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Licensed liposomal vaccines and adjuvants in the antigen delivery system

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Abstract

Liposomes (LSs) are promising nanoparticles with unique properties such as controlled nanosize, large surface area, increased reactivity, and ability to undergo modification. Worldwide, licensed liposomal forms of antibiotics, hormones, antioxidants, cytostatics, ophthalmic drugs, etc., are available on the pharmaceutical market. This review focuses on the adjuvant properties of LSs in the production of vaccines (VACs). LS-VACs have the following advantages: antigens with low immunogenicity can become highly immunogenic; LSs can include both hydrophilic and hydrophobic antigens; LSs allow to achieve a prolonged specific action of antibodies; and LSs reduce the toxicity and pyrogenicity of encapsulated antigens and adjuvants. The immune response is influenced by the composition of the liposomal membrane, physicochemical characteristics of lipids, antigen localization in LSs, interaction of LSs with complement, and a number of proteins, which leads to opsonization. The major requirements for adjuvants are their ability to enhance the immune response, biodegradability, and elimination from the organism, and LSs fully meet these requirements. The effectiveness and safety of LSs as carriers in the antigen delivery system have been proven by the long-term clinical use of licensed vaccines against hepatitis A, influenza, herpes zoster, malaria, and COVID-19.

Key words: vaccine, mRNA, liposomes, adjuvant, virosoma, lipid nanoparticles

Introduction

In recent years, the use of nanoparticles to obtain new dosage forms has gained significant interest. Liposomes (LSs) are artificial membranes and one of the promising classes of nanoparticles (Akbani and Bashiz, 2014; Sahoo et al., 2014; Rangar et al., 2014). They have unique properties such as controlled nanosize, large surface area, increased reactivity, and the ability to undergo modifications (Bulbake et al., 2017; Krasnopolskii et al., 2017). At present, it is difficult to imagine modern pharmacology without LS drugs. Licensed LS dosage forms include antibiotics, hormones, antioxidants, cytostatics, ophthalmic drugs, and other active pharmaceutical ingredients (APIs) (Allen et al., 2013; Dubald et al., 2018; Guimarães et al., 2021; Katsai et al., 2018; Shvets et al., 2016). LS nanoparticles have a number of advantages (Alavi et al., 2019; Beltran-Gracia et al., 2019; Mohamed et al., 2013; Shvets et al., 2016):

- LSs protect cells of the organism from the toxic effects of APIs;
- LSs prolong the action of APIs in the organism and protect APIs from degradation;
- LSs allow targeted specificity due to selective penetration from the blood into tissues;
- LSs change the pharmacokinetics of APIs, thereby increasing their pharmacological efficacy;
- LSs allow the development of a water-soluble form of a number of lipophilic APIs, thereby increasing their bioavailability;
- APIs can be encapsulated in the aqueous phase or within the lipid bilayer of LSs or can be bound to the LS surface, which can lead to different effects on the body.

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All the aforementioned properties explain the interest in LSs as promising adjuvant components. The major requirements for adjuvants are their ability to enhance the immune response, biodegradability, and elimination from the host organism. LSs fully meet these requirements. They consist of natural or synthetic phospholipids (egg phosphatidylcholine (EPC), soybean phosphatidylcholine, phosphatidylinositol, phosphatidylserine, etc.), with some amount of natural cholesterol, and they are easily biodegradable and harmless. Furthermore, using modern methods of lipid purification, highly purified components in which the amount of impurities does not exceed 3–10% can be obtained, and these impurities are mainly of a phospholipid nature. The components of LSs must be nonpyrogenic and nontoxic. LSs reduce the toxicity of the encapsulated antigens and adjuvants and have good biocompatibility. For 40 years, research efforts have been focused on the development of LS forms of vaccines (VACs) and adjuvants. LS-VACs have been developed against a number of diseases such as tuberculosis (Kardona et al., 2018; Sinha etal., 1997), rabies (Krasnopolsky and Pylypenko, 2021; Miao et al., 2017), and *Pseudomonas aeruginosa* (Heurtault et al., 2009).

The effects of the lipid composition of LSs containing EPC, cholesterol, phosphatidylglycerol, phosphatidylserine, and phosphatidylinositol on their adjuvant activity were studied using *Clostridium oedematiens novyi* toxoid as antigen. LS-encapsulated toxoid is more immunogenic when anionic LSs are used rather than LSs consisting only of EPC and cholesterol (Golbets et al., 1983). A previous study has demonstrated the effectiveness of the use of the LS form of tetanus toxoid consisting of EPC and cholesterol (Davis et al., 1986, 1987; Naito et al., 1998). Babych et al. (2004) showed that the LS forms of the somatic antigens of *Bordetella pertussis* and *Corynebacterium diphtheriae* induce the development of specific immunity by oral administration.

The application of gangliosides as immunomodulators and adjuvants to obtain antitumor and antiviral drugs, in particular VACs, has been discussed in previous studies (Bogdashin et al., 1986; Krasnopol'skii and Shvets, 1986). The polysialoganglioside complex isolated from the brain of *Raja clavata* was encapsulated into LSs based on EPC. LSs were intravenously administered to BALB/c mice infected with recombinant influenza F94, which was obtained by crossing the laboratory strain A/PRB/94 with influenza A virus (strain Philippines 2/82). In experiments on BALB/c mice with moderate influenza pneumonia and those with severe pneumonia caused by the influenza virus, LSs reduced the mortality and achieved the complete recovery of animals, despite the extensive lung tissue damage. Konstantinova et al. (1985) reported that specific immunological mechanisms were involved in the recovery process, as evidenced by the fact that the immunological memory persisted for 2 months after the first injection and subsequent administration of the drug to the animals. In addition, the effectiveness of LS-VACs containing gangliosides, such as monosialated ganglioside (GM3), in the stimulation of the T-cell response has been established (Grabowska et al., 2021; Gru-Degroot et al., 2015; Twihaar et al., 2020). A number of antitumor LS-VACs have been developed, for example, against prostate cancer (North and Butts, 2005; North et al., 2006; Samuel et al., 1998; Yin et al., 2021).

In recent years, many studies have focused on the LS forms of adjuvants and VACs, including review publications (Alving et al., 2016, 2020; Chatzikleanthous et al., 2021). This review focuses on licensed LS-VACs.

LS-VACs against hepatitis A and influenza (virosomes)

Hepatitis A VAC

Attempts to obtain VACs against various types of hepatitis began in the 1990s. LS-VACs against hepatitis B (Diminsky et al., 1996), hepatitis A (Ambrosch et al., 1997), hepatitis C (Engler et al., 2004), and hepatitis E (Krasnopolsky et al., 2011) have been proposed. Lipoxen Technologies Ltd. has developed the world's first hepatitis E VAC, containing the LS-encapsulated recombinant hepatitis E protein, obtained using the ImuXen technology.

Diminsky et al. (1996) obtained an LS-VAC against hepatitis B, which included recombinant hepatitis B surface antigen (HBsAg) with a size of 22 nm. LSs were prepared from dimyristoylphosphatidylcholine and dimyristoylphosphatidylglycerol in a molar ratio of 9 : 1. When this LS-VAC was used in mice, a higher titer of the specific antibody was generated compared with the free form of HBsAg and HBsAg adsorbed on the aluminum hydroxide gel.

Currently, "Epaxal®" is the only LS-VAC (virosome) against hepatitis available on the pharmaceutical market, which was licensed in 1994 and manufactured by Crucell, the Netherlands (Bovier, 2008; Pippa and Demetzos, 2017). The size of the virosomal nanoparticle is

150 nm. One dose of "Epaxal®" contains at least 24 IU of inactivated hepatitis A virus (RG-58 strain), which was grown on human diploid cells (MRC-5) and inactivated using formaldehyde (Bovier, 2008). The virosome contains viral glycoproteins as adjuvants – emagglutinin and neuraminidase (10 μg) – isolated from inactivated influenza A virus (Singapore 61/86 H1N1). In addition, one dose of "Epaxal®" contains 100 μg of phospholipids: 80 μg of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 20 μg of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), in a molar ratio 75 : 25. These lipids ensure the uptake of the hepatitis A virus antigen by immunocompetent cells, thus ensuring their immunogenicity. "Epaxal®" was obtained using the detergent removal method (Leitgeb et al., 2020). The advantages of this method are the relatively simple technology, small size of LSs, and uniformity in particle size (Leitgeb et al., 2020).

Glück (1999) showed that virosomal VACs containing hepatitis A and influenza antigens possess enhanced immunogenicity compared with alum-adsorbed VACs for hepatitis A or commercial subunits of the whole virion influenza VACs. The influenza envelope glycoproteins promote the fusion of the virosome to the membranes of nonphagocytic cells. The virosome is internalized and processed, and the antigen of the VAC is directed into the major histocompatibility complex (MHC) class I antigen processing pathway to provoke a specific antipathogen cytotoxic T lymphocyte response. However, if the virosome does not immediately fuse with the membrane of a nonphagocytic cell, the presence of hemagglutinin in the virosomal membrane ensures that the virosome maintains a compact conformation that is easily taken up by phagocytes. Furthermore, hemagglutinin contains residues that help target the virosome to sialic-acid-containing receptors on the surface of antigen-presenting cells. The uptake of virosomes by antigen-presenting cells and their subsequent degradation in the endocytic system ensure the rapid and efficient antigen presentation on MHC class II and the induction of a Th response. Thus, packaging of a pathogen antigen in a virosome promotes both humoral and cell-mediated responses (Glück, 1999; Mak and Saunders, 2006).

"Epaxal®" shows high immunogenicity and is harmless. An enzyme-linked immunoassay (ELISA) study of the level of protective antibodies in the blood of immunized animals showed that the seroconversion rate of "Epaxal®" on days 14 and 28 was 97% and 98%, respectively. After the second injection of this LS-VAC, a 100% seroconversion rate was achieved (Bovier, 2008). Thus, the use of "Epaxal®" leads to a high humoral immune response.

Influenza VACs

Influenza and its complications are responsible for high rates of morbidity and mortality in the elderly. The percentage protection of immune response in vaccination is 70–90% in young people and only about 50% in the elderly (Ben-Yehuda et al., 2003). Today, different influenza VACs are available, both licensed and at different stages of development and clinical study: live attenuated, whole-virus inactivated, split-virion, subunit, viral vector-based (based on adenovirus, arenavirus, Newcastle virus, baculovirus, herpes virus), and LS-VACs (virosomes) (Chen et al., 2021).

Studies on LS-VACs against influenza have been conducted for many years (Ben-Yehuda et al., 2003; Conne et al., 1997; Holm and Goa, 1999; Mann et al., 2009). In a previous study, the safety and immunogenicity of commercial influenza VACs were evaluated in two groups of patients (Conne et al., 1997). Trivalent VACs were used in this study. The first group was injected with a VAC containing viral hemagglutinin, whereas the second group was injected with a VAC obtained by introducing hemagglutinin into the LS membrane, consisting of EPC. One month after immunization, both VACs showed a significant increase in the mean titer of antibodies against hemagglutinin of all three components of VACs. However, a significantly higher number of patients immunized with the LS-VAC showed a more than fourfold increase in antibody titer against Singapore and Beijing virus strains compared with those administered the non-LS-VAC. The percentage of patients who had protective titer upon immunization with the LS-VAC was also significantly higher. Of particular clinical significance was the fact that the protective levels of antibodies against all three components of the VAC were observed in 68.4% of patients immunized with the LS-VAC, in contrast to 38% in those immunized with the non-LS-VAC. Ben-Yehuda et al. (2003) proposed a trivalent LS-VAC containing 15 μg of hemagglutinin of each viral strain and 33 μg of

human interleukin-2 (IL-2). They compared the immunogenicity and safety of this LS-VAC with those of the standard licensed VAC. They intramuscularly administered this VAC to elderly people (mean age of the patients 80 years). Using ELISA, the titer of protective antibodies was detected in 33% of patients in a group of the standard VAC and in 48% in the group of the LS-VAC. This study showed the harmlessness of both VACs, but the immunogenicity was higher in the LS form, whereas antibodies against IL-2 were undetected.

The influenza LS-VAC "Inflexal®V" was the outcome of long-term studies of LS-VACs containing the influenza virus or its highly purified components, which was licensed in 1997 and manufactured by Berna Biotech, Switzerland (Pippa and Demetzos, 2017). It is a polyvalent virosomal inactivated VAC that includes surface antigens of two influenza virus type A strains and one type B strain with hemagglutinin and neuraminidase subunits (Asadi and Gholami, 2021). The virus is cultivated in chicken embryo cells and inactivated using β-propiolactone, after which hemagglutinins are isolated and purified. The size of LSs in this VAC is 150 nm. One dose of the VAC contains 15 μg of hemagglutinin of each strain, 80 μg of DOPC, and 20 μg of DOPE in a 75:25 molar ratio (Herzog et al., 2009; Mischler and Metcalfe, 2002). "Inflexal®V" was obtained using the detergent removal method (Leitgeb et al., 2020; Mane et al., 2021). The advantages of this method are the relatively simple technology, small size of LSs, and uniformity in particle size (Leitgeb et al., 2020). The use of "Inflexal®V" is shown to provide a high humoral immune response. "Inflexal®V" is four times more immunogenic than traditional influenza VACs (Poon and Patel, 2020).

The use of DOPC (nonlamellar lipid) and DOPE (lamellar lipid) in VACs "Inflexal®V" and "Epaxal®" is justified by the fact that these lipids are part of the natural cell membrane. Parchekani et al. (2022) studied a model DOPC/DOPE mixture in a ratio and amount similar to the composition of "Inflexal®V" and "Epaxal®". While determining the total energy (van der Waals energy and electrostatic interaction energy), the developed elliptical LS structure became highly stable and the phospholipids DOPC and DOPE formed a bilayer LS membrane due to their geometry and physicochemical properties (Parchekani et al., 2022). The zeta potential of the model DOPC/DOPE mixture at 50 mg/ml concentration was 20.78 mV (Peters, 2013). The DOPE and DOPC lipids play different roles in the formation of LSs: the former contributes to the formation of highly curved inverted hexagonal structures, whereas the latter forms a more stable lamellar structure (Du et al., 2014).

The use of virosomes has a number of advantages (Lee and Nguyen, 2015): high efficiency in antibody production and their long-lasting circulation in the host organism; conformational stability of the antigen; protecting the antigen from degradation; and safety and suitability for all populations. In addition, virosomes can imitate viral particles. The mechanism of action of virosomes can be represented as follows:

- delivery of encapsulated antigen to cytosol of antigen-presenting cells and subsequent induction of cytotoxic T-cell response;
- phagocytosis of virosomes by immunocompetent cells;
- activation of cluster of differentiation antigen 4 positive ($CD4^+$) T helpers for the production of cytokines;
- stimulation of B cells by cytokines to produce antibodies to the viral antigen.

Tregoning et al. (2018) reported that the immunogenicity of virosomes can be increased by including suitable adjuvants in their composition. Activation of signaling pathways using the toll-like receptor (TLR), which plays an important role in protection against the influenza virus, was also attempted. Administration of TLR3, TLR9, TLR7, and TLR7/8 agonists in mice resulted in virus suppression and increased survival. Combinations of the synthesized ligands TLR4, TLR7, and TLR7/8 were effective adjuvants for recombinant influenza VACs (Zhu et al., 2021).

The protective level of antibodies in the blood is usually attained 2–3 weeks after vaccination, and the duration of postvaccination immunity is 6–12 months (Gasparini et al., 2013). Virosomes are biodegradable and nontoxic and do not lead to the production of antiphospholipid antibodies (ELISA method).

LS-VACs against malaria and herpes zoster

Numerous studies are underway to develop VACs against malaria of various antigenic and adjuvant compositions (Bonam et al., 2021). The use of LSs as carriers for adjuvants of various structures is promising. The adjuvant system AS01 is one such LS adjuvant, which was developed 20 years ago. It is an LS-based adjuvant that includes two immunostimulants, monophos-

phoryl lipid A (MPL) and saponin QS-21 (Petrovsky and Aguilar, 2004).

MPL (3-O-desacyl-4 -monophosphoryl lipid A) is a detoxified synthetic derivative of the lipopolysaccharide of the gram-negative bacteria *Salmonella minnesota* that retains the adjuvant activity of the lipopolysaccharide with minimal toxicity. Over the past 20 years, numerous studies have demonstrated the high adjuvant activity of MPL (Alpatova et al., 2020; Alving and Rao, 2008). However, the high pyrogenic activity of MPL hinders its use as an adjuvant in VACs. The inclusion of MPL into the LS membrane significantly reduces the pyrogenicity of the lipopolysaccharide. For example, MPL isolated from *S. minnesota R 595* showed less pyrogenicity in the LS form compared with the free form. The nonpyrogenic dose of free MPL was 0.32 μg/kg of rabbit weight, and the LS form of MPL at a dose of 8.1 μg/kg of rabbit weight did not show a pyrogenic and toxic reaction. The second component of the adjuvant system AS01 is saponin QS-21 isolated from an extract of *Quillaja saponaria* (soap tree). Both components are included in LS nanoparticles consisting of DOPC and cholesterol in phosphate buffer.

Currently, two VACs with the LS adjuvant system AS01 have been licensed, a recombinant malaria VAC ("Mosquirix™," manufactured by GlaxoSmithKline, Belgium) and a herpes zoster VAC ("Shingrix" manufactured by GlaxoSmithKline, Belgium).

Immunogenicity of the malarial plasmodium LS antigen was studied in monkeys using AS01 containing lipid A as an adjuvant. "Mosquirix™" (developed by Walter Reed Army Research for Institute, USA; manufactured by GlaxoSmithKline, Belgium) is a licensed protein-based recombinant LS-VAC against malaria approved in October 2021 by the WHO. Its effectiveness in protection against malarial plasmodium in infants and young children ranges from 26 to 50%. Genes of the central repeat region and T-cell epitopes of *Plasmodium falciparum*-derived circumsporozoite protein genetically fused to HBsAg are used as antigens. Both of these protein components assemble into soluble virus-like particles similar to the outer shell of the hepatitis B virus. Infection is prevented by the induction of humoral and cellular immunity with high antibody titers that prevent human liver infection by *P. falciparum*. Malaria is caused by *Plasmodium* parasites and is spread to humans through infected female *Anopheles* mosquitoes. VACs that block the transmission of parasites to the host (humans) have been proposed to reduce the spread of malaria. These VACs are aimed at preventing the transmission of parasites to other humans after mosquito bites in noninfected but immunized humans. The host produces circulating antibodies that block gamete fertilization and/or prevent ookinete development into the mosquito midgut. The VAC induces a prolonged increase in the level of functional antibodies in the host organism, and blood transfer of antibodies to mosquitoes is considered to be the most effective functional mechanism of vaccination against malaria (Carter, 2001; Huang et al., 2018, 2020a; Nikolaeva et al., 2015).

Immune-stimulating complexes (ISCOMs) play a vital role in the production of numerous VACs. In veterinary VACs, several adjuvants and antigen presentation systems, including saponins and ISCOM immunomodulatory complexes, have previously been used. Saponins enhance cellular immune responses, unlike other adjuvants, such as aluminum hydroxide, that enhance the humoral immune response.

ISCOMs are based on the combination of the antigen and the adjuvant into particles. These particles are spherical structures with a diameter of about 40 nm. They are highly stable due to the interaction of saponins and cholesterol. Saponins in ISCOM particles are quite stable, in contrast to free saponins, which are easily degraded. ISCOMs are a homogeneous population of nanoparticles containing saponins, cholesterol, and phosphatidylcholine (Bengtsson et al., 2011). The use of ISCOMs allows the simultaneous delivery of the antigen and the adjuvant to antigen-presenting cells. The antigen and the adjuvant are distributed in the cell over the endosomal and cytosolic compartments, which in turn is accompanied by the induction of T helper cells and cytotoxic T cells. Unlike most of the adjuvants, ISCOMs do not lead to depot formation and slow release of the antigen after administration and the antigen–adjuvant complex is quickly eliminated from the injection site.

A VAC against malaria based on the Pfs25 protein is also of interest, which is an effective target protein for arresting malaria transmission (Mulamba et al., 2022). The proposed VAC is a protein nanoparticle in which the Pfs25 antigen is genetically fused to the IMX313 oligomerization domain. The Pfs25 protein is represented by

a *P. falciparum* 3D7 strain sequence with three mutant potential N-linked glycosylation sites. The recombinant protein nanoparticle is expressed and secreted in the *Pichia pastoris* expression system. Using the ISCOM technology, nanoparticles that are 40 nm in size containing antigen, saponins, DOPC, and cholesterol can be obtained.

The herpes zoster VAC "Shingrix" (manufactured by GlaxoSmithKline, Belgium) can be considered a significant achievement in vaccinology. It is an intramuscular suspension of lyophilized recombinant varicella zoster lipoprotein E antigen (AgE), which is recovered using AS01 suspension as an immunological adjuvant. AgE is the most abundant glycoprotein in cells infected with the varicella zoster virus, and it is the primary target for virus-specific antibodies and T cells. The antigen is a purified and truncated form of the glycoprotein that has been truncated to 546 amino acids by inserting a stop codon into its gene before the transmembrane sequence, resulting in a soluble secreted molecule that reacts with monoclonal and polyclonal antibodies of the native protein. The truncated glycoprotein is expressed in Syrian hamster ovary cells. "Shingrix" is manufactured in two vials that separately contain the adjuvant suspension (50 μg of MPL, 50 μg of QS-21, 1 mg of DOPC, 0.25 mg of cholesterol, 0.160 mg of sodium dihydrogen phosphate, and 0.54 mg of potassium dihydrogen phosphate) and 50 μg of lyophilized AgE. As auxiliary components, 20 mg of sucrose (stabilizer), 4.385 mg sodium chloride, and 0.08 mg of polysorbate 80 are used. The VAC is stored at 2–8 °C. The adjuvant suspension is mixed with the antigen before use (the mixture can be stored for 6 h after preparation at 2–8 °C). "Shingrix" has demonstrated high protective properties in fighting herpes zoster (Cunningham and Levin, 2018; GSK; 2021).

Both MPL and QS-21 are involved in the adjuvant action of AS01 (Bagaev, 2021; Worzner et al., 2021). The LS form of MPL acts as a TLR4 receptor agonist by activating T helper 1 (Th1) cytokine production (interferon-γ (IFN-γ), IL-2, and fumor necrosis factor α (TNF-α), which are associated with phagocytosis-mediated defense against internal infectious agents. Simultaneously, the saponin fraction QS-21 initiates the activation of dendritic cells to induce T-cell-mediated immune responses.

Thus, MPL, as a TLR4 receptor agonist, activates the innate immune system upon binding to this receptor and stimulates the transcriptional activity of the nuclear factor NF-kB, which leads to the increased synthesis of anti-inflammatory cytokines and IFN-γ and subsequently to the development of the Th1 immune response. Furthermore, MPL increases chemokine production. AS01 is effective in stimulating a CD4+ T-cell-mediated immune response and is a promising adjuvant for VACs against various viruses.

Based on the results of a study of the mechanism of action of QS-21, it can be concluded that the stimulating effect of QS-21 on the development of the humoral and cellular immune response is mediated by the following mechanisms (Alpatova et al., 2020):

- impact on antigen-presenting and T cells, which leads to the activation of Th1 cytokine synthesis and promotes the elimination of intracellular pathogens;
- production of cytokines, IL-1β and IL-18, which are important for the development of the Th1 response;
- the synergistic effect of LS-encapsulated MPL and QS-21, which manifests in an early IFN-γ response, which leads to an increase in the immune response.

The ISCOM matrix is currently being explored to develop next-generation VACs for the prevention of human infectious diseases such as influenza, COVID-19, and rabies. (Zhou et al., 2021; Pedersen et al., 2011). Clinical trials of a virosomal VAC against the H5N1 strain of the influenza virus using the ISCOM matrix as an adjuvant were carried out in 60 adults, and the emergence of highly specific neutralizing antibodies was demonstrated. The antibodies were specific to the strain used in the VAC and cross-reacted with other strains to a lesser extent.

COVID-19 VACs

In 2020–2021, VACs with different structures and efficacies emerged in the global pharmaceutical market to fight the COVID-19 pandemic. The rapid development of COVID-19 VACs was predetermined by significant advances in the VAC technology over the past 10–15 years; in particular, VACs based on messenger RNA (mRNA) significantly accelerated the development of COVID-19 VACs (Buschmann et al., 2021; Pardi et al., 2020). As a consequence, a new mRNA delivery platform has been created, which has been used in both Pfizer-BioNTech and Moderna VACs against COVID-19.

The mRNA technology has been developed for *in situ* antigen expression, which represents an innovative platform for the development of VACs with the advantage

that, unlike DNA-based VACs, mRNA-based VACs do not integrate into chromosomes, thus avoiding the risks of oncogenesis and insertional mutagenesis (Brito et al., 2015; Pardi and Weissman, 2017; Pardi et al., 2018). mRNA contains information about the primary structure of proteins, is synthesized based on DNA by transcription, and is used in translation as a template for protein synthesis (Lvov and Alkhovsky, 2020). The technology of mRNA-VACs has been under development for more than two decades. In contrast to traditional viral VACs, which deliver the inactivated or attenuated form of a virus or a part of a virus (e.g. a capsular protein), mRNA-VACs deliver the genetic information to human cells. Then, the human cells produce a protein that is necessary for the immune response. The COVID-19 mRNA-VAC encodes a viral spike glycoprotein (S protein) that is used by the virus to enter human cells.

Lipid nanoparticles (LNPs) encapsulating the mRNA molecule are important components of mRNA-VACs. According to many studies, the production of lipid components and LNPs was of decisive importance in the development of mRNA-VACs. LNPs protect mRNA and transport it into cells, and hence, mRNA-VACs could not be created without LNPs (Huang et al., 2020b).

When administered intramuscularly, the LNP delivery system facilitates the capture of mRNA by host cells and its delivery into the cytosol, where the mRNA sequence is translated into the S protein in ribosomes. After posttranslational modification by host cells, the S protein appears as a membrane-bound antigen in a prefusion conformation at the cell surface, providing an antigen for B cells. Intramuscular administration of LNP-based mRNA-VACs leads to temporary localized inflammation, which promotes the recruitment of neutrophils and antigen-presenting cells to the delivery site (Schoenmaker et al., 2021).

In recent years, mRNA-VACs have been explored in the field of immunotherapy for cancer and infectious diseases due to their high efficacy and safety. mRNA is a part of a viral genome that leads to the synthesis of antigenic protein structures, in response to which specific antibodies are synthesized in the host organism (Schoenmaker et al., 2021). When mRNA is delivered to human cells by vaccination, viral S proteins are produced, and anti-S-protein-neutralizing antibodies and cellular immune responses can prevent the severe acute respiratory syndrome-related coronavirus 2 (SARS-CoV-2) infection.

Since the late 1990s, LNPs have been developed for nucleic acid delivery. The nanoparticles in COVID-19 VACs consist of a mixture of four lipid molecules: three of them stabilize the structure of LNPs, and the fourth lipid, called ionizable lipid, is crucial for the utilization of LNPs. This lipid is positively charged upon the production of LNPs, which provides the same benefits as known LS nanoparticles; however, under physiological conditions (e.g., in the bloodstream), ionizable lipids are converted to neutrally charged forms, which limits their toxic effects on the body. Moreover, the mixture of four lipids improves the stability of VACs during manufacture, transportation, and storage. Furthermore, LNPs maintain the stability of VACs in the body. By the mid-2000s, a new way of mixing and producing these nanoparticles has been developed, which used a T-shaped apparatus that mixes lipids dissolved in ethanol with nucleic acids dissolved in an acidic buffer (Jeffs et al., 2005). When these two solution streams meet, the components spontaneously form LNPs. Each developer who produces mRNA-VACs uses different variations of this drug delivery platform (Hou et al., 2021).

Thus, the COVID-19 VAC manufactured by Pfizer-BioNTech includes the following four lipid components: ((4-hydroxybutyl)azanediyl)bis(hexane-6,1-diyl)bis (2-hexyldecanoate) as an ionizable lipid, 2[(polyethylene glycol (PEG))-2000]-N,N-ditetradecylacetamide as a PEG-lipid, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), and cholesterol (Hassett et al., 2017; Jackson et al., 2020).

LNPs with mRNA are assembled in several steps:

1) rapid mixing of four lipids (ionizable lipid, DSPC, cholesterol, and PEG lipid) dissolved in ethanol with mRNA dissolved in a pH 4.0 aqueous buffer in a microfluidic mixer or a mixer with a T-shaped connection;
2) when the ionizable lipid is in the aqueous phase, it becomes protonated at pH 5.5, which is intermediate between the pKa of the buffer and the pKa of the ionizable lipid;
3) the ionizable lipid electrostatically binds the anionic phosphate backbone of mRNA, and it is hydrophobic in the aqueous medium, stimulating vesicle formation and mRNA encapsulation;
4) after the initial formation of vesicles, the pH is increased by dilution by dialysis or filtration, which leads to neutralization of the ionizable lipid by making it more hydrophobic and thus increases the

fusion of vesicles, causing further binding of the ionizable lipid to mRNA within LNPs.

The presence of the PEG lipid stops the fusion process, provides a hydrophilic outer layer for LNPs, and defines their thermodynamic stability and size, and the bilayer formed by the DSPC lies directly below the PEG lipid layer in LNPs. LNPs in the Pfizer-BioNTech VAC consist of 50% of ionized lipid, 10% of DSPC, 38.5% of cholesterol, and 1.5% of PEG lipid, and their size ranges from 100 to 170 nm.

Further development of mRNA-based drugs will lead to the synthesis of original compositions of LNPs as well as LSs. Studies are underway on the use of not only the above-described LNPs but also other LNPs in the composition of COVID-19 VACs. In South Korea, research was carried out to develop a lyophilized LS-mRNA-VAC candidate, EG-COVID (Hong et al., 2021). LSs based on 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), DOPE, and cholesterol were used to obtain the VAC candidate. The size of LSs before and after lyophilization was 191.7 ± 8.5 nm (zeta potential 54.5 ± 3 mV) and 266.7 ± 12.2 nm (zeta potential 44.4 ± 2.1 mV), respectively. In this research, the effectiveness of the VAC with the proposed composition of lipids was compared with that of a drug with LNPs similar to the Pfizer VAC. mRNA was included in LSs and LNPs, and the activity of the two VACs was compared when administered intramuscularly. EG-COVID induced sustained humoral and cellular immunity against the SARS-CoV-2 virus. The blood serum of immunized mice suppressed the SARS-CoV-2 viral infection in VERO cells (cell line isolated from the kidney of an African green monkey). According to this research, a lyophilized VAC is preferable to a liquid one, because a dried VAC can be stored and transported at higher temperatures than the Pfizer's VAC.

Gregoriadis (2021), one of the pioneers in the use of LSs as an adjuvant, substantiated the advantages of using LSs in the production of mRNA-VACs as follows:

1) LSs are biodegradable and easy to prepare and can encapsulate mRNA quantitatively;
2) LS-encapsulated mRNA is fully protected against nuclease attack in the blood circulation;
3) LS-encapsulated mRNA enters the cytoplasm of cells by endocytosis;
4) mRNA encapsulated into cationic LSs escapes the lysosomotropic pathway to end up intact in the cytoplasm;
5) within the cytoplasm, mRNA is expressed as the S protein, whereupon – by an as yet unclear mechanism – LSs or their remnants exert their immunological adjuvant action.

The biodegradability and versatility of LNPs and LSs are the major advantages of using them in the composition of VACs. Besides being a drug delivery system, nanoparticles can have significant therapeutic effects and exhibit synergism with mRNA-encoded proteins (Hou et al., 2021).

Thanks to vaccination, the global scientific community has started discussing the revolution in biotechnology and pharmacy. VACs against SARS-CoV-2 became mass drugs based primarily on new biotechnologies, mRNA (Pfizer and Moderna, USA) and adenoviral vectors (AstraZeneca, UK; Covishield, India; Sputnik V, Russia), as well as classical technologies (Coronovac and Sinovac, China) (Zhang et al., 2021b). VACs demonstrated different efficacies ranging from 85 to 95%. According to the current data, 94% fewer cases of infection symptoms are reported in patients vaccinated against COVID-19 than in unvaccinated people in a control group (Cheng et al., 2021; Polack et al., 2020). Among those who are vaccinated but still infected by the virus, 92% fewer cases of severe disease are reported.

Today, vaccination against SARS-CoV-2 is the only way to fight the COVID-19 pandemic and save human lives, and the effectiveness of LNPs has been confirmed using billions of doses of VACs against SARS-CoV-2.

The development of VACs against COVID-19 is ongoing. Novavax has developed a quadrivalent recombinant VAC against COVID-19 ("Nuvaxovid") using an insect cell line. "Nuvaxovid" is fundamentally different from the mRNA-VACs manufactured by Pfizer-BioNTech and Moderna and the viral vector VAC manufactured by Johnson & Johnson. It is a subunit protein VAC containing the SARS-CoV-2 spike protein. The technology of "Nuvaxovid" includes the following stages: selection of genes encoding particular SARS-Cov-2 antigens (spike protein); introduction of the selected genes into the baculovirus; infection of insect cells with baculovirus; replication of the virus; accumulation of the spike protein in insect cells; isolation and purification of antigen proteins; and obtaining a complex of antigen proteins and ISCOM matrix. The complex is comprised of a highly active adjuvant (saponin fraction C), a weak adjuvant (saponin fraction A), phosphatidylcholine, and cholesterol. "Nuvaxovid" induces high multifunctional cell-mediated immunity. One dose of "Nuvaxovid" con-

tains 5 μg of spike protein, 42.5 μg of saponin fraction A, and 7.5 μg of saponin fraction C (European Medicines Agency, 2021). VACs against COVID-19 using an ISCOM matrix are being studied by numerous researchers (Ayele, 2021; Smith et al., 2020; Chauhan et al., 2020; Zhang et al., 2021a). On June 7, 2022, The Food and Drug Administration Advisory Committee voted to recommend the use of "Nuvaxovid" in adults in the USA (U. S. Food and Drug Administration, 2022).

Conclusion

The history of human development has convincingly proven that vaccination is the most effective approach to the prevention of infectious diseases. The costs of immunization are negligible compared with the cost of treating infected people. Evidence of this is mRNA-VACs, which lead to antigen expression *in situ* after immunization, thereby initiating an immune response.

According to the data presented in Table 1, the effectiveness of LSs as an adjuvant system in VACs could be argued. The following are the advantages of using LS-VACs: antigens with low immunogenicity can become highly immunogenic; LSs can include both hydrophilic and hydrophobic antigens; LSs are involved in achieving a prolonged specific action of antibodies; LSs reduce the toxicity and pyrogenicity of encapsulated antigens and adjuvants.

While developing the composition of VACs, the way the immune system functions (innate and adaptive immunity) must be taken into account. The adjuvant activity of LS-VACs is based on their ability to recruit, interact, and activate antigen-presenting cells (such as dendritic cells, macrophages, and B cells) due to the physicochemical (size, charge) and immune properties (inclusion of other adjuvants and targeting ligands) of LSs. For example, the positively charged surface of cationic LSs facilitates the interaction with the negatively charged surface of dendritic cells, which are one of the primary inductors of the T-cell-mediated immune response, thus ensuring antigen delivery and uptake (Kushwah and Hu, 2011; Ness et al., 2021).

LSs significantly increase immunogenicity by enhancing antigen presentation and/or triggering the innate immune system through recognition and activation of specific cell receptors, which can lead to long-term protection against pathogenic agents (Pasare and Medzhitov, 2004). In addition, various types of cellular receptors are involved in innate immunity, such as TLR, NLR (nucleotide-binding domain leucine-rich repeat-containing receptor), and C-type lectin receptor. Each receptor contributes to the immune response that leads to the activation and differentiation of T helper cells with a possible adaptive immune response mediated by antibodies and CD8+ T cells (Pasare and Medzhitov, 2004).

Of special interest is the possibility of developing VACs based on the ISCOM matrix, especially VACs against COVID-19 ("Nuvaxovid"). The ISCOM is a homogeneous population of nanoparticles containing saponins, phosphatidylcholine, and cholesterol, which allows the reduction in the hemolytic activity of saponins and increase their stability and the possibility of interaction with various antigens. The ISCOM is based on the combination of the antigen and the adjuvant into spherical structures with a diameter of about 40 nm and can stimulate a strong immune response through the induction of Th1, Th2, and cytotoxic lymphocytes, including the secretion of cytokines (Ayele, 2021; Bengtsson et al., 2011).

Safety, physicochemical characteristics of lipids (including the level of impurities), and the level of antigen encapsulation in LSs are crucial factors in the development of LS-VACs. Only when these requirements are met, LS components of VACs can be applied. Moreover, the stability of lipid molecules in VACs, the structure of LSs, and the level of antigen encapsulation in nanoparticles must be demonstrated. LSs are effective nanoparticles for the encapsulation of hydrophobic antigens (into the bilayer of the LS membrane) as well as hydrophilic proteins (into the inner water core of LSs) or antigens associated with the surface of the nanoparticle. Antigen localization in LSs can significantly affect the immunogenicity of VACs and change the immune response accordingly (Rao et al., 2021).

The charge and size of LSs also need to be taken into account as they determine the effectiveness of the immune response. The charge of the nanoparticles significantly affects the adsorption or interaction of the antigen with LSs. A number of studies have reported that positively charged antigens are more likely to interact with cationic LSs. It is this interaction that can determine the level of antigen encapsulation into LSs (Pasare and Medzhitov, 2004). Cationic LSs show significant

Table 1. Licensed LS-VACs used for vaccination of people

Trade name and manufacturer of VAC	Antigen	LS / LNP composition	Prevention of disease
"Epaxol-Berna", Swiss Serum Vaccine Institute, Switzerland	hepatitis A antigen	DOPC, DOPE	hepatitis A
"Inflexal®V", Berna Biotech, Switzerland	hemagglutinin, neuraminidase	DOPC, DOPE	influenza
"MosquirixTM", GlaxoSmithKline, Belgium	circumsporozoite protein of *Plasmodium falciparum*, HBsAg	DOPC, cholesterol	malaria
"Shingrix", GlaxoSmithKline, Belgium	recombinant varicella zoster lipoprotein E antigen	DOPC, cholesterol	herpes zoster
Pfizer-BionTech, USA	mRNA, providing information about amino acid sequence of SARS-CoV-2 S-protein	ionizable lipid [a]; PEG-lipid, DSPC, cholesterol	COVID-19
Moderna, USA	mRNA, providing information about amino acid sequence of SARS-CoV-2 S-protein	ionizable lipid [b]; DSPC, cholesterol; PEG-2000-DMG	COVID-19

Ionizable lipid [a] – ((4-hydroxybutyl)azanediyl)bis(hexane-6,1-diyl)bis(2-hexyldecanoate); PEG-lipid – [(polyethylene glycol (PEG))-2000]-N,N-ditetradecylacetamide; ionizable lipid [b] – SM-102 (9-Heptadecanyl 8-{(2-hydroxyethyl)[6-oxo-6-(undecyloxy)hexyl]amino}octanoate); PEG-2000-DMG – 1,2-dimyristoyl-rac-glycerol,methoxy-polyethyleneglycol-2000

immunomodulating and adjuvant effects and lead to a higher immune response (Korsholm et al., 2007). The lipid composition determines the charge of LS particles; for example, neutral DSPC-based LSs (8 mV) enter the lymphocytes faster than cationic LSs (+50 mV), and positively charged LSs tend to be taken up by antigen-presenting cells to a greater extent than negatively charged or neutral ones (Tretiakova and Vodovozova, 2022). The size of LSs determines further Th1 and Th2 responses to the captured antigen (Brewer et al., 1998). The immunomodulating activity can be enhanced by including molecules such as lipid A and Gang in VACs (Bogdashin et al., 1986; Carter, 2001; Grabowska et al., 2021; Huang et al., 2018; Konstantinova et al., 1985; Nikolaeva et al., 2015; Twihaar et al., 2020).

The influence of LS carriers on antigen internalization by immunocompetent cells and the way of the immune response induction depends on the charge, size, and phase of the phospholipid bilayer, which in turn depend on the lipid composition (Tretiakova and Vodovozova, 2022). Moreover, virosomes are internalized into epithelial cells better than LSs (De Serrano and Burkhart, 2017). Hemagglutinin and neuraminidase promote the uptake of virosomes by immune cells, and sialic acid residues are overexpressed on dendritic cells. Macrophages induce rapid endosomal-mediated cellular uptake, which activates MHC class I and class II, and lymphocytes (Glück, 1999; Mak and Saunders, 2006).

LS-VACs can be administered subcutaneously, intramuscularly, or *per os*. For example, cationic LSs prepared from dimethyldioctadecylammonium form a depot at the injection site due to their size (Henriksen-Lacey et al., 2010). Besides, antigen encapsulation in LSs may reduce hypersensitivity reactions. The effectiveness of the immune response is affected by the interaction of LSs with complement and a number of proteins, which leads to opsonization (Vu et al., 2019). The interaction of LSs and other types of particles with biological fluids *in vivo* and *in vitro* leads to the adsorption of proteins that alter the chemical and physical characteristics of the particles, and this phenomenon of protein adsorption at the surface of LSs is called "protein corona" in nanomedicine (Ke et al., 2017; Vu et al., 2019). The "protein corona" may pose a challenge for the targeted intravascular delivery of LS drugs, for example, to tumor cells because LSs are rapidly covered with opsonins, which leads to complement activation and uptake by phagocytic cells (Bohlson et al., 2019). At the same time, in terms of the delivery of LS-VACs to the immune system cells, the "protein corona" can lead to a positive effect. Kaplun et al. (2000) showed that LSs containing negatively charged phospholipids and cerebroside sulfate interact with complement; therefore, it can be assumed that such LSs can activate complement, causing its depletion and possibly opsonization of LSs. Thus, any LS that has a negative charge of a certain value can modulate com-

plement action. Only a few surface-bound immunoglobulin molecules are required to activate and opsonize complement. It has been reported that natural immunoglobulin is a link between the "corona" of biomolecules and complement C3 opsonization and can determine the individual complement responses to nanoparticles. Based on these findings, it is possible to consider the effect of the surface-bound protein on the efficacy of LS carriers of antigens and adjuvants for VACs. While studying the efficacy of LS adjuvants in VACs, the pharmacotherapeutic status of LSs and their effect on the immune responses need to be considered (Grigoryeva et al., 2020). An independent issue in the development of LS drugs is the technological aspect. Previous studies have discussed in detail the techniques for obtaining LSs and the requirements for materials for their production (Krasnopolskiy et al., 2011). The possibility of using sterilizing filtration in the production of LS-VACs, in contrast to mineral sorbents and oil emulsions, is also rather important, which in turn allows for sterilizing filtration at every stage of VAC production.

The primary properties of most of the adjuvants are determined by their ability to deposit the antigen (to adsorb it on the surface or encapsulate it into nanoparticles) and their ability to stabilize the antigen and protect it against destruction and elimination, which increases the prolongation of the antigen effect on the immune system. LS-based adjuvants meet these requirements (Krasnopolsky and Borshchevskaya, 2009; Krasnopolsky et al., 2011).

LS adjuvants have found broad application in the vaccination of people and animals. Besides licensed LS-VACs against hepatitis A, influenza, herpes zoster, malaria, and COVID-19 (Table 1), LS-VACs against influenza, diphtheria, tetanus, hepatitis A and B, rabies, tuberculosis, intestinal infections, etc., are under development (Martinov et al., 2014; Pippa and Demetzos, 2017).

Conflict of interest

There is no conflict of interest.

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