Lipid-Based Nanoparticles for Delivery of Vaccine Adjuvants and Antigens: Toward Multicomponent Vaccines

Despo Chatzikleanthous, Derek T. O’Hagan, and Roberto Adamo*

ABSTRACT: Despite the many advances that have occurred in the field of vaccine adjuvants, there are still unmet needs that may enable the development of vaccines suitable for more challenging pathogens (e.g., HIV and tuberculosis) and for cancer vaccines. Liposomes have already been shown to be highly effective as adjuvant/delivery systems due to their versatility and likely will find further uses in this space. The broad potential of lipid-based delivery systems is highlighted by the recent approval of COVID-19 vaccines comprising lipid nanoparticles with encapsulated mRNA. This review provides an overview of the different approaches that can be evaluated for the design of lipid-based vaccine adjuvant/delivery systems for protein, carbohydrate, and nucleic acid-based antigens and how these strategies might be combined to develop multicomponent vaccines.

KEYWORDS: lipid nanoparticles, vaccines, delivery systems, adjuvants, COVID-19

1. INTRODUCTION

Vaccines are the most successful and cost-effective approach for the prevention of diseases, other than the supply of clean water. Traditional whole organism vaccines (e.g., polio virus) can be very effective for the control and even elimination of an infectious disease; the polio vaccine can comprise whole inactivated pathogens for injection, or attenuated form for oral administration. However, the development of alternative approaches to vaccine development based on pathogen subunits has opened up new possibilities. Subunit vaccines, which can include proteins, carbohydrates, or peptides, have demonstrated great advances in recent years. However, although these new generation vaccines typically have improved safety and tolerability profiles relative to the traditional approaches, they are often less immunogenic than conventional vaccines, due to the removal of all pathogenic features from the original organism. In contrast, nucleic acid-based (DNA and RNA) or vector-based (e.g., adenovirus) vaccines can mimic a live infection by causing the expression of antigens in situ after immunization, thereby priming both B and T cell responses. Recently, two mRNA-based vaccines created by BioNTech/Pfizer2,3 and Moderna/National Institute of Allergy and Infectious Diseases (NIAID) have become the first mRNA vaccines to receive conditional approval for human use. Adenovirus based vaccines from Astra Zeneca, Johnson & Johnson,6 and Moscow-based Gamaleya7 soon followed to address the challenges created by the COVID-19 pandemic.

There still remains several unmet medical needs for improved vaccines, particularly against challenging bacterial (e.g., Mycobacterium tuberculosis) or viral pathogens (e.g., RSV, CMV, Zika, and HIV) for which no effective vaccines or vaccines with limited efficacy have so far been developed. Additionally, more complex live attenuated (e.g., yellow fever) or viral-vectored vaccines (e.g., Johnson & Johnson against COVID-19) typically require fewer numbers of doses compared to subunit ones and might even confer protection after a single dose.8 The design of single-dose subunit vaccines remains a key goal due to the significant benefits that would be associated with their use.9 Significant challenges also remain for the potential development of therapeutic cancer vaccines, due to many inherent obstacles, including the immunosuppressive microenvironment of tumor cells.10 Tumor-derived resistance mechanisms developed during cancer progression often lead to immune escape.11 Additionally, the impaired immune system of cancer patients is an issue, often due to immune cell exhaustion, age, or the side effects of immunotherapy, which block vaccine-stimulated immune responses.12

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In order to overcome these challenges, along with a deeper understanding of pathogenicity or tumor initiation and growth, exploitation of appropriate delivery systems and adjuvants plays a pivotal role. Particularly, antigen delivery can protect the antigens from degradation, facilitate the antigen uptake from antigen presenting cells (APCs), and promote their full activation for initiating robust anti-vaccine Th1/cytotoxic T lymphocyte (CTL) responses and long-term immunological memory.

Adjuvants generally improve immunogenicity by enhancing antigen presentation and/or triggering the innate immune system via the recognition and activation of specific cell receptors, which may result in long-term protection against pathogens. The most used adjuvant for vaccine development has been insoluble aluminum salts. However, limitations have included incompatibility or ineffectiveness for some antigens, along with an inability to induce potent cell-mediated immune responses, especially cytotoxic T-cell responses, which have prompted the search for alternative adjuvants. Adjuvants have been broadly classified into delivery systems and immunopotentiators, even though many adjuvants operate as both. However, delivery systems are thought to serve as carriers for the delivery of antigens to immune cells in the body, while immunopotentiators are typically ligands of pattern recognition receptors (PRRs), such as the Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), and C-type lectin receptors (CLRs). These receptors are located on the surface, in the endosomes, or in the cytosol of APCs and can recognize specific pathogen-associated molecular patterns (PAMPs) (Figure 1). Through a variety of signaling pathways, engagement of these receptors results in quantitative and qualitative changes in immunological functions.

Adjuvants described in the literature that have been used in clinical trials are shown in Table 1.

The next generation of vaccines will likely benefit from combination adjuvant approaches, simultaneously targeting multiple branches of the immune response. In fact, adjuvants are known to act by a combination of different mechanisms including depot effect, induction of cytokines and chemokines, recruitment of immune cells, enhancement of antigen uptake and presentation, and promoting of antigen delivery to draining lymph nodes. Hence, optimal activation of immunity can be achieved by harnessing the immunostimulatory properties of different adjuvants, which in combination can boost the quality and quantity of an immune response against vaccine antigens.

Although the use of immunopotentiators in adjuvants is gradually expanding, their use can be associated with enhanced adverse events unless an appropriate formulation is used to control these effects. An additional challenge has been the use of freely soluble immune potentiators, which are easily difusible and can quickly move from the site of administration into systemic circulation, potentially causing severe adverse effects. In an effort to overcome these issues various nanocarrier approaches such as insoluble aluminum salts, liposomes, emulsions, and polymeric nanoparticles have been suggested to improve stability, reduce toxicity, target delivery, and improve pharmacokinetics and the biodistribution profiles of immunopotentiators.

Liposomes are currently considered one of the most flexible and successful delivery systems, due to their many advantages, including biocompatibility, encapsulation capabilities, high loading capacity, and surface modification approaches that are available, to generate desired functions. A wide range of lipid-based products are currently available for the treatment of various diseases, including vaccines against hepatitis A (Epaxal), influenza (Inflexal V), shingles (Shingrix), and COVID-19 (mRNA-1273 and BNT162b2 by Moderna and BioNTech, respectively). The validation of the RNA platform through inclusion in licensed vaccines will undoubtedly foster the development of new vaccines formulated with lipid nanoparticles.

Although different reviews have been published on liposomes over the recent years, this work aims at providing an overview of the recent advances and challenges in the design of modern vaccine delivery systems based on lipid nanoparticles, with a focus on functionalization and combinatorial strategies for the incorporation of protein, DNA, and RNA.
and thus form a bilayer. Hydrophobic tails constitute the inner region of the membrane heads orient themselves toward the aqueous medium and the vesicles. Unilamellar liposomes (ULVs) are formed when a

ogy, into two major types, unilamellar and multilamellar component vaccines.

of biocompatible and biodegradable phospholipids at the Babraham Institute, University of Cambridge, and Liposomes were discovered by Alec D. Bangham in the 1960s

with adjuvants or small molecules for cell-specific targeting

phosphorothioate-guanine oligodeoxynucleotides; DDA, dimethyldioctadecylammonium; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DTaP, diphtheria, tetanus, and pertussis; GLA, glycoprotein A; HIV, human immunodeficiency virus; HPV, human papillomavirus; LPS, lipopolysaccharide; MenC, meningococcal serogroup C; MPL, monophosphoryl lipid A; poly(I:C), polyinosinic, polycytidylic acid; QS21, Quillaja saponaria LPS, lipopolysaccharide; MenC, meningococcal serogroup C; MPL, monophosphoryl lipid A; poly(I:C), polyinosinic, polycytidylic acid; QS21, Quillaja saponaria

Table 1. Key Adjuvants Available in Vaccines on the Market or Evaluated in Clinical Trials

<table>
<thead>
<tr>
<th>Adjuvant</th>
<th>Company/Discovery</th>
<th>Classification/Composition</th>
<th>Mechanism of Action</th>
<th>Disease Tested</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alum</td>
<td>A. Glenny 1926</td>
<td>Insoluble salts</td>
<td>Immune cell recruitment, inflammasome, Th2 responses</td>
<td>DTaP, hepatitis A, B, influenza, HPV, pneumococcus, COVID-19</td>
<td>19–22</td>
</tr>
<tr>
<td>MF59</td>
<td>Chiron</td>
<td>Oil-in-water emulsion/Tween80, Span85, squalene</td>
<td>Immune cell recruitment</td>
<td>Influenza</td>
<td>23–25</td>
</tr>
<tr>
<td>AS01</td>
<td>GSK</td>
<td>Liposome/MPL, QS21, DOPC, cholesterol</td>
<td>TLR4, immune cell activation, Th1 responses</td>
<td>Malaria, tuberculosis shingles</td>
<td>26</td>
</tr>
<tr>
<td>AS03</td>
<td>GSK</td>
<td>Oil-in-water emulsion/Tween80, α-tocopherol, squalene</td>
<td>Immune cell recruitment</td>
<td>Influenza</td>
<td>27</td>
</tr>
<tr>
<td>AS04</td>
<td>GSK</td>
<td>Aluminum salt-based combined adjuvant/ MPL + Alum</td>
<td>TLR4, Th2 responses</td>
<td>HPV, hepatitis B</td>
<td>28</td>
</tr>
<tr>
<td>AF03</td>
<td>Sanofi Pasteur</td>
<td>Oil-in-water emulsion/Span80, polyoxyethylene cysteyl stearyl ether, mannnitol, squalene</td>
<td>Immune cell recruitment</td>
<td>Influenza</td>
<td>29</td>
</tr>
<tr>
<td>Virosomes</td>
<td>First described by Almeida et al. in 1975</td>
<td>Microbe-based lipid membrane delivery systems</td>
<td>Promote antigen presentation, Th1 and Th2 responses</td>
<td>Hepatitis A, influenza</td>
<td>33–35</td>
</tr>
<tr>
<td>RC-529</td>
<td>Corixa</td>
<td>Synthetic glycolipid</td>
<td>TLR4</td>
<td>Hepatitis B</td>
<td>36, 37</td>
</tr>
<tr>
<td>MPL</td>
<td>E. Ribi</td>
<td>Chemically modified LPS</td>
<td>TLR4</td>
<td>Pollen allergies</td>
<td>38</td>
</tr>
<tr>
<td>AS015</td>
<td>GSK</td>
<td>Liposome/AS01, CpG</td>
<td>TLR9, TLR4, immune cell recruitment</td>
<td>Lung cancer, melanoma</td>
<td>39</td>
</tr>
<tr>
<td>AS02</td>
<td>GSK</td>
<td>Oil-in-water emulsion/MPL, QS21</td>
<td>TLR4, Th1 responses</td>
<td>HIV, TB, malaria</td>
<td>27</td>
</tr>
<tr>
<td>AS37</td>
<td>Novartis</td>
<td>TLR7a with a benzazaphthyridine chemical scaffold, adsorbed to Alum</td>
<td>TLR7</td>
<td>MenC</td>
<td>40</td>
</tr>
<tr>
<td>MPL + SE</td>
<td>E. Ribi</td>
<td>Oil-in-water emulsion</td>
<td>TLR4, Th1 responses</td>
<td>Leishmaniasis, influenza</td>
<td>41</td>
</tr>
<tr>
<td>GLA + SE</td>
<td>Infectious Disease Research Institute</td>
<td>Oil-in-water emulsion</td>
<td>TLR4</td>
<td>Leishmaniasis, influenza, RSV</td>
<td>42</td>
</tr>
<tr>
<td>CAF01</td>
<td>Statens Serum Institut</td>
<td>Cationic liposomes/DDA, TDB</td>
<td>Depot effect/immune cell recruitment</td>
<td>TB, chlamydia</td>
<td>43–45</td>
</tr>
<tr>
<td>Matrix-M</td>
<td>B. Morein</td>
<td>Quillaja saponins formulated with cholesterol and phospholipids into nanoparticles</td>
<td>Immune cell recruitment, Th1 and Th2 responses</td>
<td>COVID-19, influenza</td>
<td>46–48</td>
</tr>
<tr>
<td>R484</td>
<td>3M Drug Delivery Systems</td>
<td>Imidazoquinoline</td>
<td>TLR7/8</td>
<td>Influenza, melanoma</td>
<td>49</td>
</tr>
<tr>
<td>Advax</td>
<td>Vaxine</td>
<td>Microcrystalline plant-based polysaccharide (delta-insulin) particle</td>
<td>Th1 and Th2 responses</td>
<td>Influenza, hepatitis B, malaria, West Nile virus, COVID-19</td>
<td>48, 50–52</td>
</tr>
</tbody>
</table>

“AS01, adjuvant system 01; Alum, aluminum; CAF01, cationic adjuvant formulation 01; COVID-19, coronavirus disease 2019; CpG, cytosine-phosphorothioate-guanine oligodeoxynucleotides; DDA, dimethyldioctadecylammonium; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DTaP, diphtheria, tetanus, and pertussis; GLA, glycoprotein A; HIV, human immunodeficiency virus; HPV, human papillomavirus; LPS, lipopolysaccharide; MenC, meningococcal serogroup C; MPL, monophosphoryl lipid A; poly(I:C), polyinosinic, polycytidylic acid; QS21, Quillaja saponaria; RSV, respiratory syncytial virus; SE, stable emulsion; TB, tuberculosis; TDB, trehalose 6,6-dibehenate. In some of the adjuvants the full adjuvant mechanism of action is not fully clarified yet. Initial evidence is presented.

2. BASICS OF LIPOID-BASED FORMULATIONS: DISCOVERY, CHARACTERISTICS, AND MANUFACTURING

Liposomes were discovered by Alec D. Bangham in the 1960s at the Babraham Institute, University of Cambridge, and consist of biocompatible and biodegradable phospholipid bilayers. Phospholipids are amphipathic and are characterized by having a lipophilic tail and hydrophilic head on the same molecule. During liposome manufacturing, the polar heads orient themselves toward the aqueous medium and the hydrophobic tails constitute the inner region of the membrane and thus form a bilayer.

Liposomes are usually defined, according to their morphology, into two major types, unilamellar and multilamellar vesicles. Unilamellar liposomes (ULVs) are formed when a single bilayer of phospholipids surrounds the aqueous core. Small unilamellar vehicles (SUVs) with size less than 100 nm and the large unilamellar vehicles (LUVs) with size up to a few micrometers are most extensively used. Multilamellar liposomes (MLVs) consist of several concentric bilayers separated by aqueous compartments. The number of bilayers, the lipid composition, and the manufacturing method are among the factors impacting on the liposome size.

Not all the lipid-based nanocarriers have a contiguous bilayer that would qualify them as liposomes. Example are the lipid nanoparticles (LNPs) which assume a micelle-like structure, encapsulating therapeutic molecules in a nonaqueous core. LNPs represent the newly emerging gold standard for nucleic acid vaccine delivery as they are more effective than classical lipid-based particles.

Nowadays there is a plethora of lipid combinations that can be considered for the development of lipid-based formulations (Table 2). Common components of liposomes and LNPs are the neutral phospholipids such as 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1-α-phosphatidylcholine (HSPC),
and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), which give the structures of lipid bilayer and cholesterol, which enhances membrane stability. For the case of liposomes, negatively (1,2-dioleoyl-sn-glycero-3-phosphate (DOPA), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS)) or positively (dimethyldioctadecylammonium (DDA), 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP)) charged lipids are used that can modulate liposome structure and surface properties. Generally, charged liposomes demonstrate high stability, which relies on the presence of surface charge, which induces electrostatic repulsion to prevent aggregation and flocculation. Critical components of LNPs are the ionizable cationic lipid (e.g., DLinDMA) and PEG lipid for achieving high RNA encapsulation efficiencies and the steric barrier effect, respectively. Ionizable cationic lipids have been introduced in the early 2000s, and they have the ability to change their pH according to the pH of the environment. Thus, they have a positive charge at acidic pH and become neutral at physiological pH.

Lipid formulations can be produced by a wide variety of techniques. Thin film hydration method (original Bangham method), detergent depletion, solvent injection, reverse-phase evaporation, and emulsion methods are some of the conventional preparation methods. Among them, thin film hydration represents the simplest and oldest method used. In this method, lipids are first dissolved in a suitable organic solvent and dried down to yield a thin film at the bottom of the flask. The lipid film is then hydrated using an appropriate aqueous medium to produce a liposomal dispersion. The resulting particles are usually highly polydisperse, varying in size and shape. Significant drawbacks of the conventional preparation methods are their non-scalability for industrial production, vesicle size variability, organic solvent residuals, and low encapsulation efficiencies. To address these common issues, microfluidics systems have been more recently used. The different microfluidic technologies have been extensively reviewed elsewhere. Generally, microfluidics uses intersecting microchannels for the highly controlled mixing of nanoliter volumes of two or more miscible solvents (commonly an aqueous phase mixed with a water miscible alcohol such as methanol, ethanol, or isopropanol). During the mixing, the change in polarity promotes nanoprecipitation and the formation of lipid-based nanoparticles. As with other manufacturing techniques, optimization of critical process parameters (flow rate, flow ratio, temperature) and material parameters (aqueous buffer selection and composition, solvent) is essential as they impact the properties of the final product. The adoption of microfluidics as part of production process offers the advantages of robust particle size control and high reproducibility across production scales and hence the ability to support scale-independent and/or continuous operation.

3. LIPOSOMES AS VACCINE-ADJUVANT DELIVERY SYSTEM

Liposomes are one of the three classes of adjuvants that are currently included in licensed vaccines (aluminum salts and oil-in-water emulsions are the others). A key advantage of liposomes over the others is that they can be effective carriers of both hydrophilic and hydrophobic molecules such as antigens and immunopotentiators. Undoubtedly, Alum and emulsions adjuvants represent key benchmarks for adjuvant development since they have been administrated in millions of doses worldwide. Aluminum adjuvants act primarily to increase antibody production and are therefore suitable for vaccines targeting pathogens killed primarily by antibodies. However, there are diseases such as malaria, tuberculosis, and HIV where more potent Th1 cellular immune responses are required and cannot be achieved by Alum. Oil-in-water emulsions have an essential role in the

<table>
<thead>
<tr>
<th>Feature/Type of nanoparticle</th>
<th>Liposome</th>
<th>Lipid nanoparticle</th>
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<tbody>
<tr>
<td>Easy surface modification</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Loading of hydrophilic and hydrophobic molecules</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Biocompatible and biodegradable</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Improve stability of therapeutic agents</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Controlled drug release</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Large scale production and sterilisation</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Manufacturing cost</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Physical and biological stability</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Avoidance of organic solvents during manufacturing when desired</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Drug leakage</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Toxicity</td>
<td>+</td>
<td>+</td>
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</tbody>
</table>
development of flu vaccines. Their adjuvant action is strongly correlated with a significant increase of humoral immune responses. Use of emulsions as carriers of multiple immunopotentiators has not always been the most preferable approach. The adjuvant activity of adjuvant system AS01 and AS02 was compared in a human malaria challenge study. Both adjuvant candidates contained the same immunopotentiators, formulated in a liposome or emulsion form for AS01 and AS02, respectively. The vaccine antigen, RTS,S induced higher cell-mediated immune responses and appeared to induce greater clinical protection with AS01 than with AS02. Therefore, in this situation, the delivery of both immunopotentiators in liposome was necessary for achieving synergistic adjuvant activity of the immunopotentiators.

Liposomes as immunological adjuvants were introduced by Gregoriadis and Allison in 1974. During their studies, they observed that negatively charged liposomes containing dicetyl phosphate (DCP) could promote robust immune responses against diphtheria toxoid. One of the primary advantages of liposomes compared to Alum and emulsions is plasticity. Liposome surfaces can be modified with appropriate ligands for altering their immunological profile. In contrast, emulsions are very fragile and have limited capacity to allow surface modifications. Moreover, liposome physicochemical properties such as size, surface charge, and lipid composition can be customized toward desired immune profiles, by choosing also their preparation method.

Liposome vaccine adjuvant activity is based on their ability to attract, interact, and activate APCs (e.g., dendritic cells (DCs), macrophages, and B cells) based on their physicochemical (size and charge) and immunogenic properties (incorporation of other adjuvants and targeting ligands). For instance, the positively charged surface of cationic liposomes favors interactions with the negatively charged surface of DCs, facilitating antigen delivery and uptake. DCs are of major importance as they are among the main inducers of T cell-mediated immune responses through major histocompatibility complex (MHC)-mediated antigen presentation to the T cell receptors (TCRs). Modified liposomes with appropriate targeting ligands can lead to stimulation and activation of cells through PRRs, which results in maturation of APCs (co-stimulatory molecules and cytokine signals) and antigen processing and presentation (Figure 2). These signals provided by APCs determine the T and B cell polarization to ensure that they act appropriately against the pathogen.

AS01 is an example of a liposome-based vaccine adjuvant system containing two immunostimulants: monophosphoryl lipid A (MPL) and the saponin QS-21. MPL is the detoxified derivative of Salmonella Minnesota lipopolysaccharide and stimulates activation of innate immunity via TLR4. QS-21 is a saponin extracted from the bark of the South American tree Quillaja saponaria Molina, fraction 21. QS-21 promotes antigen-specific antibody responses but also stimulates potent T cell responses, most notably in non-human species. Liposomes allowed the co-delivery of the MPL and QS-21 to the same immune cell populations in the draining lymph node, enabling their synergistic action, thus promoting vaccine immunogenicity. Another saponin and lipid-based adjuvant is ISCOMATRIX, which consists of cage-like structures of phospholipid, saponin, and cholesterol. Its adjuvancy is based on its ability to promote cross-presentation of antigens, epitope spreading, and antibody affinity maturation.

Figure 2. Induction of humoral and cellular immunity by liposomal delivery. Liposomes are taken up by immature APCs and present antigen by MHCII to naïve CD4+ T cells. CD4+ T cells become activated and proliferate to Th1 and Th2 subtypes. CD4+ T cells activate B cells through IL-4, IL-5, and IL-10 to produce antibodies against the antigen. Antigens can also be found in the cytosol of DC, which allows them to be presented by MHC I, directly activating cytotoxic T lymphocytes. In this case, Th1 cells produce IFN-γ and IL-2, which favor cellular activation and hence cytotoxic T cell responses (cellular response).
4. COMBINATORIAL ADJUVANT STRATEGIES

Adjuvants can be derived from many types of molecules including vitamins, carbohydrates, peptides, proteins, antibodies, aptamers, and enzymes. The loading mode and the physicochemical properties of liposomes such as surface charge, membrane fluidity, and size (reviewed extensively elsewhere) are of major importance as they influence the efficiency of the system. The approach to be employed depends on the nature of the molecules, the physicochemical properties of liposomes, and the final application of the system. A combination of surface functionalization and modification techniques has been used to formulate nanoscale multifunctional liposomal formulations including simple coadministration, encapsulation, surface decoration through electrostatic complexation or covalent conjugation (Figure 3).

Examples of various adjuvants/ligands–liposomes combinations are presented in Table 3.

![Figure 3](image-url)  
**Figure 3.** Different approaches can be utilized for the incorporation of antigens, adjuvants, and targeting ligands into/onto liposomes. (A) Encapsulation: hydrophilic molecules such as proteins, antigens, DNA, and RNA can be added into the aqueous core of the liposomes during manufacturing. Hydrophobic molecules can be incorporated into the lipid bilayer. (B) Electrostatic binding: different targeting moieties and antigens can electrostatically bind the oppositely charged lipids on liposomes surface. (C) Conjugation: targeting ligands as proteins, peptides, antibodies, and small molecules can be linked on the surface of preformed liposomes by covalent conjugation to functionalized lipid anchors for targeted delivery.

4.1. Coadministration of Lipid-Based Nanoparticles, Antigens, and Other Adjuvants. A simple physical mixture of antigen with preformed liposomes is a significantly more convenient approach compared to alternatives. Although there are concerns that simple mixing of adjuvants with antigens may result in their dissociation after entering the body, this method has been successful in many cases, including for marketed products. This approach has been applied in many human vaccines containing mixture of protein antigens with aluminum hydroxide or aluminum phosphate in suspensions. After mixing, rapid interaction between the aluminum and various groups on antigens led to their association and adsorption. The simple mixing approach is used in the Mosquirix and Shingrix vaccines against malaria and shingles, respectively, where the AS01 adjuvant is mixed with the antigen prior to administration.

Thoryk et al. explored the requirements for administering vaccine formulations containing LNPs as adjuvants with the recombinant antigen hepatitis B virus surface antigen (HBsAg) and ovalbumin (OVA). They observed that the coadministration of LNPs with antigens at the same injection site and the same time can boost the antigen specific B-cell and T-cell immune responses. Notably, the immune responses achieved with LNPs were higher than those elicited by aluminum-based adjuvant. The coadministration of HBsAg antigen with LNPs resulted in high levels of antigen specific CD4+ and CD8+ T-cell responses, which were 3- to 5-fold higher than that those induced when HBsAg was coadministered with aluminum adjuvant. Similar results were obtained with OVA. When examining the effect of combining LNPs and aluminum in a single formulation, they observed an enhanced B-cell response but a reduced T-cell response.

Similar studies by Swaminathan et al. demonstrated that LNPs coadministered with a synthetic TLR9 agonist, immune-modulatory oligonucleotides, IMO-2125 (IMO), significantly enhanced immune responses to HBsAg and OVA in comparison to IMO alone.

Previous work by Yanasarn et al. showed that protein antigens admixed with DOPA-based negatively charged liposomes induced strong, functional antibody responses. In addition, antigen-specific CTL responses were obtained, which prevented the growth of antigen-expressing tumor cells in mice. They suggested that the adjuvanticity of negative liposomes may be related to their ability to up-regulate the expression of molecules involved in the activation and maturation of APCs and to slightly facilitate the uptake of the antigens by APCs.

4.2. Encapsulation. Encapsulation into liposomes can be applied on all molecules regardless of their degree of hydrophobicity. However, the encapsulation method and the degree of encapsulation strongly depend on the physicochemical properties of the molecules in question (polarity, partition coefficient). Hydrophobic compounds can be easily incorporated into liposomes during the manufacturing process, with high encapsulation efficiency. The lipophilic nature of these molecules favors their direct incorporation into the lipid bilayer, by addition into the dissolved lipid organic phase. On the other hand, hydrophilic compounds such as nucleic acids, antigens, and peptides can be added into the hydrophilic core of liposomes during manufacturing. When the thin film hydration method is used, the molecule to be entrapped can be included in the aqueous media (for hydrophilic molecules) or in the lipid film (for lipophilic molecules). When a microfluidic device is used, hydrophilic adjuvants can be dissolved in the aqueous phase and lipids into the organic phase. After simultaneous injection of both streams into the chip, liposomes are formed with hydrophilic compounds in the aqueous interior.

There are conflicting results related to whether the antigen encapsulation offers significant benefits in a vaccine formulation. There are studies demonstrating that the antigen location (entrapment vs surface adsorption) does not impact vaccine biodistribution, APCs recruitment, and antibody response. On the other hand, it has been reported that antigen location can result in different outcomes of the immune response. For instance, Barnier-Quer et al. proved that adhesion of influenza hemagglutinin (HA) on the surface of cationic liposomes induced slightly higher antibody responses than encapsulation of HA within the same carrier, due perhaps to the immediate availability of the surface-exposed antigen for
B cell recognition and stimulation, while a disruption of vehicle is needed when antigen is encapsulated. Studies also linked the induction of cell-mediated immune responses with the antigen loading mode. For applications related to mucosal administration and tumor-targeting, where anionic or neutral liposomes are preferred, encapsulation may be the chosen method for antigen loading due to absence of strong electrostatic forces between negatively charged antigens and liposomes, which does not favor the strong surface binding. In contrast, cationic liposomes can be used for encapsulation and/or adsorption, which may be ideal for initial and prolonged exposure of antigen.

Hence, antigen location may be an important factor in modulating the immune responses of antigens delivered by nanoparticles, yet it remains unclear if it is absolute requirement, since in some cases adjuvant delivered in particles mixed with soluble antigen has been shown to be effective in driving strong immune responses. Additional factors are the route of administration and the physicochemical properties of the formulation, which may also contribute to the immune outcome.

One of the promising areas for TLR mediated therapeutics is to utilize them as vaccine adjuvants where there are no available vaccines (including viral, bacterial, and parasitic infections). Although TLRs combinations are proven to be effective for enhanced immunity, they usually suffer from instability. One strategy is offered by the formulation of TLRs agonists in a stable form within delivery systems to improve their bioavailability and bioactivity. TLRs agonists have been encapsulated into liposomes and have demonstrated enhanced immunostimulatory activity. In recent years, different combinations of adjuvants have been explored for testing their potential for synergistic enhancement of the immune response when administered within liposomes.

Bayyurt et al. coencapsulated two nucleic-based TLRs agonists, the TLR3 polynosinic:polycytidylic acid (poly(I:C)) and TLR9 cytosine-phosphorothioate-guanine oligodeoxynucleotides (CpG) along with OVA protein antigen into DSPC:cholesterol neutral liposomes to evaluate potential synergistic immune activity. They demonstrated that combinations of TLRs into the same particle achieved simultaneous antigen and adjuvant delivery to APCs and triggered long-lasting, sustained humoral and cellular anti-OVA specific immunity. Notably, the coencapsulated group yielded more pronounced Th1-biased anti-ovalbumin immunity with 2-fold less antigen compared to a separately encapsulated liposome formulation. Immune responses were sustained and successfully protected immunized mice from tumor development. Andrew et al. investigated the effect of coencapsulation of TLR9 ligand CpG with microbial adjuvant listeriolysin O (LLO) and model antigen OVA into pH-sensitive liposomes consisting of L-α-phosphatidylethanolamine (egg DSPE) and cholesteryl hemisuccinate (CHEMS). Combination of adjuvants in liposomes demonstrated higher CTL response, enhanced number of CD4+ and CD8+ IFN-γ secreting T cells, as well as an increased Th1-type antibody response compared with immune responses obtained with OVA-containing LLO-liposomes.

In another study, anionic liposomes consisting of dipalmitoylphosphatidylcholine (DPPC), 1,2-dipalmitoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (DPPG), and cholesterol were selected for combining the TLR4 agonist glucopyranosyl lipid adjuvant (GLA) and the TLR7 agonist imiquimod (IMQ). TLR7 ligand was in the interior of the liposome and the TLR4 ligand intercalated into the lipid bilayer. Administration of the combined system with the recombinant malaria antigen, PbCSP, demonstrated that the synergistic adjuvant was able to induce higher Th1-biased immune responses compared to immunity obtained with each TLR agonist alone. Combination of TLRs ligands led to the simultaneous triggering of two innate sensors, resulting in an enhanced immune response. Similar conclusions were reached by Rueda and colleagues, who reported that incorporation of different adjuvants into liposomes activated different pathways, inducing a more potent immune response compared to the use of single TLR agonist. They demonstrated that Fc receptor-targeted liposomes, coencapsulating the TLR7/8 R848, TLR3 poly(I:C), TLR4 lipopolysaccharide (LPS) along with a multiepitope antigen, induced higher immune responses than two or one TLR agonist. Inclusion of the three distinct immunostimulatory...
TLR ligands adjuvants into nontargeted liposomes demonstrated higher efficiency in comparison to the Fc-targeted liposomes containing only one TLR.\textsuperscript{129}

4.3. Surface Modifications. Incorporation of adjuvants on the surface of liposomes can be achieved through electrostatic interactions or covalent attachment to lipid anchor or to PEG. An important aspect in tailoring a liposomal system is the optimization of ligand density on the liposomes surface. Previous studies have shown that cellular uptake is increased with an increase of ligand density on the surface of particles.\textsuperscript{160–164} However, issues such as aggregation and suppressed therapeutic effect may result from increasing ligand density beyond an optimum level.\textsuperscript{165}

4.3.1. Electrostatic Binding. For surface incorporation through electrostatic binding, opposite electric charges are required between the liposomes and adjuvants/antigens. The strength of electrostatic forces between them determines the degree of adsorption. Regarding the antigens, protein antigens with isoelectric points (pIs) below 7.4 are negatively charged; thus they adsorb onto cationic liposomes at physiological pH.\textsuperscript{166} Cationic liposomes have been demonstrated to induce a stronger immunogenicity than that of neutral and anionic liposomes.\textsuperscript{167} It is reported that chemokine induction is involved in the strong adjuvanticity of cationic liposomes. Comparative studies between neutral, negative, and cationic charged liposomes by Yan et al. revealed that only cationic liposomes were able to up-regulate the CCL2 chemokine gene upon stimulation of DC, and this induction was mediated through the ERK pathway both \textit{in vitro} and \textit{in vivo}.\textsuperscript{168}

The superiority of cationic liposomes could also be attributable to their capability to adsorb negatively charged antigens, thus improving the antigen presentation to APCs and providing a depot effect at the site of injection,\textsuperscript{170,169} followed by a sustained release to the draining lymph node. Additionally, cationic liposomes interact with negatively charged macromolecules in plasma causing \textit{in vivo} aggregation, which limits their drainage from tissues.\textsuperscript{171} Studies by Henriksen-Lacey et al. investigated the importance of antigen–liposome interactions on the antigen depot effect and immunogenicity. Their biodistribution studies demonstrated that more than 50% of the initially coadministered Ag85B-ESAT-6 antigen dose was detected when administered with DDA-based cationic liposomes, whereas only 11% of the initial antigen dose was detected when administered with neutral liposomes. Notably, more than 80% of the initial DSPC: TDB dose was still present at the SOI at this time point, suggesting that antigen adsorption to the liposomes is a prerequisite for antigen retention at the SOI, with poorly adsorbed antigen behaving as free antigen, draining rapidly.\textsuperscript{170,172} Further studies from the same group revealed that DDA:TDB liposomes ensured a more prolonged antigen presentation compared to neutral DSPC:TDB liposomes, which translated into higher Th1 (IFN-γ) and Th17 (IL-17) immune responses.\textsuperscript{170} Nucleic acids are also negatively charged (e.g., TLR3 agonist poly(I:C) which is a synthetic analogue of dsRNA, TLR9 agonist CpG which is synthetic DNA). Their hydrophilic nature, due to the negatively charged phosphate groups, is an obstacle to their cellular uptake, as they cannot cross the lipophilic and negatively charged membranes, due to the charge repulsion.\textsuperscript{173} Thus, cationic liposomes have been explored as a delivery system to facilitate their entry into cells.

Nucleic acids electrostatically bind to liposome surfaces, forming stable lipoplexes that facilitate their uptake into cells through endocytosis. Cationic lipids neutralize the anionic nucleic acid molecules, enabling their delivery across the cellular membrane. The cationic charges also facilitate the interaction and escape of nucleic acid-loaded liposomes to the cytoplasm.\textsuperscript{185} Studies demonstrated that nucleic acid-based adjuvants bound on cationic liposomes are more potent than nucleic acids or liposomes alone. The nanoparticulate presentation protects the adjuvants from extracellular degradation and enhances their entry into the endosomal compartment, where TLR3 and TLR9 are expressed, thereby enhancing vaccine immunity.\textsuperscript{133,135,137,140,141}

Hansen et al. explored the effect of adsorption of TLR3 agonist poly(I:C) on cationic adjuvant formulation (CAF01). CAF01 is a liposomal adjuvant composed of a DDA-based cationic lipid-synthetic analogue of dsRNA, TLR9 agonist CpG, and liposomes.\textsuperscript{138} CAF01 enhanced the antigen-specific T cell responses that reduce tumor burden in mice. Also, formulating poly(I:C) with CAF01 abrogated a systemic inflammatory response to poly(I:C), with no systemic reactions. It is speculated that the electrostatic interactions between poly(I:C) and CAF01 prevent poly(I:C) from being released upon injection, but instead it is taken up by relatively few immune cells as a complex of lipids, innate receptor ligand TDB, poly(I:C), and antigen.\textsuperscript{139}

In a recent study, we demonstrated that decoration of liposomes with GBS67–CpG conjugate can greatly enhance protein immunogenicity. In particular, the group B Streptococcus (GBS) 67 antigen conjugated with CpG using maleimide chemistry was most potent. Conjugation can impair protein epitopes in some cases; on the other hand chemical ligation between antigen and TLR9 agonist CpG has been proven to be essential for achieving strong immunopotency.\textsuperscript{174–176} Protein–CpG conjugates were adsorbed on the surface of DSPC:cholesterol:DDA cationic liposomes to a very high degree (>90%).\textsuperscript{177} Following intramuscular immunization, surface-decorated cationic liposomes with GBS67–CpG formed a vaccine depot at the injection site and induced a significant increase of functional immune responses against GBS compared to the simple coadministration of GBS67, CpG, and liposomes.\textsuperscript{138}

4.3.2. Covalent Attachment. Adjuvants can also be covalently attached on liposome surfaces. Molecules can be functionalized with various targeting ligands based on three kinds of reaction, i.e., formation of amide bond between carboxyl and amino groups; disulfide bond formation by reaction of pyridyldithiols and thiol group; and thioethers bond formation by reaction of maleimide and thiol groups.\textsuperscript{170} Glycosylation of liposomes through carbohydrate (e.g., mannose, glucose) insertion has proven to be important for the design of advanced lipid-based formulations. Many human cells possess different glycoproteins and glycolipid receptors, which recognize bacterial glycans including mannose on the cell walls of infectious agents (bacteria, fungi, viruses, etc.). Therefore, the presence of carbohydrate moieties on liposomes surface can increase their selectivity by binding to glycoprotein or glycolipid receptors, resulting in efficient delivery of...
therapeutic agents. Carbohydrate-based structures when used as part of the liposome formulation can interact with lectins. Carbohydrate–lectin interactions have been exploited for the design of drug delivery systems.

The mannose receptor (CD206) is a CLR expressed by most tissue macrophages, DCs, and specific lymphatic or endothelial cells. The targeting of this receptor is a viable and attractive strategy for the delivery of carbohydrate-containing imaging/diagnostic agents as well as the intracellular delivery of therapeutics for many infectious diseases, such as tuberculosis, pneumonia, HIV, and influenza. DC-SIGN and Langerin receptor (CD207) are two other mannose-binding CLRs. Langerin receptor is mostly expressed on Langerhans cells (LC), which populate the skin epidermis and all stratified epithelia. It is also found on dermal DCs and resident DCs in the skin-draining lymph nodes. The DC-SIGN receptor, on the other hand, is highly expressed on immature conventional DCs, particularly on the mucosa and the dermis. Therefore, CLR targeting is seen as a way to increase vaccine delivery particularly via intradermal administration, aiding the development of needle free delivery systems (such as patches or similar).

Mannose receptor-mediated targeting has already been studied for DNA and RNA delivery in antitumor immunotherapy, in the formulation of an HIV DNA vaccine, and also for the delivery of self-amplifying mRNA (SAM) vaccines. Mannose-coated particles were obtained by modifying the zwitterionic lipid components, polyethylene glycol (PEG), via ester bonds with cholesterol or via conjugation to cholesterol amine. Although mannose is already sufficient to trigger the DC-SIGN receptor and favor uptake from DC, we have recently observed that balance between oligomannose length and amount of PEG used for liposome stabilization (see next paragraph) can be exploited to further enhance the immunogenicity of SAM vaccines. These mannosylated liposomes hold the promise to be suitable for the development of skin delivery systems. Wamhoff et al. described the design of liposome formulations covered with a glycosimetic ligand for the lectin receptor Langerin, to selectively target Langerhans cells, for the development of novel vaccination strategies. They demonstrated for the first time the CLRs-mediated targeting of nanoparticles to individual immune cell subsets using glycomimetics.

Mannosylated liposomes have been combined with CpG in an effort to design a novel liposomal adjuvant system. Kuramoto et al. reported higher production of IL-12 and IFN-γ after intravenous administration of mannosylated cationic liposome/CpG complex in mice, in comparison to those obtained with bare CpG/cationic liposomes lipoplex or naked CpG. Conjugation of hydrophilic compounds (e.g., CpG, resiquimod) with a lipid allows their direct incorporation onto the lipid bilayer. Lai et al. designed a new liposome platform by combining mannose and CpG on the surface of liposomes through their lipidation. Mannosylated-CpG liposomes showed superior ability to stimulate the activation of DCs over the use of mannosylated liposomes. Assessment of antitumor activity of the system in a B16 melanoma model demonstrated a systemic up-regulation of antitumor immunity and down-regulation of immunosuppression, leading to significant tumor growth inhibition.

However, enhanced immunogenicity by adjuvant combinations depends on the approach used but most importantly on the adjuvant selection, as certain adjuvants may not work synergistically. For instance, Wilkinson et al. tested the effect of DSPE lipid-TLR7/8 agonist resiquimod conjugate incorporated into DDA:TDB (CAF01) cationic liposomes. Their studies demonstrated that conjugation ensured the presence of resiquimod with DDA:TDB liposomes in contrast with simple mixing, but no notable benefit on the immunogenicity was obtained compared to a physical mixture. Although no clear evidence was provided to support the adoption of the resiquimod conjugated formulation over the simple CAF01 cationic liposome formulation, lipid tailoring of resiquimod and its analogues shows advanced adjuvant effects compared to their free forms when liposome formulations were not used. In contrast, studies by Chen and Huang demonstrated that palmitoylation of a human papilloma virus E7 peptide at the N-terminal modulated its immunogenicity. Palmitoylation increased E7-specific CTL responses 2-fold over unconjugated peptide. Lipidation of proteins and peptides has been explored for adjuvant development with several examples reported recently. Lipidated forms of proteins and peptides were demonstrated to be more immunogenic than nonlipidated forms. One example is the Neisseria meningitis group B vaccine Trumenba, which features two recombinant lipoprotein antigens, each incorporating an N-terminal lipid moiety with TLR2 agonist activity. This activity was lost with removal of the O-linked fatty acids, similar to removal of all lipids, demonstrating that lipid moiety plays an adjuvant role.

Coupling of TLR9 agonist CpG or TLR7/8 agonist 3M012 with ferritin-hemagglutinin (HA) particle elicited stronger antibody responses than matched-dose mixtures or unconjugated ferritin-HA alone. Interestingly, ferritin-HA-3M012 conjugate and ferritin-HA-3M012 mixture achieved the same immunogenicity, with the conjugate containing 500-fold less 3M012 than the mixture. The superiority of conjugation over the mixture is attributed to the more precise and powerful stimulation effect of the adjuvant.

4.3.3. Pegylation. A challenge often linked with the use of liposomes is their short circulation time when administered intravenously. In order to overcome this issue, liposomes are often modified with PEG hydrophilic polymer moieties. Pegylated liposomes (or “stealth” liposomes) were found to be more stable than nonpegylated liposomes, as pegylation hinders the adsorption of protein opsonins onto liposome surfaces, which results in increased clearance of liposomes by the mononuclear phagocytic cells in the liver and spleen.

For the case of cationic liposomes where the depot effect is involved in their immunostimulatory mechanism, pegylation may not be beneficial, depending on the PEG density and length used, as higher PEG concentrations can lead to adverse effects. Work by Kaur and others demonstrated that pegylation of DDA:TDB cationic liposomes resulted in masking of the cationic charge and subsequently reduction of antigen adsorption on the liposome surface. More importantly, high levels of PEG (25%) led to the faster drainage of liposomes from the injection site, thus blocking the depot effect and reducing the Th1-driven immune response. Similar conclusions were reached by Roces et al., who investigated the effect of biotin–avidin complex on the retention of DDA:TDB liposomes in the lymphatics and the induced immune responses. It had been previously demonstrated that biotin–avidin complexes can improve the accumulation and retention of liposomes into the draining
lymph nodes. For the formulation of biotinylated liposomes, DSPE-PEG2000-biotin (20%) was added into the lipid mixture prior to preparation. HS6 tuberculosis antigen was adsorbed on the liposome surface (>90%), and avidin was intramuscularly injected 2 h prior to immunization. Their experiments demonstrated that the formation of avidin–biotin-coated liposome complexes resulted in higher accumulation of liposomes and their associated antigen in the draining lymph nodes, compared to DDA:TDB liposomes, followed by reduction of Th1-driven immune response. Thus, the presence of PEG in the formulation not only controlled the biodistribution of the vaccine but also switched the type of T cell responses from a Th1 toward a Th2.

On the other hand, it has been demonstrated that an optimized degree of pegylation may reduce depot formation, enhance passive drainage to the lymph nodes, and eventually improve immunogenicity. In general, liposomes with a brush-like coverage (5−15% PEG) and with a molecular mass of 2000 Da have a more stable steric barrier and prolonged circulation time. Studies by Schmidt et al. demonstrated that pegylation (10%) of CAF09 (DDA:monomycoloyl glycerol (MMG) analogue 1/poly(1:C)) with DSPE-PEG2000 resulted in drainage of a larger dose fraction to the lymph nodes, followed by an induction of stronger CD8+ T cell responses in the blood upon subcutaneous immunization, as compared to nonpegylated CAF09. Goswami et al. observed that reduction of PEG from 2% to 0.3% resulted in increased mannose exposure on the LNPs but also resulted in a lower SAM encapsulation efficiency. Thus, although pegylation increases liposome circulation time, the immunological profile of these particles needs to be balanced relative to the ligand/PEG content in order to achieve particle stability, high encapsulation, biodistribution, and presentation of attached ligands.

Additionally, the free end of PEG can be harnessed for chemical ligations with different targeting ligands, such as peptides, proteins, antibodies, carbohydrates, and polysaccharides. The interaction between ligands on nanoparticles and the cellular receptors induced receptor-mediated endocytosis, which allows internalized nanoparticles to successfully release the therapeutic material. Zhao et al. formulated SPC/Cholesterol/DOTAP/DSPE-PEG2000-mannose liposomes (Lip E7/CpG) for the delivery of CpG TLR9 agonist and HPV16 E7 peptide. Mannose was conjugated to the free end of DSPE-PEG2000 prior to liposome formation. CpG and E7 peptide were encapsulated into liposomes. Evaluation of the therapeutic efficacy on large (volume over than 200 mm³) TC-1 grafted tumors revealed that Lip E7/CpG was able to induce significant tumor inhibition, compared to the empty liposomes and free E7/CpG. The antitumor effect was a result of enhanced CD4+ and CD8+ T cells and IFN-γ-producing cells in spleens and tumors and enhanced HPV-specific CTL responses.

Table 4 summarizes the advantages and disadvantages of all the combinatorial strategies discussed.

### 5. LIPOSOMES FOR POLYSACCHARIDE DELIVERY

Liposomes have also been considered for the development of glycoconjugate vaccines. Glycoconjugate vaccines aim to improve the immunogenicity of polysaccharides antigens by the addition of protein antigens in an effort to provide B and T cell epitopes to increase the vaccine performance. Recently, Jones et al. proposed a faster approach to glycoconjugates production designing a dual-functioning liposomal formulation. In particular, polysaccharides from *Streptococcus pneumoniae* have been encapsulated into liposomes while the protein antigen is surface exposed on the liposomal surface. Each antigen complements each other as it is associated with a separate progression state of the disease. The end result is a glycoconjugate mimic that offers a simpler, noncovalent form of polysaccharide–protein colocalization and scalable route to full serotype vaccine coverage.

In a different study, Li et al. used liposomes for the co-delivery of 20 *Streptococcus pneumoniae* polysaccharides and two pneumococcal proteins antigens, GIP0 (an α-glycosylphosphate oxidase) and PncO (a bacteriocin ABC transporter transmembrane protein), both of which have been shown to provide protection in sepsis and pneumonia challenge models. Protein antigens were used as His Tagged antigens to decorate the surface of liposomes through a modified lipid component DOGS-NTA-Ni. *Streptococcus pneumoniae* polysaccharides were encapsulated into liposomes. Evaluation of the system in animal models of sepsis and pneumonia demonstrated a broad vaccine coverage against 70 serotypes of *Streptococcus pneumoniae*, some of which are not covered by the currently available pneumococcal vaccines. The same group introduced the next generation of glycoconjugate-like *Streptococcus pneumoniae* vaccine comprising 24 different serotypes encapsulated in liposomes which have been surface engineered only with PncO protein. They demonstrated that the novel vaccine formulation induced significant titers of CPS antibodies for all serotypes with comparable performance to PCV13 and PPSV23, the two current licensed pneumococcal vaccines. A similar system was designed using alternative noncovalent attachment methods for carrier or antigen proteins, such as the streptavidin–biotin-based strategy without losing its potency highlighting the adaptability of the designed system.

Said Hassane et al. designed liposome diepitope constructs composed of synthetic oligosaccharides mimicking the O-antigen of the *Shigella flexneri* 2a lipopolysaccharide (B-cell epitope) and influenza hemagglutinin peptide HA 307–319 (Th epitope). Surface decoration of liposomes with B and T
cell epitopes was achieved through coupling to the TLR2 agonist Pam3CAG which was incorporated in liposomes during preparation. In mice, these synthetic liposomes were shown to elicit antibody responses against the native lipopolysaccharide and protected mice against *Shigella flexneri* 2a challenge.223

6. LIPID-BASED PARTICLES FOR MRNA DELIVERY

In recent years, mRNA (mRNA)-based therapeutics have arisen as a very promising class of biologics. RNA technology can be used for the expression of any protein of interest directly in vivo, resulting in induction of both humoral and cytotoxic T cell responses. Additionally, mRNA can be recognized by mammalian cells through different PRRs (e.g., TLR3, TLR7, TLR8) giving to mRNA adjuvant properties.225 Since RNA is naturally unstable, delivery systems have been employed for its protection and delivery to targeted sites. Several classes of lipid-based nanocarriers have been studied for RNA delivery, the most notable ones being liposomes, lipid-based nanoparticles, and lipid nanoemulsions. Special focus has been given on LNPs containing ionizable cationic lipids as they have small size, serum stability, low surface ζ potentials at physiological pH, and cationic charge at acidic pH values (e.g., in endosomes) leading to improved RNA encapsulation efficiency due to electrostatic interactions.226–228

6.1. mRNA Vaccines. In recent years, several mRNA vaccines (Figure 4) entered clinical studies (reviewed else-

Figure 4. Schematic representation of RNA vaccines. Synthetic RNA constructs encoding viral proteins are packaged in LNPs that serve as delivery vehicles protecting them from degradation and promoting cellular uptake (1). LNPs encapsulating RNA are taken up by the cells through endocytosis (2). Endosomal escape allows release of the RNA into the cytosol (3). Constructs are immediately translated by ribosomes to produce the protein of interest (4), which undergoes subsequent post-translational modification and protein processing for MHC presentation (5).

<table>
<thead>
<tr>
<th>Table 5. Composition of LNPs in Licensed COVID-19 Vaccines</th>
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<tr>
<td>vaccine</td>
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<tr>
<td>BNT162b2</td>
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<td>mRNA-1273</td>
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4All ALC-0159, 2-[(polyethylene glycol)-2000]-N,N-ditetradecyldiamine; ALC-0315, (4-hydroxybutyl)azaendiylibis(hexane-6,1-diylibis(2-hexyldекanoate); DSPC, 1,2-distearoyl-sn-glycero-3-phosphocholine; PEG2000-DMG, 1,2-dimyristoyl-rac-glycero-3-methoxy(polyethylene glycol) 2000; SM-102, (heptadecan-9-yl 8-[(2-hydroxyethyl)(6-oxo-6-(undecyloxy)hexyl)amino]octanoate).
Along with these vaccines, other mRNA based candidates are under clinical trials and numerous are also at the preclinical stage. Examples are the CVnCoV and LUNAR-COV19/ARCT-021 developed by CureVac and Arcturus Therapeutics/Duke—NUS Medical School, respectively. CVnCoV comprises LNP-formulated, nonchemically modified, sequence engineered mRNA encoding full-length spike protein. CVnCoV is currently being studied in a phase 3 clinical trial. LUNAR-COV19/ARCT-021 is a self-transcribing and replicating RNA (STARR)-based vaccine encoding an alphavirus-based replicon and the SARS-CoV-2 full-length spike glycoprotein. STARR combines self-replicating RNA with LUNAR technology which is a lipid-mediated delivery system. This candidate is currently in phase 2 clinical trials. Although full data related to exact formulation composition of CVnCoV and LUNAR-COV19/ARCT-021 have not yet been disclosed, LNPs consist of DSPC, cholesterol, PEGylated lipid, and a cationic lipid. Preliminary data from both candidates are very promising demonstrating high tolerability and safety profile.

In addition to the wild type and prefusion stabilized mutant of the S-protein, a furin cleavage-site mutant (GSAS) and a double mutant form (2P/GSAS) have been recently tested in animal models for their capacity to elicit neutralizing antibodies (nAbs). The lead 2P/GSAS candidate was further assessed in dose-ranging studies in mice and cynomolgus macaques and for efficacy in a Syrian golden hamster model. Similar to the clinically tested vaccines, these preclinical candidates were based on classic lipid nanoparticles composed of ionizable lipids, phosphatidylethanolamine, cholesterol, and PEG.

Noteworthy, the exacerbating effect of some antiviral vaccines (e.g. dengue and RV) toward the disease, known as antibody-dependent enhancement, has been observed causing the failure of clinical trials. There is debate among the scientific community whether similar events could be caused by vaccination to fight SARS-CoV-2. While the large use of the RNA vaccines in the population has so far been shown to be safe and well tolerated, attention must be paid to longer term safety associated with this vaccination in different age groups. Also, there is growing interest in understanding if differences achieved in the observed protection of the mRNA vaccines depending on males and females could be related not only to divergent pathogenic mechanisms but also to the diverse metabolic processing of vaccine components, including liposomal ingredients.

6.2. mRNA Therapeutics. Although current clinical efforts to use mRNA as a drug are mainly situated at the level of prophylactic and therapeutic vaccines (reviewed extensively elsewhere), recent preclinical studies have addressed the feasibility of using mRNA to encode the production of therapeutic antibodies directly in vivo. In 2008, Hoerr et al. first introduced the concept of using mRNA encoding for antibodies. Later in 2017, Pardi et al. reported the success of using mRNA encoding antibodies formulated in LNP. In these studies, the light and heavy chains of VRCo1, a neutralizing antibody against HIV-1, were used. A single dose of 30 μg of mRNA-LNP encoding the VRCo1 Ab induced high levels of functional antibodies able to protect humanized mice from HIV-1 challenge. mRNA-LNPs encoding VRCo1 demonstrated superiority over the recombinant purified protein VRCo1 mAb (2-fold higher antibody serum titers than 600 μg of the corresponding recombinant protein). In subsequent studies by Thran et al. the same concept was repeated using a rabies, a botulism, and a lymphoma model. Single injections of mRNA—LNP were sufficient to establish rapid, strong, and long-lasting functional antibody titers leading to full protection against virus challenge or intoxication and eradication of neoplastic cells in murine models, with no toxicity observed. Similarly, Kose et al. noted high antibody expression levels in mice after intravenous administration of lipid nanoparticles with encapsulated mRNA encoding antibody against chikungunya, a mosquito-transmitted virus that causes systemic infection in humans, which is characterized by acute onset of fever and severe polyarthralgia. Antibodies were able to protect mice against viral infection and virus-associated arthritis and also induced protective concentrations of serum antibody in macaques.

A recent study by Van Hoecke et al. reported the local delivery of mRNA encoding a bispacific single-domain antibody format in the lungs using cationic liposomes. Particularly, they developed N1-methylpseudouridine-containing mRNA encoding His-tagged bispacific VH (RiboBiFE; bispacific Fc-receptor engaging) of which one part is directed against influenza A M2e (matrix protein 2 ectodomain) and the other part against the mouse FcγRIV. A liposome formulation composed of DOTAP and cholesterol was used as the delivery system for the pulmonary delivery of the RiboBiFE constructs. After intratracheal administration in mice, an expression of the RiboBiFE in the lungs was observed, which was detectable for at least 2 days. In addition, a single dose of mRNA-nanoparticles encoding the RiboBiFE was able to protect mice from an influenza A virus challenge.

In 2017, the FDA approved CD19 chimeric antigen receptor (CAR) T cell therapy for the treatment of relapsed or refractory acute lymphoblastic leukemia and large B cell lymphoma. On the basis of these successes, CAR T cell therapy is now being explored for the treatment of several other cancers, including glioblastoma and refractory multiple myeloma. Although viral vectors are currently used for CAR T cell engineering, they suffer from limitations including manufacturing, cost, and in vivo translation. LNP-mediated mRNA delivery has been explored for human CAR T cell therapy in an effort to generate safer, less expensive CAR T cells. Billingsley et al. synthesized a library of 24 ionizable lipids, formulated them into LNPs, and screened them for luciferase mRNA delivery to Jurkat immortalized T cells. This screening revealed seven formulations capable of enhanced mRNA delivery over lipofectamine. Among them, C14-4 formulation showed superiority for its potent transfection and low cytotoxicity. C14-4 LNPs encapsulating CAR mRNA were evaluated for their ability to generate functional CAR T cells demonstrating less T cell toxicity and similar amounts of CAR surface expression in comparison to electroporation-based mRNA delivery, the currently used method for the delivery of mRNA to T cells. In a coculture assay with acute lymphoblastic leukemia cells, LNP-engineered CAR T cells demonstrated the same potent cancer cell killing ability as both electroporation and virally engineered CAR T cells. Thus, LNPs are a promising alternative strategy for mRNA-based ex vivo engineering of CAR T cells.

7. CONCLUSIONS

Vaccines represent a global priority for the improvement of healthcare worldwide, with adjuvants playing a vital role in further vaccine development. Although big advances have

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already been made in vaccine adjuvants, there are still important challenges to address. There is a critical need for the development of adjuvants that can break the immune tolerance and induce strong T cell responses for potential use in cancer vaccines. In addition, adjuvants that are able to induce potent immune responses in immunologically hyporesponsive populations, such as the elderly, immunocompromised, and other chronically sick individuals, are required.\textsuperscript{265} Combinatorial adjuvant strategies, e.g., AS01, have appeared as a viable approach to overcome these obstacles. Vaccine scientists can now choose between a larger panel of compounds and technologies to design and develop formulations to generate potent immune responses, suitable for different pathogens, considering that each antigen–adjuvant combination is unique.\textsuperscript{266}

Lipid-based nanoparticles play an important role in this context as they have intrinsic adjuvant properties. Lipid nanoparticles can be easily decorated with small molecules or glycans targeting the vaccine to subsets of specific immune cells and increasing the engagement of receptors, ultimately enhancing the potency of the immune response. In addition, adjuvants or antigens of different physicochemical parameters can be encapsulated into lipid nanoparticles. Combination of these features renders this class of particles highly valuable tools to deliver a variety of vaccine antigens, including proteins, carbohydrates, and nucleic acids to combat both pathogens and cancer.

Recent licensing of RNA vaccines against COVID-19 has resulted in a further advancement in the field, since LNPs are key to ensure antigen delivery. This class of vaccine is currently under use mainly in adults but has been clinically validated in adolescents,\textsuperscript{267} showing it to be an important weapon among the tools available to fight the ongoing pandemic. Along with vaccine delivery, the use of lipid nanoparticles to deliver antibodies is emerging, further expanding their application from preventive to treatment. Although current clinical studies are mainly focused on the potential of RNA for vaccine development, RNA encoding antibodies hold the promise to overcome obstacles related to production and purification processes as also posttranslational modifications of protein-based antibodies.\textsuperscript{256}

8. FUTURE DIRECTIONS

The future of adjuvant research is heading toward the development of novel combination adjuvants with special focus on the use of PRRs agonists and particulate adjuvants.\textsuperscript{253,268} It is possible to achieve a synergistic immune stimulation by formulating TLRLs in combination or potentially with non-TLRLs, delivered at the target site by nanoparticle formulations. Lipid-based nanoparticles have been demonstrated to be a modulable and flexible platform for the design of multicomponent adjuvant systems.

Nowadays, a range of combinatorial strategies for incorporation of various immunopotentiators, targeting molecules, and antigens are available, leading to the formulation of products with multiple adjuvants/antigens components. Furthermore, an arsenal of strategies for encapsulation or external presentation of different class of antigens (proteins, carbohydrates, nucleic acids) has also been developed. These approaches can be combined in the future to generate novel and more potent vaccines.

Although, some form of association of the antigen with the nanoparticle and other adjuvants could be more beneficial than simple admixing, this may substantially increase the complexity of manufacturing. The challenges become greater when the designed delivery system becomes more complex, with the addition of surface modification, and with coatings and/or ligands. The use of synthetic coatings and ligands may affect the biocompatibility, biodistribution, and toxicity profile of liposomal formulations and will require careful evaluation of the interaction of the nanoparticles with biological tissues and cells.\textsuperscript{269,270} A balance between efficiency, production cost, and simplicity is needed with the cooperation of experts from all the involved fields to advance more efficacious multi-component vaccines.

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All authors contributed equally to the planning, writing, and correction of this review.

Notes

The authors declare the following competing financial interest(s): R.A. and D.T.O. are employees of the GSK group of companies. D.C. was a Ph.D. student at the University of Strathclyde (UK) at the time of the study and supervised by GSK. Epxalax and Inflexal are trademarks of Crucell. Shingrix is a trademark of GSK. Trumemba is a trademark of Pfizer. mRNA-1273 is a trademark by Moderna, and BNT162 is a trademark by BioNTech-Pfizer.

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■ ABBREVIATIONS

ALC-0159, 2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide; ALC-0315, (4-hydroxybutyl)azanediyl)bis(hexane-6,1-diyl)bis(2-hexyldecanoate); Alum, aluminum; APC, antigen presenting cell; AS01, adjuvant system 01; BBB, blood–brain barrier; CAF, cationic adjuvant formulation; CAR, chimeric antigen receptor; CD, cluster of differentiation; CHEMS, cholosteryl hemisuccinate; CLR, C-type lectin receptor; COVID-19, coronavirus disease 2019; CpG, cytosine-phosphorothioate-guanine oligodeoxynucleotide; CPS, capsular polysaccharide; CTL, cytotoxic T lymphocyte; DC, dendritic cell; DCP, dicetyl phosphate; DDA, dimethyl-
dipotadecylammonium; DEX, dexamethasone; DOPA, 1,2-dioleoyl-sn-glycero-3-phospho-L-serine; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; DPPC, dipalmitylphosphatidylcholine; DPPG, 1,2-dipalmityl-sn-glycero-3-phospho-L-rac-glycerol; DSPC, 1,2-distearylsn-glycero-3-phosphocholine; egg DSPE, l-α-phosphatidylethanolamine; DTAp, diphtheria, tetanus, and pertussis; FA, folate; GBS, group B Streptococcus; GA, glycyrrhetinic acid; GLA, glucopyranosyl lipid adjuvant; GNR, gold nanorod; HA, hemagglutinin; HBsAg, hepatitis B virus surface antigen; HIV, human immunodeficiency virus; HPV, human papillomavirus; HSPC, 1,α-phosphatidylcholine; IL, interleukin; IMQ, imiquimod; LC, Langerhans cell; LLO, listeriolysin O; LNP, lipid nanoparticle; LPS, lipopolysaccharide; LUV, large unilamellar vehicle; MCH, major histocompatibility complex; MLV, multilamellar liposomes; MMG, monomonoeycol glycerol; MPL, monophosphoryl lipid A; mRNA, messenger RNA; MUC1, mucin 1; NLR, NOD-like receptor; ODN, oligodeoxynucleotide; OVA, ovalbumin; PAMP, pathogen-associated molecular pattern; PEG, polyethylene glycol; PEG200-DMA, 2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide; PEG2000-DMG, 1,2-dimyristoyl-1α-phosphatidylglycerol-3-methoxypolyethylene glycol-2000; p.i, postinjection; PLGA, poly(D,L-lactic-co-glycolic acid); PNA, peanut agglutinin; poly(I:C), polyinosinic:polycytidylic acid; PRR, pattern recognition receptor; RBD, receptor binding domain; RES, reticuloendothelial system; RLR, RIG-1-like receptor; RSV, respiratory syncytial virus; SAM, self-amplifying mRNA; SM-102, (heptadecan-9-y1 8-(2-hydroxyethyl) (6-oxo-6-(undecyloxy)hexyl)amino)octanoate; SOI, site of injection; SPC, soybean phosphatidylcholine; SUV, small unilamellar vehicle; TB, tuberculosis; TCR, T cell receptor; TDB, trehalose 6,6-dibehenate; Tf, transferrin; TIR, transferrin receptor; TLR, Toll-like receptor; TNF-α, tumor necrosis factor α; ULV, unilamellar liposome; VEGFR 2, vascular endothelial growth factor receptor 2; WHO, World Health Organization

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