCT04951323), in association with other vaccination strategies designed for other diseases (i.e., NCT06038617, NCT06020118), testing the efficacy and the administration setting of the boosters (i.e., NCT05057182, NCT05213455). The use of these vaccines is associated with rare side effects that include heart conditions (i.e., pericarditis, myocarditis, cardiomyopathy) [80,81]. The causes of this phenomenon are still under investigation even though different hypothesis were formulated, including the induction of an auto immune response triggered by the spike proteins probably against α-myosin, exaggerated cytokine production, and reactogenicity to the mRNA [80]. For this reason, different observational trials were activated to study this phenomenon (i.e., NCT05157191, NCT06103123, NCT05282498, NCT06113692). Also, different companies are focusing on developing this technology. A recent phase I/II clinical trials were recently established by Sanofi Pasteur to evaluate the safety and immunogenicity of an RSV and RSV/metapneumovirus mRNA vaccine (NCT05639894, NCT06686654, respectively). The vaccine formulation consisted of two different LNPs (i.e., LNP containing CL-0059 or CL-0137) loaded with RSV mRNA, and administrated as a single intramuscular injection of 3 doses of RSV mRNA in healthy adult participants. Unfortunately, we were unable to gather more information about this therapeutic, but this technology is currently tested also against influenza. Moderna has developed a series of seasonal vaccines based on mRNA encoding for the influenza virus surface glycoprotein HA encapsulated in LNP. mRNA-1010 is a quadrivalent mRNA vaccine containing mRNA that encodes HA for four influenza A and B strains (A/H1N1, A/H3N2, B/Victoria and B/Yamagata). A phase I/II clinical trial of this vaccine in healthy adults (NCT04956575) was completed, and the vaccine demonstrated therapeutic performances similar to the standard dose of the FDA-approved inactivated seasonal influenza vaccine (Afluria) [82]. mRNA-1010 is currently in phase III ongoing studies in adults (NCT05827978, NCT05415462) and older adults (NCT05566639). Additionally, a phase I/II study of the basic mRNA-1010 vaccine and mRNA-1020 and mRNA-1030 vaccines, which contain mRNA encoding NA antigens in addition to HA-encoding mRNA, was completed, but no results have been released (NCT05333289). Sanofi has developed several monovalent mRNA-LNP vaccines containing mRNA that encodes either the full-length HA protein of different influenza strains or NA antigens. Some of these vaccines are currently in Phase I clinical trials, where they are being evaluated against the quadrivalent recombinant influenza vaccine (RIV4). These studies include NCT05829356 (A/H3N2) and NCT05426174. Another monovalent mRNA-LNP nanovaccine, DCVC H1 HA mRNA vaccine, which encodes the full-length H1 HA protein of influenza A/H1N1, was developed at the Duke CIVIC Vaccine Center (DCVC) and is currently being compared to the licensed quadrivalent influenza vaccine (Fluzone Quadrivalent) in a phase I clinical trial (NCT05945485). In this effort, it is worth to mention also the work of Gennova in generating new mRNA vaccines against Sars-CoV2 (GEMNOVAC-19) and the Omicon variant (GEMNOVAC–OM). Gennova's technology differs from Pfizer-BioNTech and Moderna LNP in several key aspects. Firstly, the payload is a self-amplifying mRNA [83], which allows for a lower dose to be used during administration. Unlike non-amplifying mRNA, the open reading frame of samRNA encodes a replicase composed of four nonstructural proteins. These proteins work together to form an RNA-dependent RNA polymerase, which enables the mRNA to self-amplify within the cell. Secondly, GEMCOVAC can be lyophilized, remaining stable for up to 12 months when stored at 2–8 °C. This characteristic makes it particularly attractive for warm countries like India, where it was conceived and produced. Lastly, GEMCOVAC is administered intradermally using a needle-free injection system with a device known as Tropis (PharmaJet) [84]. As claimed by Gennova [85], the mRNA is absorbed [86] to the surface of the nano-lipid emulsion, rather than being encapsulated as in other mRNA vaccines that use LNPs, simplifying the manufacturing process and limiting material losses. Additionally, this procedure enhances scalability and facilitates technology transfer, which is essential for the global dissemination of mRNA-based technology and addressing the inequitable distribution of vaccines worldwide. GEMCOVAC–OM recently showed promising results in a phase II/III clinical trial as a booster

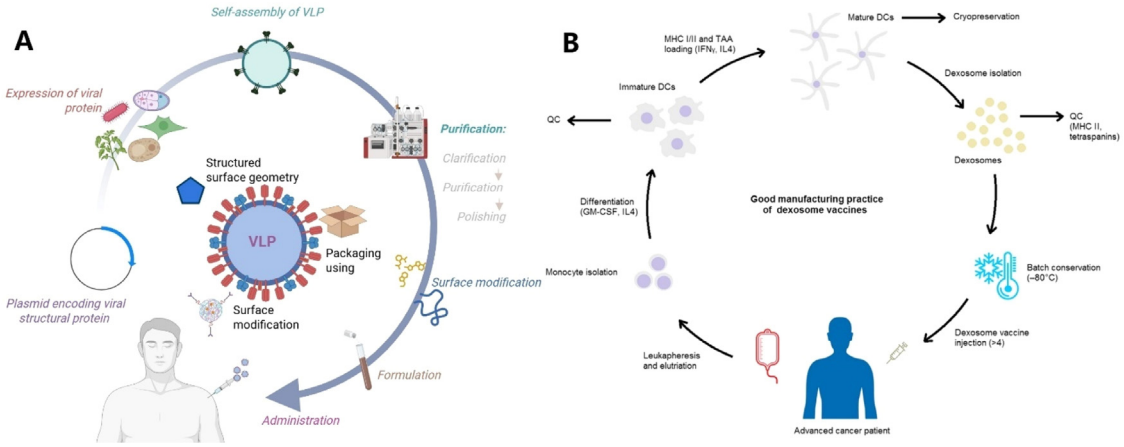


Fig. 4 – Nanoparticle production. (A) Workflow for VLP production: from plasmid encoding and protein expression to self-assembly, purification, surface modification, packaging, formulation and administration. Figure generated with Biorender; (B) Dexosome production cycle for clinical usage. Reprinted with permission from [182], copyright© The Author(s) 2020.

after vaccination with other COVID vaccines [84], receiving Emergency Use Authorization as a single dose booster specifically targeting the Omicron strain. LNPs are also at the base of the clinical translation of Abogen Bisciences vaccine targeting different KRAS mutations overexpressed in cancer (cancer vaccine ABO2102 currently under evaluation in Phase I, NCT06577532). The system rely on a proprietary payload based on a highly efficient RNA circularization cis-splicing system (Cis-System) [87] that allows for a more efficient translation process a significant advantages in the production since less impurities are generated.

A significant trend in the field is the development of a universal vaccine aimed at eliciting a multi-target humoral response against all known influenza subtypes. A collaborative effort by Hensley's groups resulted in a promising vaccine candidate expected to provide universal protection against all influenza subtypes. This breakthrough involved isolating mRNA encoding the HA antigens from all 20 known influenza A and B virus subtypes and encapsulating them in LNP. Preclinical studies in small rodents (mice and ferrets) have demonstrated the vaccine's efficacy in providing protection against both matched and mismatched influenza virus strains [88]. Advantages and disadvantages of different types of nanovaccines described along with examples undergoing clinical trials can be found in Table 1.

4. Virus-like particles (VLP)

4.1. *Impact on the scientific community*

As alternative to novel mRNA vaccines, protein nanovaccines still represent an optimal tool to prevent pathologic conditions. These technologies have been designed to substitute current vaccination based on attenuated or inactivated pathogens that showed major limitations in terms of both safety and large-scale administration. A typical workflow to generate VLP is shown in Fig. 4A. VLPs are nanoparticles (20–200 nm) [89] composed of one or more often multiple viral proteins, eventually resembling the pathogen structure. However, they lack of the genetic material necessary for viral replication and, for this reason, they are considered safe [90]. VLPs are more reactogenic compared to soluble recombinant antigens due to their unique structure containing viral structural proteins resembling the morphology of the parental virus [91]. They can be generated in different *in vitro* systems, including bacteria, insect, plant and mammalian cells [91,92]. They showed high cellular and humoral immunogenic properties thanks to their highly ordered and variable antigenic structure and to the presence of adjuvants that usually enrich their formulations. Their structure is prone also to carry small therapeutic and imaging molecules [93]. VLPs are divided into two main types based on their structural characteristics: enveloped VLPs and non-enveloped VLPs. Enveloped VLPs (eVLPs) are more intricate in structure because they include a lipid membrane sourced from the host cell, which provides opportunities to incorporate adjuvants and foreign antigens. The makeup and origin of this envelope are influenced by the virus family, the natural process of virion assembly, and the specific host system used for production [94]. The envelope of eVLPs contains surface antigens, primarily glycoproteins, which enhance immunogenicity by acting as adjuvants [91]. Additionally, they can be categorized based on the number of protein layers forming their structure, namely single- or multilayered VLP. Usually, VLP are built on the molecular scaffold of self-assembly proteins that can eventually being genetically or chemically modified with antigens. Viral capsid or core proteins can be used for this purpose, as shown with bacteriophages [95]. For example, the capsomers of hepatitis B virus (HBV) were used as scaffold to generate a universal vaccine against influenza A virus [96]. In this effort, four conserved sequences common to different strains of the virus were inserted in the VLP after expression in *Escherichia coli* (*E. coli*). To favor the self-assembly of the system, a molecular link formed by glycine and serine was included to

connect two different capsomers. As a result, a chimeric virus named Tandiflu1 with no genetic materials was engineered demonstrating 100% protection against two major strains of H1N1 virus in a preclinical *in vivo* model.

4.2. *Clinically approved VLPs*

HBV was also the target of the first FDA-approved VLP Recombivax-HB® (Merck) and Engerix-B® (GSK) available since 1986 and 1989, respectively. Both these vaccines are composed of recombinant highly purified small surface of HBV antigen (HBsAg) expressed in large quantities in Saccharomyces cerevisiae, and are based on the self-assembly properties of these molecules. The major difference between these vaccines lies in the concentration of the HBsAg (designed to have a concentration of 20 and 10 μg/ml for Engerix-B® and Recombivax HB®, respectively) and the type of aluminum adjuvants used. Recombivax-HB® contains aluminum hydrophosphate sulphate, while Engerix-B® is formulated with aluminum hydroxide. Both of them were considered extremely safe [97] and effective [98] and opened new scenarios for the development and approval of other VLP-based vaccines against different diseases including: HBV (Sci-B-Vac™/VBI Vaccines), malaria (Mosquirix™/GSK) and HPV (Gardasil®, Gardasil 4®, Gardasil 9®/Merck and Cervarix®/GSK). Gardasil 9®, also known as 9vHPV, is an advanced 9-valent version of the Gardasil® and Gardasil 4® vaccines, offering broader protection against HPV types. Compared to its predecessors, Gardasil 9® provides additional protection against five more HPV types (31, 33, 45, 52 and 58) responsible for approximately 20% of cervical cancers, in addition to HPV types 16 and 18 (linked to cervical and anal cancers), and prevents the genital warts caused by HPV types 6 and 11. The vaccine is composed of highly purified VLPs containing the recombinant major capsid (L1) protein of the HPV types, which are adsorbed onto an amorphous aluminum hydroxyphosphate sulfate adjuvant. The mean particle size was ~50 nm, with the shape and size being influenced by the specific virus type. By focusing only on the completed trials with results generated in the last 5 years, VLP were used against very different diseases including mosquito-borne (NCT05065983) and warfare agents [99], acute gastroenteritis caused by norovirus (NCT02153112, NCT02669121), HPV (NCT00943722, NCT02576054), Zika Virus (NCT04033068), Meningitis (NCT04490018), H1N1 2009 pandemic influenza (NCT01072799), COVID-19 (NCT04773665, NCT04962893) and Influenza (NCT03814720). These vaccines (comprising phases I-III) resulted generally safe and effective in generating neutralizing antibodies [99–104]. A general view of the VLP approved in the last 2 decades is shown in Table 2.

4.3. *VLP: Three important examples*

Between current clinical trials evaluating this technology, it is worth mentioning the first clinical testing of VLP developed harnessing a plant pathogen engineered against influenza (Medicago) [92]. This technology is based on the ability of *Agrobacterium tumefaciens* to transfect *Nicotiana benthamiana*, an indigenous Australian plant similar to tobacco. After infection, *Nicotiana benthamiana* can express antigenic sequences inserted in the bacterium genome and accumulate these proteins in its leaves. The maintenance and the engineering of this technology is scalable and cost-effective and the isolation of the transgenic product is relatively easy, making the production cycle quick (30–40 d) [120]. As result of this effort, it was generated an enveloped VLP comprising a lipid bilayer bearing a mix of recombinant H1, H3 and two B HA proteins to generate a quadrivalent influenza vaccine (QIV). Plant-derived quadrivalent VLP influenza vaccine demonstrated high lot-to-lot consistency (NCT03321968). This vaccine was tested in two phase III clinical trials investigating the efficacy of this technology in adults and elderly [92]. The results demonstrated solid therapeutic effects and relatively low occurrence of serious adverse effects, even though the primary goal of the first trial (70% of efficacy in the adults) was not met. Following these trials, a similar plant-based VLP technology was developed to generate an anti-Sars-CoV2 vaccine (Covifenz®), that eventually received FDA approval in Canada, but the production company canceled the authorization [121]. Each dose of Covifenz® VLP vaccine contains 3.75 mg VLP of SARS-CoV-2 spike protein and 0.25 ml AS03 adjuvant (NCT04894435). Finally, it is worth mentioning the well-characterized RSV-F vaccine [122] developed against RSV. This technology is based on the ability of RSV fusion surface glycoprotein (RSV-F) to self-assemble in VLP [123]. This protein mediate virus internalization into target cells and it is consistently expressed in different strains of the virus. RSV-F can be transfected via baculovirus in Sf9 insect where it is expressed and purified in high amounts. This recombinant RSV-F spontaneously assembled into protein nanoparticles with a morphology consistent with F oligomers arranged as rosettes [124]. HPLC size exclusion chromatography (SEC) analysis revealed 98% purity of RSV-F particles primarily composed of polypeptidic units of the antigen with an average molecular weight per particle between 1000–8000 kDa [123]. RSV-F can assume three different conformations determining particle characteristics: prefusogenic F (particle size of 40 nm and PDI of 0.17) [123], prefusion F (size of 23 nm and PDI of 0.15), and postfusion F (size of 28 nm, and highly variable PDI [125]). The purified recombinant RSV-F exists in the form of multiple RSV-F trimers (19.6 nm in length) consisting of oligomers formed by hatpin-shaped rods with the wider end projecting away from the centers. RSV-F nanoparticles are assembled with an average of four trimers per particle. The vaccine based on the RSV prefusogenic F nanoparticle showed to be effective, safe and immunogenic in healthy adults [126] (NCT01290419). In particular, intramuscular immunization of healthy adults (18–49 year old) was well tolerated without dose-related increases adverse events, independently from the presence of aluminum phosphate adjuvant. The RSV-F nanoparticle vaccine induced virus neutralization and was proven safe and effective also in comparison with palivizumab. Fries et al. [127] reported the findings of a clinical trial (NCT01709019) involving healthy patients older than 60. Participants received either the licensed inactivated trivalent influenza vaccine TIV concurrently with RSV-F vaccine or a placebo. The safety profile of all vaccine formulations was deemed acceptable, as there was

Table 2 – VLP approved in the last 2 decades.

Name	Company	Desease	Expression system	Antigen	Adjuv.	Ref
DTB-HEP B®	P.T. Bio Farma	HBV	*P. past.*	HBsAg	$Al(OH)_3$	[105]
Engerix-B®	GSK	HBV	*S. cer.*	HBsAg	$Al(OH)_3$	[106]
Enivac HB®	Panacea Biotec	HBV	*P. past.*	HBsAg	$Al(OH)_3$	[107]
Gene Vac-B®	Serum Inst. Of India	HBV	*H. polym.*	HBsAg	$Al(OH)_3$	[108]
Recombivax-HB®	Merck	HBV	*S. cer.*	HBsAg	AAHS	[109]
PreHevbrio®	VBI vaccines	HBV	CHO cells	HbsAg, Pre-S1, Pre-S2	$Al(OH)_3$	[110]
Hecolin®	Xiamen Innovax Biotec	HEV	*E. coli*	Portion of HEV genotype 1 capsid protein	Aluminium adjuvant	[111]
Cervarix®	GSK	HPV	Hige five cells	HPV 16/18 L1	$Al(OH)_3$	[112]
Gardasil®	Merck	HPV	*S. cer.*	HPV6/11/16/18 L1	AAHS	[113]
Gardasil 4®	Merck	HPV	*S. cer.*	HPV16/18/31/45 L1	AAHS	[114]
Gardasil 9®	Merck	HPV	*S. cer.*	HPV6/11/16/18/31/33/45/52/58 L1	AAHS	[115]
Walrinvax®	Walvax and GSK	HPV	*P. past.*	HPV16/8 L1	AS04	[116]
Inflexal V®	Crucell	Influenza	Cell free	Influenza virosome	-	[117]
Nuvaxovid®	Novavax	COVID-19	Sf9	Spike antigen	Matrix-M	[118]
Covifenz®	Medicago	COVID-19	*N. bent.*	Spike antigen	AS03	[119]

no evidence of systemic toxicity or serious adverse effects. Additionally, participants exhibited a rapid immune response, with detectable antibodies appearing as early as 7 d after administration. The concurrent use of the vaccines did not interfere with their efficacy, as both vaccines demonstrated high effectiveness. The tolerability and immunogenicity of the RSV-F nanovaccine was also demonstrated after intramuscular administration to healthy young women [128,129] (NCT01960686; NCT01704365). The effectiveness of the RSV-F nanovaccine in protecting newborns was assessed in a phase II trial [18] (NCT02247726) involving administration of a single intramuscular injection to pregnant women in their third trimester. This study demonstrated promising results in terms of antibody responses. The transfer of vaccine-generated maternal RSV-specific IgG through the placenta ranged from 90% to 120%, with a half-life of around 40 d However, these findings were not corroborated in a global phase III trial [130] (NCT02624947]). Another study was conducted during the phase III trial (NCT04605159), which demonstrated that infants born to vaccinated mothers were protected from RSV disease by a single dose of the investigational RSV Maternal vaccine (healthy pregnant women aged 18–49, intramuscular administration). The findings suggest a reduction in the likelihood of developing any and severe RSV-associated respiratory tract disease in the vaccinated cohort relative to the placebo group. However, the candidate vaccine was associated with an elevated risk of preterm birth [131]. Study of revaccination with the RSV-F nanovaccine was well tolerated in the phase III clinical trial (NCT04886596), yet did not result in an augmented efficacy benefit against severe RSV [132]. A further study NCT03529773 demonstrated that RSV prefusion F revaccination was immunogenic, with a peak of geometric mean fold rises that were lower than those observed following the initial vaccination [133]. It is worthy of note that an intranasal RSV vaccine, RSV/6120/ΔNS2/1030s, has been developed. This is a cDNA-derived live vaccine candidate that has been attenuated by the deletion of the interferon antagonist NS2 gene and the genetically stabilized 1030s missense polymerase mutation in the polymerase, which confers temperature sensitivity [134]. A phase I placebo-controlled study revealed effectiveness of a single intranasal vaccine dose demonstrating the significantly higher RSV plaque-reduction neutralizing antibody titers compared to placebo recipients (NCT03387137). mRNA-based RSV vaccine, mRNA-1345, in the phase II–III trial (NCT05127434) demonstrated no apparent safety concerns, and a reduced incidence of RSV-associated lower respiratory tract disease and RSV-associated acute respiratory disease was observed following a single dose of the mRNA-1345 vaccine among adults aged 60 and older [135]. The same outcomes were observed in a phase I clinical trial (NCT04528719) involving healthy adults aged 18 to 49 y. Following a single injection of mRNA-1345, an acceptable safety profile was noted, accompanied by a durable neutralizing antibody response. These findings support the continued development of the treatment [136]. Finally, it worth mentioning the technology of UVAX Bio LLC to generate a vaccine against HIV. This technology is based on the 1c-SApNP® platform including 20 copies of native-like, prefusion-stabilized trimers or trimmed glycans from the envelope of the virus called UVAX-1197 and UVAX-1107, respectively. These vaccines have been recently tested in heathy volunteers (NCT06541093) in a completed study, but the results were not released yet. Although more examples of VLPs will be detailed later, numerous published reviews delve into the diverse methodologies employed to generate VLPs. We highly recommend consulting these papers for a comprehensive overview [91,137,138].

5. Outer membrane vesicle nanovaccines

5.1. *Impact on the scientific community*

Outer membrane vesicles (OMVs) are usually referred as nanostructures (20–450 nm, depending on the bacterial source [139]) naturally released from the membrane of

Gram-negative bacteria [140]. Chatterjee and Das discovered OMV in the 60's investigating the biology of Vibrio Cholera [141], more and more evidences showed that the exfoliation of the external wall is a common feature of many gram-negative pathogens and the investigation of these structure for vaccination purpose accompanied these investigation since the beginning. These vesicles in fact are enriched with pathogens antigens and cannot replicate, making them ideal candidates for this purpose. OMV formation requires the detachment of the outer membrane from the peptidoglycan layer and the inner membrane, which are linked by various lipoproteins (Fig. 3C) [142]. Among the mechanisms underlying membrane vesiculation, several key factors have been identified: (1) a reduction in lipoprotein abundance, which decreases crosslinking between the membrane and underlying layers; (2) defects in the Tol-Pal system, which is responsible for connecting the outer membrane to the inner membrane; (3) alterations in peptidoglycan structure; (4) increased membrane turgor caused by the accumulation of misfolded proteins or peptidoglycan fragments and; (5) elevated phospholipid content, which influences membrane curvature [143]. Recent evidences suggested that vesicles release can occur also in Gram-positive bacteria and micro-organism insensitive to this staining [144]. The vesicles are believed to originate via distinct process following biochemical pathway of outer membrane blebbing or stress-induced lysis [145].

Envelope components and in particular integral proteins, lipoproteins and peptidoglycans are usually present in the structure of these formations, while the presence of other components (i.e., cytoplasmic protein and nucleic acid) has been usually observed as cargo and it depends on the vesicle formation routes. Finally lipopolysaccharide and lipooligosaccharides implement the components profile and all of these elements have immunomodulatory properties [145]. OMV play a multifaceted role in bacterial physiology and interaction with their environment. They facilitate nutrient [146] sensing and binding, in particular iron sensing [147]. They also have defensive properties by acting as a decoy neutralizing phages [148], antibiotics [149] and host defense factors [150], while demonstrating bactericidal properties for other microorganisms [151]. Furthermore, OMV are involved in DNA transfer, allowing genetic material exchange among bacteria, although the mechanisms of uptake remain partially understood [145]. While OMV are natural product their yield product does not meet the required standards for public immunization and therefore different techniques are used to increase their synthetic efficiency. These methods include genetic modification affecting the pathways responsible for outer membrane ultrastructure maintenance (i.e., Tol-Pal system or the gene responsible for lipoprotein enrichment), chemical and physical stresses (i.e., detergent extraction, sonication, extraction agents), and biological stresses (peptide or antibiotics).

5.2. *Clinical application of OMV nanovaccines*

OMV perfectly recapitulate the expectations of the scientific community regarding the research and development of nanovaccines. Their consistent geometry significantly enhances the antigen presentation process, and they serve as antigenic reservoirs that can be boosted further and modified through genetic engineering approaches. The OMV vaccine represents a groundbreaking advancement in immunization technology, offering numerous advantages for both safety and efficacy [152]. Non-replicating in nature, OMVs aim to provide multiple and safer vaccinations to the patients. Their proven safety profile can be enhanced through LPS detoxification, making them a reliable choice for immunization. OMVs intrinsically elicit robust innate and adaptive immune responses, making them effective in combating various pathogens. Additionally, their stability allows for the maintenance of integrity during transport and long-term storage, facilitating widespread distribution. The platforms used for OMV production reached high yield and purity, thanks to optimized production efficiency and well-established purification processes. Furthermore, the versatility of this platform enables the combination of OMVs with different antigen types, paving the way for personalized and targeted vaccine strategies [152]. This technology was particularly isolated and investigated to develop vaccines against meningitis and recently showed the ability to boost the effect of other vaccines against this disease [153]. OMV vaccines were first approved for use against the *Neisseria meningitidis* serogroup B strain (MenB). VA-MENGOC-BC™, approved in Cuba in 1987, was effective against a specific MenB serotype and included serogroup C, achieving an efficacy rate of approximately 85% [154]. MenBVac was utilized in France and Norway; although it demonstrated a rapid decline in immune response, it also provided protection against other strains of this pathogen [143]. A similar technology, MeNZB™, was employed to address a MenB outbreak in the early 2000s. Bexsero™ (also known as 4cMenB), the only OMV-based vaccine currently authorized by the FDA and EMA, is administered to young individuals aged 10 to 25. It consists of the MeNZB™ vaccine enhanced with additional recombinant proteins, offering protection against 66%–91% of MenB strains and showing cross-protection against *Neisseria gonorrhoeae* [154]. At least two clinical studies (NCT04722003, NCT04094883) were completed in the last 15 years to evaluate the efficacy of this technology Bexsero™ in protecting against this sexually transmitted infective disease showing consistent response and good tolerability to the vaccine. These technologies can be produced from the specific strains responsible for outbreaks, marking a significant advancement in the field, particularly due to the high variability and low immunogenicity of the major antigen of this bacterium, PorA. Along with this, antigen specific OMV vaccines (menPF-1) were also designed and successfully tested (NCT01640652) to target the ferric enterobactin receptor (FetA) that in some case might result a better antigen for training the immune system against MenB. Recently, Intravacc company announced the completion of a phase I clinical trial to test the safety of their new OMV platform against COVID19 (NCT05604690). This vaccine exploits OMV from N. meningitis as well implemented with the stabilized spike protein of SARS-CoV-2. Despite the use of the traditional protein antigen that usually has lower impact on public opinion, the vaccine demonstrated good tolerability and mucosal protection when administered intranasally.

Recent developments in the field are devoted at generating multifunctional vesicles. For example, Lu et al. [155] developed a novel "Antigen Presentation Signal Enhancer" (APSE) based on bacterial OMV by surface-modifying these vesicles with anti-PD-L1 antibodies. In this case, APSE not only delivered tumor antigens but also boosted co-stimulatory signals in antigen-presenting cells (APCs) due to the immunogenic nature of OMVs. The antibody modification binded PD-L1, potentially restoring CD80 function and enhancing co-stimulatory signals, thereby improving anti-tumor immune activation.

6. Other nanovaccine designs and new perspectives

6.1. *Gold nanovaccines*

Thanks to the extensive work performed in material science, it is now possible to generate nanoparticles from any source, including natural, biological, organic and inorganic materials. Some of these materials have shown anti- [156] and pro-inflammatory [157] properties that can be harnessed to generate immune-modulating technologies. In particular, gold nanoparticles (AuNP) have been shown to possess adjuvant properties [158]. In this context, Emergex Vaccines Holding together with the Center for Primary Care and Public Health in Switzerland established a phase I clinical trial (NCT04935801) named naNODENGUE testing the safety and reactogenicity of a new nanovaccine against the Dengue flavivirus, principal responsible for the Dengue fever. The system is based on AuNP coated with different peptides derived from the viral envelope glycoprotein III (EDIII), covering the 4 pathological serotypes (Denv1–4) of this pathological agent. This investigation was performed on healthy volunteers and focused on the effects generated by two different doses of the vaccine administered via intradermal injection, also referred as to DengueTcP^{TM}. Even though the results of the study are not yet published, Emergex confirmed the positive outcomes of the naNODENGUE study [159]. Both the doses administered showed high tolerability accompanied by a robust activation of CD8 memory and effectors T cells against the different serotypes of the virus. The company also claimed that DengueTcP^{TM} did not induce anti-Denv antibody increase, phenomenon at the basis of the ADE that affects other vaccines for this disease (see the Section 2). However, the vaccine was efficient in activating T-cells that could migrate in the infected tissues and neutralize the virus. The ability of AuNPs to generate an immune response in pre-clinical models was already tested with success. Quach et al. showed that AuNPs coated with EDIII peptides to induce an immune response against Dengue virus [22]. Their preclinical work represents a milestone in the field since confirmed the ability of AuNPs to act as adjuvants via activation of the complement system. They also demonstrated that the efficacy of the system was particle concentration- and size-dependent with the larger particles (80 nm) more effective than the smaller (40 and 20 nm). Further histologic analysis demonstrated also the safety of the treatment.

6.2. *Ferritin nanoparticles*

Protein nanoparticles emerged as a promising tool to effectively induce an immune response against EBV by targeting gp350 used to functionalize the carriers. Two candidate proteins were first investigated for their ability to mount the antigen and induce an immune response: ferritin and encapsulin [36]. Both these proteins can spontaneously generate nanoparticles of 24 and 60 subunits, respectively. Given their spontaneous self-assembly into highly ordered nanoparticles, amenability to transgenic production, biocompatibility, and ease of functionalization with antigens, it can be speculated that protein cages like ferritin and encapsulin emerge as promising candidates for generating reactogenic technologies. These similarities to VLP suggests optimal matrix properties for effective antigen presentation and immune system stimulation, offering an exciting platform for the design of next-generation nanovaccines. Ferritin, the most investigated platform, is a protein evolved to store iron and controlling the balance between soluble to insoluble form "on demand." Ferritin is ubiquitously present between almost all the living beings [4] and can self-assemble in small hallow nanoparticles generating well dispersed carriers highly stable in different environmental conditions. More importantly, the surface of these particles can be functionalized easily with different antigens via chemical and genetic functionalization. For this reason, ferritin was chosen as preferential platform because of the ability of developing solid functionalization protocols to attach gp350 on its 3-fold axes. This approach was previously successfully tested in developing anti-influenza vaccine by genetically attaching HA at the interface between 3 converging subunits of ferritin, generating a physiologically relevant viral spike [160] (Fig. 5). In particular, the investigation of the protein structure allowed to identify an Asp residue in proximity of the n-terminal of each subunit that, considering its accessibility to the solvent, allowed for a successful modification with the ectodomain of HA. Affinity to anti-HA antibody analysis in combination with electron microscopy confirmed the successful of nanoparticle assembly. Based on data obtained in preclinical models, a clinical study is being conducted to evaluate the safety and immune response of an EBV nanovaccine based on gp350-ferritin nanoparticles (NCT04645147).

During the development of UFluA, a universal influenza vaccine, an even more sophisticated technology was employed. The central concept was to utilize the conserved stem region of influenza HA as an antigen component of the nanovaccine, aiming to counteract antigenic drift and elicit a stronger, more durable immune response. In this endeavor, scientists genetically modified the HA sequence to remove the immunodominant head region and enhance trimerization [161]. The resulting modified stem sequence was fused to the sequence of *Helicobacter pylori* (*H. pylori*) non-heme ferritin, which possesses the ability to self-assemble into 24-subunit spherical nanoparticles with octahedral symmetry and a hollow core. Consequently, symmetrical spherical nanoparticles were obtained, with eight trimeric viral spikes projecting from the system's surface. The expression of

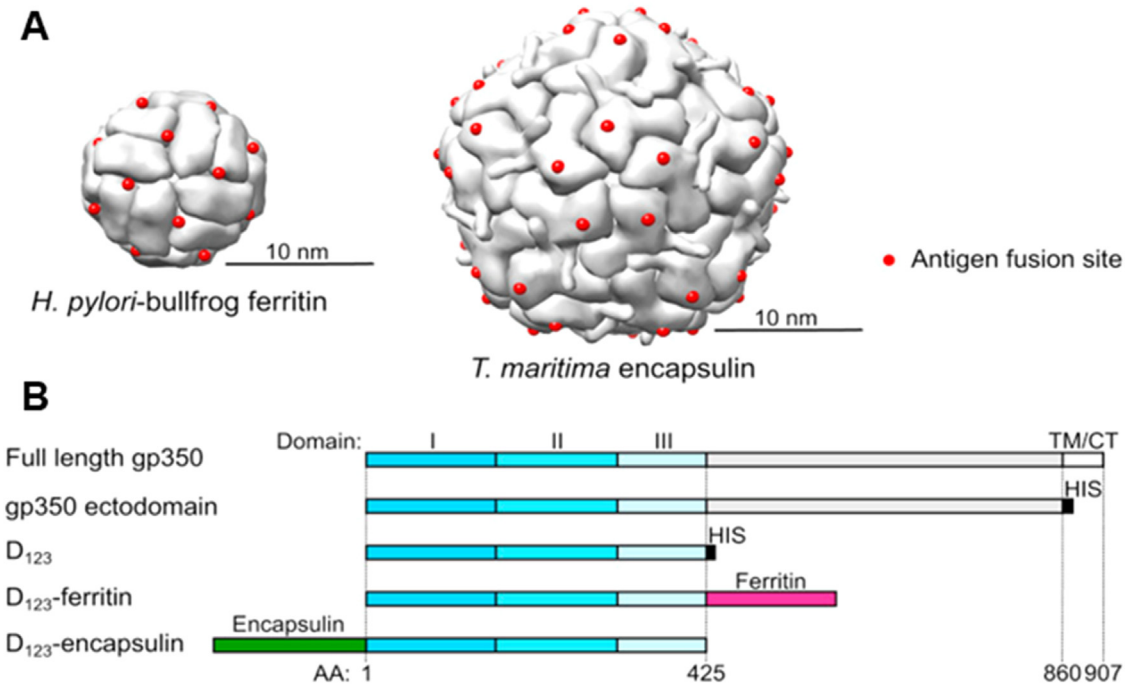


Fig. 5 – Molecular Design of gp350-Based Nanoparticles: (A) Hybrid ferritin model (left) and encapsulin (right) assemblies showcasing engineered insertion sites for exogenous antigens as depicted by red spheres; (B) Schematic representation delineating full-length gp350, its truncated variants, and gp350-based nanoparticles. The domains I, II, and III of gp350 are color-coded in dark blue, sky blue, and pale blue, respectively. The structurally undefined region and transmembrane (TM)/cytoplasmic tail (CT) are shaded in gray and white, respectively. The hexa-histidine tag (HIS), ferritin, and encapsulin are delineated in black, red, and green, respectively. The amino acid (AA) positions relative to full-length gp350 are indicated at the bottom. Reprinted with permission from [36], copyright© 2015 Elsevier Inc.

protein components and the self-assembly of nanoparticles were carried out in human freestyle HEK 293 cells (293F). A phase I clinical trial of UFluA in healthy adults and non-pregnant women (NCT05155319) is currently underway. The same technology with the mRNA encoding HA was utilized to create the H1ssF-3928 (influenza H1 HA stabilized stem ferritin nanoparticle vaccine) and H1ssF-3928 mRNA-LNP vaccines by Vaccine Research Center of the National Institutes of Health. H1ssF-3928 contains the stem domain from Influenza A/New. In a phase I trial (NCT03814720), this vaccine demonstrated favorable safety and tolerance, inducing a broad antibody response against the HA stem. Currently, a phase I clinical trial of the H1ssF-3928 mRNA-LNP vaccine is ongoing (NCT05755620). Recent trends in the field are exploring the use of *H. Pylori* ferritin to implement this technology with multiple epitopes to provide long-lasting protection against pathogens that tend to mutate including influenza [162] and Sars-CoV-2 [163]. This platform offers several advantages including enhanced immunogenicity, broad and durable protection, stability, biocompatibility, potential for scale production [164–166].

6.3. *Exosomes, dexosomes and proteolipid vesicles*

The search for ideal nanovaccine platforms is vast, requiring efficient antigen delivery paired with adjuvant properties. Many materials can be engineered in the nanoscale to provide these features. For example, relatively new trends in the field [167] are investigating novel methods to generate high yield of proteolipid vesicles directly from the cell membrane [168]. In this scenario, the control of the vesicle structure results fine-tuned and addressable to different needs including implementing the carriers with new functions via molecular biology tools. In this scenario, Pan et al. [169]. generated cell membrane vesicles via extrusion of cancer cells previously transfected with CD40L and PD1 to elicit a strong immune response based on vaccination and immune therapy. However, we believe that exosomes might represent the most promising candidates in this category thanks to their ability to mediate cellular communication both in healthy and in diseased cells [170]. Their high biocompatibility stems from their inherent blend of lipid and protein components [171], mirroring the successful composition of other nanovaccines. Additionally, they demonstrate a natural capacity to deliver biological cargo, including mRNA, combined with a high ability to negotiate the passage through biological barriers [172]. Even though not inherently strong adjuvants, they can be readily combined with adjuvant molecules, or molecules impairing lysosomal entrapment [173], while in some cases they showed specific and strong immunization properties [174]. Interestingly, recent evidence suggests they may possess intrinsic inflammatory properties, further enhancing their potential application in this field [175,176]. Exosomes are small extracellular vesicles (up to150 nm in diameter) secreted

by various eukaryotic cells as a result of the fusion of the endosomal compartment with the plasma membrane [177]. Exosomes serve as key mediators of intercellular communication, transporting diverse cargo including a large variety of RNA molecules and proteins between distant tissues and organs. While their early discovery met significant issues in isolation and characterization hampering their potential use in clinics, recent advances in the field improved the production yield and allowed for tailoring their packaging, surface and targeting properties [168,171]. More importantly, currently exosomes can be synthesized *in vitro* through extensive cell cultures or via new methods based on cell membrane isolation and reconstitution in proteolipid vesicles with similar delivery properties [168,178]. Within the large portfolio of cells that can generate exosomes, DC are probably the most investigated. Dexosomes (DEX) are exosomes secreted from DC and they maintain many proteins of these cells including antigens, peptide-MHC-I complex, and costimulatory molecules that can result in T-cell activation [179]. In this process, DC activation with cytokines [180] or other molecules [181] is paramount both for boosting exosome secretion and tailoring their composition. The clinical utility of these carriers is underpinned by their biocompatibility and biological compatibility. Monocytes can be directly isolated from patients, and then manipulated *ex vivo* to induce their differentiation into mature DCs. Following rigorous quality control measures, these cells can be induced to generate immunogenic DEX that can be injected back into the patients for immunization [182]. Good manufacturing practice of DEX production is schematically presented on Fig. 4B

To date, the results of four clinical trials (three phase I and one phase II) have been published, with one trial (NCT01159288) completed, but with no data yet available. DEX based vaccines were designed to treat cancer including advanced melanoma [183], non-small cell lung cancer [184]. In these trials, DC were used to load MAGE peptide in DEX. The result showed that the vaccinations was well-tolerated with events of delayed-type hypersensitivity (DTH) reaction against MAGE peptides. In another trial [185], exosomes containing carcinoembryonic antigen (CEA) peptides were isolated from patients with CRC (stage IIIb-IV) and used with or without co-treatment with GM-CSF. In this case, besides DTH, the patients treated with exosomes in combination with GM-CSF showed also CEA-specific CTL response. Despite an acceptable safety profile, clinical trials revealed moderate efficacy of DEX in eliciting an immune response. This suggests limitations that can be addressed in future studies. Several factors likely contributed to the observed efficacy including: (1) Advanced cancer stage where antigen-specific T cells are scarce [186], (2) elevated activity of immunosuppressive Tregs in these patients [184], (3) missing immunotherapy combinations which could synergize with DEX to enhance CTL activation, and (4) early trial limitations [186]. Additionally, optimal adjuvants, known to boost immune responses, were not employed. By addressing these limitations in future studies, researchers can potentially improve the efficacy of DEX as an immunotherapy.

6.4. *PLGA nanovaccines*

Poly(lactic-co-glycolic acid) (PLGA) nanoparticles have emerged as a promising platform for biomedical applications due to their versatility [187,188]. These carriers can accommodate and deliver a wide range of therapeutic agents, including proteins, peptides, nucleic acids, and small molecules, to specific cells or tissues [189]. PLGA nanoparticles have been utilized in various fields, such as drug delivery, tissue engineering, cancer therapy, and treatment of cardiovascular and neurodegenerative diseases [190]. Recent advancements in this technology are showing the versatility of this polymer in enhancing the biocompatibility or the targeting properties of other materials including metal, synthetic and biological particles [191–193]. PLGA particles can be internalized by APCs, while enabling controlled drug release through hydrolytic degradation into nontoxic metabolites, making them ideal for vaccine delivery systems. The properties of PLGA nanoparticles, such as size and release profile, can be customized by modifying the monomer ratio, though antigenic instability remains a challenge [194].

Surface modification of PLGA nanoparticles represent a crucial for enhancing their efficacy as vaccine carriers. Common strategies include PEGylation, which improves stability and colloidal properties [195], surfactants, lipids, proteins, peptides, antibodies, and nucleic acids to improve targeting and cellular uptake [196,197]. Specific coatings such as sodium alginate and Eudragit L100 have been shown to protect protein antigens in simulated gastric and intestinal fluids, making them promising for oral vaccine delivery [198] (Fig. 3D). Various methods for synthesizing PLGA nanoparticles have been developed, including emulsion-evaporation, nanoprecipitation and microfluidic techniques [190]. They are easy to functionalize to enhance their efficacy, imaging and diagnostic properties [199,200], and PLGA was approved by both FDA and the European Medicine Agency (EMA) as part of different medications and medical devices even though their large production in the nanoscale is not trivial [201]. Thanks to all these properties, PLGA was extensively tested as antigen carrier for many disease including infections [202], autoimmune diseases [203] and cancer [204]. The ability of this polymer in improving current vaccination approaches is currently under evaluation in a phase I clinical investigation testing the efficacy of PLGA scaffolds (WDVAX) embedded with cancer cell patient-derived lysates designed for metastatic melanoma patients (NCT01753089). Recently, a phase I clinical trial [205] (NCT04751786) started to explore a PLGA carrier named PRECIOUS-01 encapsulating three peptides sequences derived from the cancer-testis antigen NY-ESO-1 along with the activator for invariant natural killer T cells threitolceramide-6 to protect the patients against tumors overexpressing this antigen. New trends in the field leverage this versatile platform to enhance cancer immunotherapy strategies. Advanced-stage solid tumors and metastases often overexpress mucin 16 (MUC16) with immature glycans, such as the Tn antigen, resulting in a unique glycoprotein profile absent in healthy tissues and suitable

for targeted approaches. Freitas et al. [206] utilized PLGA nanoparticles to present a library of MUC16-Tn via single-pot enzymatic glycosylation, creating multivalent constructs that mimic cancer heterogeneity. These glycoconstructs exhibited strong binding to the Macrophage Galactose-type Lectin receptor, which is highly expressed on immature APCs, facilitating precise immune cell targeting. The nanovaccine candidate demonstrated low cytotoxicity and successfully induced immunization both *in vitro* and *in vivo*.

7. Generation of nanoadjuvant

7.1. Background and current research in the field

Adjuvants are essential vaccine (and nanovaccine) components, that are formulated together with antigens to boost the immune response, increase antigen stability and bioavailability. A variety of adjuvants have been already licensed or are undergoing clinical trials. The mechanism of action of approved adjuvants varies greatly, for example, aluminum salts (Alum), that are used as a component of HAV and HBV Twinrix, HBV Engerix-B, HPV Gardasil and Gardasil-9 vaccines [207], provide immune niche formation where antigens accumulate and immune cells are recruited, activate inflammatory NLRP3 response and promotes IL-1β secretion [207,208]. Delivery systems, such as oil-in-water emulsion, liposomes and LNPs, act by protecting antigen cargo, providing slow and prolonged antigen release, extending the elimination time, and increasing antigen bioavailability. Oil-in-water emulsions stimulate preferentially Th2-mediated immune response [209]. Some oil-in-water emulsions contain immunostimulatory components: MF-59 adjuvant (component of the approved influenza vaccine FLUAD) combines delivery system and immunostimulatory activities because it contains squalene, Tween 80 and Span 85, affecting pattern recognition receptors (PRR) and therefore resulting in recruitment and activation of DCs, T- and B-cells [210]. AS-03, licensed as a component of HPV vaccine Cervarix® and HBV vaccine Fendrix contains immunostimulants squalene and alpha-tocopherol, activating human monocytes and macrophages [211].

The liposomal adjuvant AS-01 incorporates the immunostimulants monophosphoryl lipid A (MPLA) and saponin QS-21. MPLA serves as an agonist for Toll-like receptor 4 (TLR4), facilitating the maturation of DCs and driving a robust Th1-mediated immune response [212,213]. Meanwhile, QS-21 activates the inflammasome and enhances the secretion of pro-inflammatory cytokines [214]. This adjuvant was FDA-approved as a component of herpes zoster vaccine SHINGRIX, moreover, tuberculosis vaccine containing AS-01 has shown promising results in clinical trials (NCT01755598, NCT01755598). Research on saponin-based nanoparticles with TLRs showed great potential, as these constructs generated tailored immune responses for specific diseases. Recent studies in mice demonstrated their ability to enhance the effectiveness, durability, and breadth of COVID-19 and HIV vaccines, highlighting their versatility across different antigens [215]. Immunostimulants themselves are used as nanoadjuvants as well. Some examples include single-stranded DNA sequences with unmethylated deoxycytidylyldeoxyguanosine dinucleotides (CpG ODNs) that interact with intercellular TLR9, activating Th1-mediated immune response. In particular, CpG ODN 1018, licensed as a component of hepatitis B HEPLISAV-B vaccine, activate Th1 $CD4^+$ cells to secrete inflammatory cytokines IFN-γ, tumor necrosis factor-alpha (TNF-α) and interleukin-2 (IL-2) [216,217].

The development of nanoadjuvants marks a significant advancement in vaccine technologies for enhancing immune responses via engineering of ad hoc delivery systems. These innovations address challenges like low immunogenicity and targeted delivery, offering solutions for diseases such as cancer and infectious conditions. Nanoadjuvants, like saponin-based self-nanoemulsifying systems, improve humoral and cellular immunity, increasing antibody titers and cytokine production in tumor vaccine models [218]. Squalene-based nano-emulsions have shown high efficacy in vaccines against foot-and-mouth disease [219]. Additionally, chitosan-coated nanoemulsions and silicone oil-based nanoadjuvants support intranasal vaccines, promoting mucosal immunity, stability and adjuvant properties [220,221], while manganese-based liposomes boost DNA vaccine transfection efficiency and immunogenicity [222]. Polyanhydride nanoparticles and pentablock copolymer micelles were shown to address age-related immune impairments with minimal inflammation [223]. Despite challenges like safety evaluations, advances in kinetically activating nanoadjuvants demonstrate promise in cancer immunotherapy by reducing immune cell exhaustion [224], underscoring nanoadjuvants' transformative potential in vaccine development.

7.2. ISCOMATRIX

Novavax is at the forefront of developing next-generation protein nanovaccines, utilizing antigenic nanoformulations enhanced by their proprietary Matrix-M nanoadjuvant. The debate between mRNA and protein vaccines remains a pressing issue. In light of public concerns surrounding mRNA vaccines, Novavax is dedicated to delivering vaccines that offer comparable therapeutic efficacy in safeguarding against and alleviating infection symptoms, while potentially exhibiting fewer side effects, despite the need for further data on their products [225]. According to their website [226], Novavax vaccines consist of two types of nanocomponents. The first component is the Matrix-M, which is a nanoparticle composed of lipids (cholesterol and phospholipids) and surfactants organized in a honeycomb-like structure measuring 40 nm. The surfactants are specifically represented by saponins derived from fractions A and C of soapbark tree extract and are referred to as Matrix-A and C. Matrix-M represents a direct evolution of the immunostimulating complex matrix known as ISCOMATRIX technology, which was initially developed by Morein et al. in 1984 [227]. In the initial prototype of the system, saponins, cholesterol and phospholipids were combined with surface antigens (i.e., influenza, measles, rabies), forming micelle-like structures (40 nm) wherein the hydrophobic regions of the antigens were embedded within the surfactant nanostructure.

Cholesterol served a role beyond structural support, as its interactions with the saponins protected these molecules against hydrolysis. The system demonstrated a high ability to enhance the immunogenicity of antigens exhibiting poor reactogenicity when administered alone. The system improved antigen presentation, induced chemokine and cytokine secretion, resulting in the recruitment of immune cells in the draining lymph nodes, and favored the activation of both the innate and adaptive immune systems, providing an ideal combination of immune modulatory and antigen delivery properties [228]. Interestingly, the scientists who developed ISCOMATRIX hypothesized that the efficacy of the system was likely due to its ability to promote the cytoplasmic delivery of antigens through fusion with the cell and endosomal membrane [231]. After cytoplasmic diffusion, the antigen could be processed eventually for MHC-I, activating $CD8^+$ CTL cells. Several phase I-II clinical trials were conducted before 2015 to evaluate the efficacy and safety of vaccines developed with ISCOM technologies. These trials, which have since been terminated or completed, tested ISCOM against various types of cancers (NCT02054104; NCT00195901), particularly advanced stages of melanoma (NCT01341496; 00,518,206) in combination with different CTA like NY-ESO1. However, these trials did not demonstrated any significant benefits in using this technology. ISCOMATRIX was also tested in combination with influenza peptides against flu (NCT00851266), hepatitis C [228], and with a VLP against HPV (NCT00851643) in combination with aluminum hydroxysulfate. In the case of the HPV trial, it was compared with Gardasil, showing higher efficacy and good tolerance, although we did not find any follow-up to this trial. In a phase I trial (NCT01477580), ISCOM showed higher efficacy in generating immunity against the four serotypes of Dengue virus when combined with recombinant truncated viral envelope proteins compared to aluminum-hydroxide adjuvant or unadjuvanted antigens. However, immunity significantly decreased after 6–8 months, and despite being generally well-tolerated, the system was associated with a higher occurrence of adverse events, including grade 3 toxicity [229].

7.3. *Matrix-M*

Matrix-M is the result of years of studies dedicated to refining and improving the ISCOMATRIX formulation. Despite the optimization of the system, the clear difference between ISCOMATRIX and Matrix-M is that the former was designed to incorporate antigens, while the latter is used in combination with a protein nanoparticle (Fig. 3E). The antigens are usually generated via the insect Sf9 cell/baculovirus expression vector system technology (BEVS) [230] and then they are mounted on a central core of polysorbate80. Novavax developed various vaccines, and currently, most trials are focused on investigating the efficacy and safety of COVID vaccines. After extensive research in mice and baboons [231], the anti-COVID-19 vaccine NVX-CoV2373 (a.k.a. Nuvaxoid™) was authorized for emergency use against different variants of SARS-CoV-2, representing the first protein vaccine for the prevention of this disease [232]. The vaccine was generated from a recombinant form of the spike protein modified in the furin-like cleavage motif by substituting four amino acids and substituting two more amino acids (K986P and V987P) with 2 prolines. These modifications were fundamental to confer the system chemical and temperature stability. The system was expressed in BEVS and was isolated as spike protein homotrimer particles with a size smaller than 30 nm [231]. Eventually, the antigenic components of NVX-CoV2373 were formulated with polysorbate80, forming particles of 60 nm. More recently, in the U.S., a new version targeting the variant XBB.1.5 was released to the public. Interestingly, it was recently shown that mixing boosters, especially applying an mRNA vaccine booster after a protein vaccine primary shot, might increase the immunity period [233]. In this context, Novavax's vaccine might be the key to generating more efficient vaccination approaches. Like the mRNA vaccines, this technology was first developed for other diseases including RSV (NCT01290419), avian influenza (NCT01897701), Ebola (NCT02370589), autoimmune diseases (NCT05236491), and recently a phase III study has been announced to investigate a new combination vaccine to induce protection against COVID and flu (NCT06291857). NanoFlu vaccine (also known as qNIV), developed by Novavax consists of a protein nanoparticle containing HA from four influenza strains (A/H1N1, A/H3N2, B/Victoria and B/Yamagata). The system was generated as described above by harnessing on baculovirus transfection of Sf9 to produce HA proteins that are eventually embedded in a polysorbate 80 particle. The HA proteins self-assemble into trimers, which are then purified. During chromatography, detergent exchange to polysorbate 80 induces cross-linking of the trimers, resulting in nanoparticle formation [234]. These nanoparticles, which are 30–35 nm in size, were eventually formulated with the saponin-based Matrix-M adjuvant. Phase I/III clinical trials of NanoFlu have been completed, and the results demonstrated the vaccine's effectiveness. In particular, a recent Phase III randomized, observer-blinded study showed that NanoFlu induced a humoral and cellular immune response that was similar or even superior to that of the approved quadrivalent seasonal influenza vaccine (IIV4) in older adults [235]. Despite some adverse events, the vaccine was well tolerated and had a favorable safety profile [235]. Phase I/II studies of a combination vaccine consisting of the SARSCoV-2 recombinant spike (rS) nanoparticle and the quadrivalent HA nanoparticle influenza vaccine (qNIV), both formulated with Matrix-M adjuvant, are currently ongoing (NCT04961541, NCT05519839). This combination vaccine is produced in the same way as the NanoFlu vaccine, except that the trimer of the full-length coronavirus spike protein is also incorporated into the nanoparticle. In mouse models, the prototype vaccine provided high protection efficacy against stringent lethal SARS-CoV-2 and homogenous H1N1 influenza co-infection and achieved a 100% survival rate. According to the results of the NCT04961541 study, HAI antibody titers and the incidence of adverse events were comparable to those of the qNIV vaccine.

8. Experimental frontiers in the oral nanovaccines

In this review, we primarily focused on nanovaccines that have recently been released to patients or at least tested in clinical trials. Other reviews [236–238] can provide a more in-

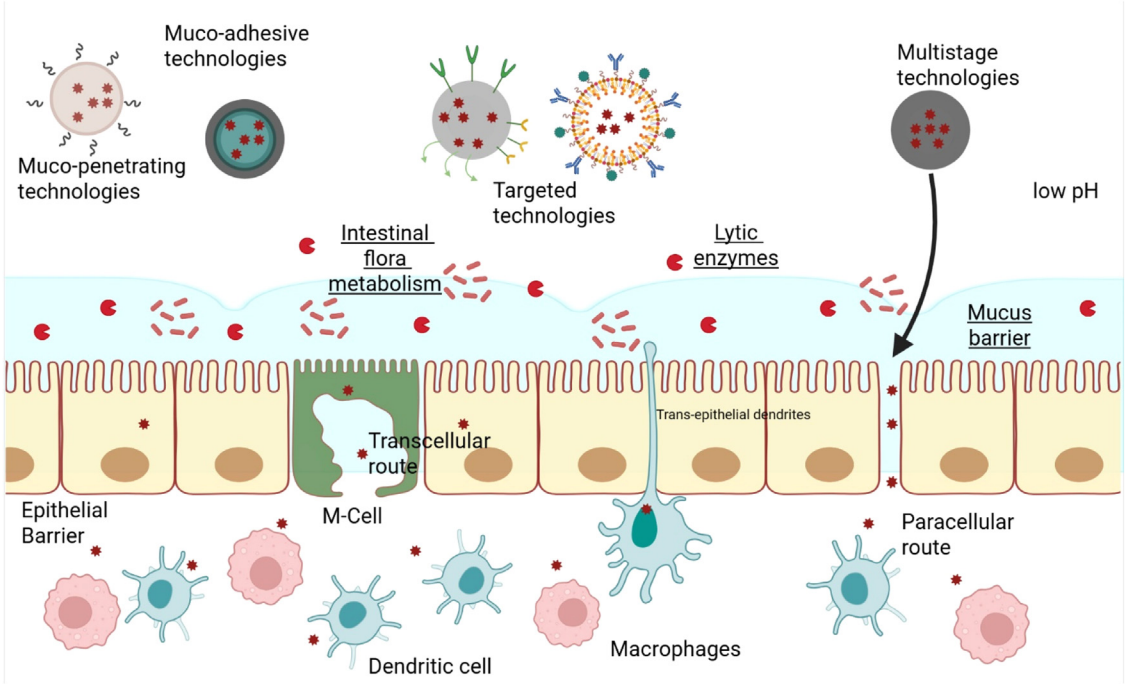


Fig. 6 – Overview of GIT biological barriers and strategies for overcoming them in oral drug delivery. The figure highlights the mucus barrier, intestinal flora metabolism, and enzymatic activity as key challenges. It also illustrates various absorption pathways, including transcellular, paracellular and M-cell-mediated routes, alongside advanced technologies such as muco-adhesive, muco-penetrating, targeted and multistage systems designed to enhance drug stability, penetration and efficacy in the GIT environment. Figure Adapted from [241], and generated with Biorender.

depth overview of the general advancements in the field. We chose to narrow our research to clinically tested nanovaccines to present successful examples of these technologies, with the hope of assisting the readers in further advancing this area. However, discussing new frontiers of nanovaccines, we believe special emphasis should be placed on formulations designed for oral delivery for several reasons. Firstly, the engineering of an oral nanovaccine (like any other drug) has a milder impact on public opinion [239], since oral medications are generally perceived as less invasive than parenteral administrations. This is particularly relevant given the intense emotional response generated by the release of nanovaccines designed for SARS-CoV-2 under emergency conditions [240]. It is also important to emphasize that the engineering of nanovaccines for oral administration represents also a significant advancement in pharmaceutical development. The enteral route presents specific biological barriers, including low pH, digestive enzymes, mucus, epithelial barriers, and intercellular junctions, as well as microbiota metabolism [239,244] (Fig. 6). While ad hoc strategies can be conceived for different conditions, research on oral nanovaccines is polarized on developing approaches to tackle CRC, which exhibits high global mortality rates. Conventional treatments—such as surgery, chemotherapy and radiotherapy—have significant limitations, making immunotherapy a promising alternative by leveraging the host immune system. However, the immunosuppressive tumor microenvironment of CRC often compromises the efficacy of these approaches. Given its location, oral medication or vaccination is considered one of the best strategies to address this disease. A notable example of the technological efforts required to create such vaccines is provided by the seminal work of Zhang et al. [242]. They exploited new advances in regulating the periodic oscillatory genetic circuits to induce *E. coli* to release nanovaccines in the gastrointestinal tract (GIT). In this work, *E. coli* was engineered to cyclically grow and lyse (synchronous lysis), releasing a nanoprotein cluster composed of the BMC-AH1-A5 antigen, which protects mice against the growth of CT26 CRC cells. The bacteria were first encapsulated in a chitosan-modified sodium alginate hydrogel to protect them in the GIT environment and promote their adhesion, colonization, and proliferation. During controlled lysis, the bacteria released the nanovaccine, which could be internalized by DCs, inducing an anti-tumor response. The controlled release of antigens from the bacteria was crucial for developing an effective immune response, mitigating the loss of the nanovaccine due to degradation. The gut microbiome is increasingly recognized as a significant factor in regulating CRC progression and treatment efficacy. Emerging evidence suggests that gut microbiota can enhance the effectiveness of immunotherapy. For instance, *Lactobacillus rhamnosus* (LGG) [243] and *Bifidobacterium longum* (*B. longum*) [244] exhibited anti-cancer and anti-inflammatory properties and can enhance DC activity. However, their efficacy, particularly against CRC, may be hindered by the various

biological barriers in the GIT. For these reasons, as previously mentioned, they should be encapsulated or coated with biocompatible materials for protection. To enhance further their properties, probiotics can be administered to promote their proliferation, survival, and therapeutic efficacy. Recent studies have shown that combining nanovaccines with engineered probiotics may represent a successful strategy against colon cancer [245]. LGG and *B. longum* were coated with a lipid membrane and demonstrated the ability to prevent colon cancer growth *in vivo*. Their effect was further enhanced when administered alongside cancer nanovaccines (subcutaneously injected) derived from lysates of cancer cells formulated in PLGA carriers. Additionally, there are notable examples of multifunctional nanovaccines that combine treatment with immune training against CRC. *In situ* vaccination aims to overcome limitations by generating autologous neoantigens directly within the tumor using methods such as radiation, chemotherapy, or phototherapy. This can help circumventing challenges associated with traditional therapeutic vaccine development, including antigen identification, neoantigen generation, and tumor heterogeneity. In particular, a recent study presented a novel oral nanovaccine referred to as Ce6/R837@Lp127NPs [246], designed to elicit an *in situ* vaccination effect against this tumor. This innovative approach integrated a silk fibroin-based nanoparticle core loaded with chlorin e6 (Ce6) as a sonosensitizer and imiquimod (R837, a toll-like receptor 7 agonist) as an immunoadjuvant, coated with plant-extracted lipids and pluronic F127 for enhanced stability and mucus penetration. Sonodynamic therapy (SDT) is a promising trend in the field, offering features such as tumor targeting, safe application, and high tissue penetration. The system effectively permeated CRC tissues upon ultrasound irradiation, leading to the generation of reactive oxygen species that induced tumor cell death. This process released neoantigens that, together with R837, stimulated DC maturation and promoted cytotoxic T lymphocyte infiltration into the tumor. The combination of SDT with immune checkpoint blockade (PD-L1 antibodies) further enhanced systemic antitumor immunity, significantly inhibiting orthotopic tumor growth and eradicating distant tumors while positively modulating the intestinal microbiota. This work highlights a robust and patient-friendly platform for personalized cancer vaccination via oral administration, showcasing its potential for clinical application in CRC treatment.

9. Conclusions

Nanovaccines hold immense potential for preventing diverse diseases, as demonstrated by technologies currently undergoing clinical evaluation. This review highlights the extensive efforts in human testing and the clinical translation of the most recent nanovaccines, emphasizing the importance of constant innovation and exploration in advancing healthcare. While significant progress has been made, further research is needed to adapt these technologies to a wider range of conditions, particularly those with poorly defined target antigens. This effort necessities of a multidisciplinary root including biologists immunologists, clinicians and considering the large development informatics tools also computational biologists and bioinformaticians [247]. To develop novel translational nanovaccines, it is essential to evaluate each disease with distinct antigen delivery platforms, despite the challenges this may pose for scientific coordination and comparison. Lipid and protein nanoparticles are current frontrunners due to their safety and ability to induce immune responses. Regardless of the encapsulated antigen (mRNA or protein), carriers that destabilize the endosomal compartment, such as Matrix-M, have shown higher efficacy. This also opens opportunities for exploring exosomes as a future platform, despite the challenges in generating nanovaccines using this method. The rapid release of novel mRNA vaccines has raised public concerns, leading to polarized opinions within the scientific community. For seasonal, non-lethal illnesses, traditional protein antigen delivery nanovaccines may be more favorable. In contrast, for more lethal conditions like cancer, intensive research into non-viral mRNA delivery platforms is imperative. The nature of the disease, its mortality rate, and the public's perception of different technologies are critical considerations for future development. At the forefront of the field are technologies for oral administration, CRISPR/Cas9 delivery, and biomimetic platforms. (e.g., extracellular vesicles and OMV). These innovations have the potential to revolutionize vaccine delivery and efficacy, as well as providing more accessible vaccination methods with minimal impact on public opinion [242,248].

Conflicts of interest

The authors declare that there is no conflicts of interest.

CRediT authorship contribution statement

Vera S. Egorova: Formal analysis, Writing – original draft, Data curation. **Ekaterina P. Kolesova:** Formal analysis, Data curation, Writing – original draft. **Maya V. Voronina:** Writing – original draft, Formal analysis, Data curation. **Evgenya R. Denisova:** Formal analysis, Writing – original draft, Data curation. **Anastasiia O. Syrocheva:** Formal analysis, Data curation, Writing – original draft. **Tatiana Pallaeva:** Data curation, Writing – original draft, Formal analysis. **Mazdak G. Hakemi:** Formal analysis, Writing – original draft, Data curation. **Andrey A. Zamyatnin Jr.:** Writing – review & editing, Supervision, Funding acquisition, Data curation, Conceptualization, Formal analysis. **Konstantin I. Ivanov:** Formal analysis, Writing – original draft, Data curation. **Dmitry Kostyushev:** Formal analysis, Data curation, Writing – original draft. **Sergey Brezgin:** Data curation, Formal analysis, Writing – original draft. **Anastasiia Kostyusheva:** Data curation, Formal analysis, Writing – original draft. **Alessandro Parodi:** Supervision, Writing – review & editing, Visualization, Funding acquisition, Conceptualization, Project administration.

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