

## NAVIGATING THE LANDSCAPE OF ADJUVANTS FOR SUBUNIT VACCINES: RECENT ADVANCES AND FUTURE PERSPECTIVES

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### ABSTRACT

The development of effective subunit vaccines relies on the incorporation of adjuvants to enhance immune responses and improve vaccine efficacy. This paper provides a comprehensive review of the various adjuvants employed in subunit vaccine development, with an emphasis on liposome-based, carbohydrate-based, polymer-based, and nanoparticle-based adjuvants. Additionally, the general concept of vaccine adjuvants, their classification into different types, and the underlying molecular mechanisms by which they exert their immunostimulatory effects are discussed. The use of adjuvants in subunit vaccine development has revolutionized immunization strategies by enhancing vaccine efficacy and inducing robust immune responses. Further research is needed to understand the safety profiles of adjuvants, elucidate the underlying molecular mechanisms, and optimize the adjuvant formulations. By harnessing the power of adjuvants, we can advance the development of effective subunit vaccines against infectious diseases and malignancies, thereby contributing to global health outcomes.

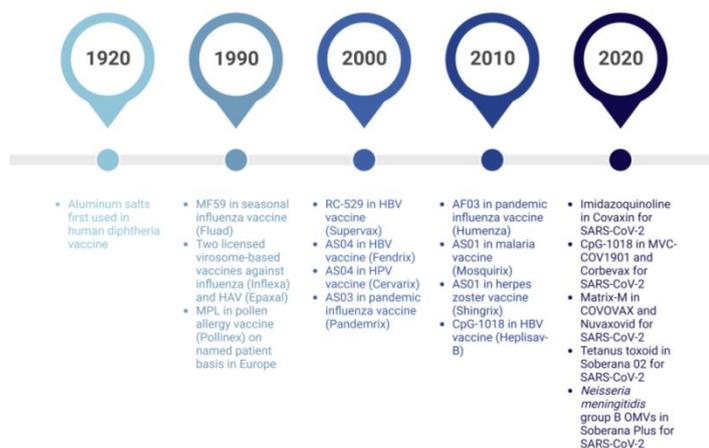
**Keywords:** Adjuvants, Delivery systems, Immune response, Subunit vaccines

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### INTRODUCTION

Subunit vaccines are a new generation of vaccines that utilize constituents derived from pathogenic bacteria, parasites, or viruses to elicit adaptive immune responses against pathogens [1]. These pathogenic constituents, commonly denoted as antigens, predominantly manifest as discrete proteins or artificial peptides [2]. Extensive clinical investigations have been conducted over the past few decades to evaluate the efficacy of protein and peptide antigen-based vaccines, and a number of these formulations are now commercially accessible worldwide (fig. 1). While conventional subunit vaccines predominantly exploit antigens that are markedly safer and highly purified than their whole-organism-based counterparts [3], their reduced immunogenicity is attributed to their diminished size and the absence of pathogen-associated molecular patterns (PAMPs) required for optimal antigen recognition [4]. Consequently, to induce or augment an immune response, non-immunogenic substances, referred to as adjuvants, are commonly incorporated into vaccine formulations [5].

Adjuvants can be classified based on various criteria, including physicochemical characteristics and modes of action [6]. A widely accepted categorization scheme is predicated on their mechanisms of action, which divides adjuvants into two primary groups: delivery systems (particulate) and immune potentiators [7], as shown in table 1. Another noteworthy class of adjuvants is mucosal adjuvants, which share certain attributes with the aforementioned categories. In the case of delivery system adjuvants, antigens are combined with an adjuvant that principally serves as an antigen transporter. Furthermore, these adjuvants can elicit a localized proinflammatory response by activating the innate immune system, thereby attracting immune cells to the inoculation site [8]. Specifically, the antigen-adjuvant complex activates pathways governed by pattern recognition receptors (PRRs) by mimicking PAMPs. This activation results in the stimulation of innate immune cells and subsequent production of cytokines and chemokines. Immune potentiators also directly engage in the same pathway [9] (fig. 2).



**Fig. 1: Timeline of vaccine adjuvant development and licensing [5], AS, adjuvant system; MPL, monophosphoryl lipid A; HAV, hepatitis A virus; HBV, hepatitis B virus; HPV, human papillomavirus; WHO, World Health Organization; OMVs, outer membrane vesicles**

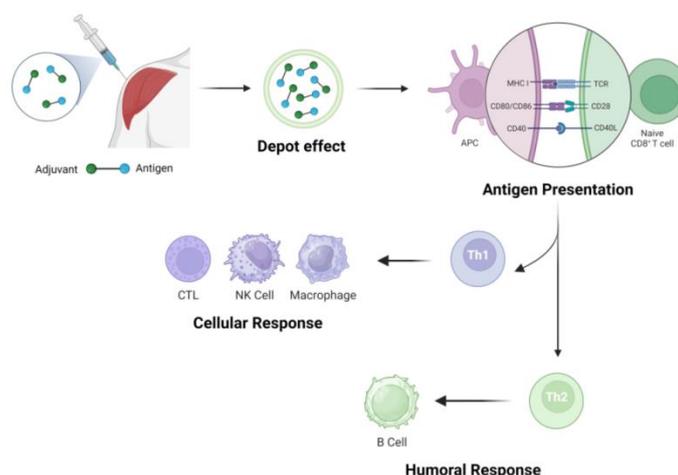


Fig. 2: Mechanisms of action of adjuvants [8]

Table 1: Classification of adjuvants according to their main mechanism of action [5]

Adjuvant groups	Types of adjuvants
Delivery systems	
Mineral Salts	Aluminum salts
Emulsions	Freund's adjuvants MF59 AS03
Microparticles	Virus-like particles Virosomes PLA/PLGA
Immune potentiators	
TLR1/2 agonists	L-pampo, MALP-2, Pam2CSK4 and Pam3CSK4
TLR3 agonists	Poly(I: C) (polyinosinic: polycytidylic acid) Poly-ICLC
TLR4 agonists	Monophosphoryl lipid A (MPL)
TLR5 agonists	Flagellin
TLR7/8 agonists	Imiquimod (R837; 1-(2-methylpropyl)-1H-imidazo [4,5-c]quinolin-4-amine) and resiquimod (R848, 4-amino-2-(etoximetil)-a,a-dimethyl-1H-imidazo [4,5-c]quinoline-1-ethanol)
TLR9 agonists	CpG ODNs
Combined adjuvants	AS01 and AS02 AS04
Mucosal adjuvants	Cholera toxin (CT) Heat-labile enterotoxin (LTK3 and LTR72) Chitosan

Multiple factors warrant careful consideration when selecting a vaccine adjuvant, with safety being a paramount concern. An ideal adjuvant should exhibit a commendable safety profile, ensuring minimal adverse effects and optimal tolerability. Furthermore, it should be easy to produce, possess favorable pharmaceutical attributes, such as appropriate pH, osmolality, and endotoxin levels, and maintain long-term stability during storage. Economic feasibility is also a crucial consideration [10]. Accommodating all these prerequisites while simultaneously upholding the adjuvant's safety represents a formidable challenge. Consequently, only a limited number of vaccine adjuvants have been incorporated into currently employed vaccine formulations.

The existing repertoire of adjuvants includes thiomersal, alum, complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), Montanide, adjuvant 65, and lipovant. However, it is important to note that these adjuvants frequently exhibit toxic and reactogenic properties [11]. Consequently, one of the primary obstacles encountered in subunit vaccine research is the need to engineer adjuvants that are devoid of toxicity or possess minimal toxicity [8].

This review aims to comprehensively examine the currently used subunit vaccine adjuvants and to evaluate ongoing studies on the properties and possible future use of new adjuvants. Five databases

were used to search for relevant articles, including Google Scholar, PubMed, Scopus, Web of Science, and Elsevier, published from 2013-2023. The following keywords were used: "subunit vaccine adjuvants", "subunit vaccines", and "vaccine adjuvants".

#### Vaccine adjuvants: an overview

Adjuvants are agents that, when employed in conjunction with vaccine antigens, elicit a heightened and more potent immune response than that elicited by the vaccine alone [12]. Incorporating adjuvants into vaccines serves several purposes: (i) enhancing the immunogenicity of antigens, (ii) diminishing the requisite antigen dosage or immunization frequency necessary to confer protective immunity, and (iii) bolstering the efficacy of vaccines in specific populations such as newborns, the elderly, or individuals with compromised immune systems [13].

Over the years, aluminum hydroxide and aluminum phosphate gels, commonly referred to as alum, have found extensive application as adjuvants in various licensed vaccines, serving as a safer alternative to Incomplete Freund's adjuvant because of the elimination of several cytotoxic properties [14]. However, Alum exhibits certain limitations, including susceptibility to freezing and drying, inconsistent and modest humoral immune responses, and specific safety concerns, which compromise its overall immunostimulatory

efficacy [15]. The advent of delivery systems has played a pivotal role in advancing the development of efficient vaccine adjuvants, primarily by facilitating antigen uptake by antigen-presenting cells (APCs) or augmenting the influx of APCs. A notable advantage of particulate formulations for vaccine delivery is the preservation of antigens from proteolytic degradation, which enhances the cellular uptake of the vaccine [16]. Furthermore, the inclusion of immunopotentiators, such as PRRs, along with the delivery system, can greatly contribute to generating a robust immune response by recognizing pathogen-associated molecules and facilitating increased antigen uptake. Consequently, the delivery system safeguards the encapsulated antigen from the host's *in vivo* environment, ensuring sustained release to evoke a durable antigen-specific immune response. Immunostimulatory adjuvants in the form of PRRs provide a stimulatory dose of linked PRR ligands, further enhancing antigen uptake and facilitating preferential presentation to APCs. Thus, this integrated approach enables prolonged antigen delivery with heightened immunogenicity [17].

The incorporation of adjuvants is of particular significance when formulating vaccines targeting the elderly population. This significance arises from the physiological phenomenon of immunosenescence observed in this group of individuals, which leads to diminished immune responses following natural infections or immunization interventions [18]. In such scenarios, the inclusion of adjuvants can serve as a valuable strategy for overcoming this limitation in vaccine efficacy.

In the context of vaccine development for diseases such as cancer, chronic infections (e. g., HIV, Hepatitis C Virus, Tuberculosis, and herpes simplex virus (HSV)), and emerging pathogens such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the ability of an adjuvant to elicit a qualitatively robust immune response is of paramount importance [19]. A comprehensive understanding of the immunobiology encompassing Toll-like receptors (TLRs), other PRRs, immunoregulatory cells, and the pivotal role of specific T helper (Th) cell responses in resolving distinct diseases lays the foundation for ongoing advancements and optimizations in vaccine design [20].

#### Molecular mechanisms of vaccine adjuvants

Currently, six adjuvants (Alum, AS04, MF59, AS03, AS01, and CpG ODN) are approved for use in human vaccines. This achievement has been facilitated by the extensive structural characterization of multiple adjuvants and identification of diverse PRRs and co-stimulatory ligand receptors, which have substantially enhanced our understanding of the underlying molecular mechanisms governing adjuvant function. Acquiring a comprehensive understanding of the mode of action of adjuvants is critically important for the design of vaccines capable of inducing pathogen-specific effector responses and establishing durable memory responses. Moreover, this understanding is invaluable during the developmental and regulatory phases of adjuvant safety [21]. The possible mechanisms by which adjuvants exert their adjuvant activity are discussed below.

#### Delivery systems

Adjuvants employed as delivery systems in subunit vaccines, including liposomes, immune-stimulating complexes (ISCOMs), and nanoparticles, have demonstrated remarkable efficacy in eliciting protective immunity [16]. These adjuvants play a crucial role in preventing the rapid degradation of proteins and peptides *in vivo*, consequently augmenting the dose effectiveness of the vaccine antigen. Notably, the co-administration of antigens with cationic liposomes elicits more robust antigen-specific immune responses than neutral or anionic liposomes [22]. Liposomes, serving as effective vaccine delivery systems, function as carriers in adjuvants, such as AS01, a liposome-based formulation comprising monophosphoryl lipid A (MPLA) and QS-21 [23]. Furthermore, enhanced saponin-based tensoactive adjuvants, namely ISCOM, ISCOMATRIX, and Matrix-MTM, have emerged as particulate antigen delivery systems with potent immunostimulatory activities [24]. ISCOMs adopt a cage-like structure with diameters ranging from 40 to 50 nm, consisting of saponins, cholesterol, and phospholipids. Similarly, ISCOMATRIX exhibits a structurally analogous formation,

albeit without the inclusion of the antigen itself (which can be formulated with ISCOMATRIX to prepare an ISCOMATRIX vaccine) [25]. Both ISCOMATRIX and ISCOM possess attributes encompassing antigen delivery and immunostimulatory properties, making them versatile tools for vaccine development [26].

#### Depot effect

The depot effect pertains to the gradual and sustained release of antigens at the injection site, which provides continuous stimulation to the immune system. This mechanism facilitates improved antigen uptake by APCs and is associated with the induction of high antibody titers. Initially, the adjuvant activity of alum was primarily attributed to its depot effect; however, recent evidence suggests that this effect is not the sole mechanism underlying its adjuvant activity [27].

In a mouse model, alum rapidly induced an inflammatory milieu characterized by the upregulation of inflammatory chemokines, leading to the recruitment and clustering of neutrophils at the injection site. Additionally, alum promotes neutrophil death, resulting in the release of neutrophil extracellular traps (NETs) composed of extracellular DNA, which play a substantial role in the adjuvant action of alum [28]. Oil-in-water emulsions such as Emulsigen®, water-in-oil emulsions such as the cationic adjuvant formulation (CAF)01 (consisting of a cationic liposome composed of dimethyldioctadecylammonium/ $\alpha,\alpha'$ -trehalose 6,6'-dibehenate or DDA/TDB), and biodegradable micro- and nanoparticles have also exhibited adjuvant activity mediated by the depot effect in mouse models [29].

#### Activation of immune signalling

Adjuvants can activate various PRRs and initiate signal transduction pathways through TLRs, nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and other crucial cellular pathways. The effectiveness of the yellow fever vaccine YF-17D, a live attenuated virus vaccine, can be attributed to its ability to activate multiple TLRs, including TLR2, 7, 8, and 9, on or within dendritic cells (DCs) in mice [30]. YF-17D also activates DCs derived from human monocytes and plasmacytoid DCs (pDCs) [30]. Intracellular NLRs such as NOD1 and NOD2 recognize diaminopimelic acid (DAP)-containing muropeptides from gram-negative bacteria, whereas NOD2 detects the muramyl dipeptide (MDP) component present in all bacterial peptidoglycans. The adjuvant activity of mucosal adjuvant Cholera Toxin (CT) is mediated through the NOD2 receptor [31]. Adjuvants induce a cascade of signal transduction pathways to exert their effects at both innate and adaptive levels. Intramuscular injection of MPL or AS04 in mice leads to NF- $\kappa$ B activation in muscles and local draining lymph nodes [32]. Synthetic derivatives of MPL activate TLR4 and selectively stimulate the p38 MAPK pathway, which is closely associated with optimal induction of IFN- $\gamma$ -induced protein 10 (IP-10), TNF- $\alpha$ , and IL-10 in mice [33].

#### Induction of cytokines, chemokines, and IFNs

In their microarray analysis, the impact of three potent human vaccine adjuvants, MF59, CpG ODN, and alum, on gene expression in mouse muscles was investigated [34]. They identified a shared set of 168 genes, referred to as "adjuvant core response genes," that encode cytokines, chemokines, innate immune receptors, IFN-induced proteins, and adhesion molecules. These genes play crucial roles in orchestrating immune responses. The induction of nonpathogenic inflammatory responses by these adjuvants creates a local immunocompetent environment that contributes to their adjuvant activity. Among the three adjuvants, MF59 exhibited the strongest capacity to induce adjuvant core response genes, leading to an enhanced and rapid influx of MHC-II+ and CD11b+ cells at the injection site and a more efficient transport of antigens to the draining lymph nodes [35]. Both alum and MF59 were found to induce chemokines involved in cellular influx, including CCL2, CCL3, CCL4, and CXCL-8, thereby facilitating the recruitment of immune cells to the injection site [36].

#### Induction of humoral immunity

The regulation of antibody responses is significantly influenced by innate immune responses, which affect the magnitude, quality, and

persistence of these responses. The magnitude of the antibody response is particularly crucial in providing protection against various infections, such as diphtheria, hepatitis A, Lyme disease, tetanus, yellow fever, polio, rabies, and pneumococcal infections (Pulendran and Ahmed, 2011). For infections such as RSV and meningococcal infections, both the magnitude and quality of antibodies and cell-mediated responses are of vital importance. To achieve enhanced immune responses, adjuvant systems, such as AS01, are employed in vaccines against malaria (RTS, S), herpes zoster (HZ/su), tuberculosis (TB), and HIV. Additionally, AS03 is utilized in several influenza vaccines, including trivalent inactivated H1N1 influenza, H5N1 pre-pandemic influenza, and candidate H7N1 and H7N9 pandemic influenza vaccines. Moreover, AS04 has been incorporated into licensed HPV-16/18 and HBV vaccines. These adjuvant systems are recognized for their ability to augment antigen-specific T cell and antibody responses [37]. Their incorporation into vaccines holds promise for enhancing the protective immunity against various infectious diseases.

Immunological memory is a defining characteristic of the adaptive immune system that functions as a crucial element in the establishment of protective immunity against infectious diseases. The germinal center (GC) reaction is at the core of memory development and orchestrates the generation of long-lasting immunological memory. The regulation of GC differentiation, affinity maturation, and the formation of enduring memory responses relies on the induction of specific key molecules, including CD40 inducible T-cell costimulator (ICOS), IL-21, programmed death-ligand 1 (PD-1), CD95, IRF4, and B-cell lymphoma 6 protein (Bcl-6) (Pulendran and Ahmed, 2011).

TLRs expressed on various cell types within GC, such as GC B cells, follicular dendritic cells (FDCs), and T cells, exert a profound influence on the initiation and progression of antibody responses. Notably, the utilization of nanoparticles, which bear resemblance to virions in size and incorporate TLR ligands, such as MPL and R837, in conjunction with H5N1 hemagglutinin, has been demonstrated to enhance the persistence of GCs. This prolonged persistence of GCs significantly affects the differentiation of memory B cells, thus playing a critical role in the establishment of enduring antibody responses in murine models [37].

#### Induction of cellular immunity

Activation of TLRs, including TLR3, TLR4, TLR7, TLR8, and TLR9, has been associated with the promotion of Th1-biased immune responses, whereas TLR2/TLR1, TLR2/TLR6, and TLR5 signaling pathways have been shown to induce Th2-biased immunity. In mice, CD11c<sup>+</sup>CD11b<sup>-</sup>CD8 $\alpha$ <sup>+</sup>dendritic cells localized in the marginal zones of lymph nodes (LNs) have been identified as key regulators of Th1 responses and effective cross-presentation of antigens *in vivo* and *ex vivo* [40]. In humans, BDCA1<sup>+</sup>(CD1c<sup>+</sup>) and BDCA3<sup>+</sup>(CD141<sup>+</sup>) DCs, corresponding to murine CD8 $\alpha$ <sup>-</sup> and CD8 $\alpha$ <sup>+</sup>DCs, respectively, play a role in the cross-presentation of extracellular antigens [40].

The use of poly (I: C), a TLR3 agonist, enhances major histocompatibility complex class I (MHC-I) expression and type I interferon (IFN) production, thereby promoting antigen cross-presentation to CD8<sup>+</sup>T cells and antigen-specific cytotoxic T lymphocytes (CTLs). Conversely, alum, an adjuvant commonly used in vaccines, favors Th2 responses characterized by robust antigen-specific IgG1 and IgE production while failing to induce CD8<sup>+</sup>T cell immunity and even inhibiting Th1 immune responses in mice [41]. Squalene-based oil emulsions have demonstrated potent induction of both Th1 and Th2-mediated immunity while maintaining good tolerability [42].

Specific adjuvants, such as QS-21, MF59, and CFA, have been shown to induce Th1-biased or mixed Th1/Th17 and Th1/Th2 immune responses. In TB vaccines, experimental cationic adjuvant formulations combined with immunostimulators such as TDB stimulate robust cellular and humoral immune responses as well as the generation of polyfunctional memory T cells and Th1- and Th17-biased immune responses in mice [43]. A summary of the mechanisms of action of the different adjuvants is shown in table 1, while table 2 presents the adjuvants currently undergoing clinical trials.

**Table 2: Adjuvants currently in use in Phase I, II, and III vaccine trials [8]**

Adjuvants	Experimental vaccines
AS01	<i>Haemophilus influenzae</i> vaccine <i>Moraxella catarrhalis</i> vaccine Tuberculosis vaccine HIV vaccine
CpG ODN	Cancer vaccine for patients with melanoma (Phase I)
Flagellin	Plague vaccine (Phase I) Influenza vaccine (Phase I/II)
PolyI: C12U (Ampligen)	H5N1 influenza vaccine (Phase III) Cancer vaccine (Phase I/II)
CAF01	<i>Chlamydia trachomatis</i> vaccine (Phase I) Tuberculosis vaccine (Phase I)
IC31	Tuberculosis vaccine (Phase I/II)
ISCOMATRIX	Tumor cell vaccine (Phase I) Melanoma (Phase II)

#### Subunit vaccine adjuvants

##### Liposome-based adjuvants

Liposomes, which are bilayer lipid or phospholipid vesicles, primarily consist of amphiphilic lipids and phospholipid molecules that represent natural components at various scales. However, the incorporation of additional components such as sterols, polypeptides, antioxidants, and polymers allows for the modulation of the bilayer structure, prolongation of blood circulation half-life, enhancement of tolerance against reactive oxygen species, and development of targeted strategies for these lipid vesicles [44, 45]. Liposomes improve the encapsulation, release, and delivery of bioactive compounds to target cells and tissues, thereby enhancing their stability and efficacy [45]. For ease of cellular endocytosis, two-layered liposomes are favored in the formulation, with the inclusion of cholesterol and polyethylene glycol providing stabilization and protection against immune cell attack, respectively [46]. The first demonstration of liposomes in mRNA vaccines dates back to 1978 when rabbit globin mRNA sequences were delivered to mouse lymphocytes [46]. Over the past few decades, liposomes and lipid nanoparticles have been developed to enhance subunit vaccines against various infectious diseases, including TB [47]. However, to maximize liposome efficacy, several factors must be optimized, including liposome size, surface charge, and lipid bilayer composition [48]. Depending on the desired effect, ligands such as drugs, peptides, cytokines, RNA, nucleotides, and antibodies can be conjugated onto or loaded within liposomes using diverse strategies [49].

The development of liposomal vaccines has been driven by the objective of targeting specific immune cell types to elicit tailored immune responses [50]. As an adjuvant or delivery system, cationic liposomes have shown promise in enhancing the potential of diverse subunit vaccines owing to their strong interactions with negatively charged immune cells [47]. When combined with other immunostimulatory factors like TDB (trehalose 6, 60-dibehenate), monophosphoryl lipid A (MPL), trehalose dimycolate (TDM), and Poly I: C, cationic liposomes demonstrated robust electrostatic interactions with APCs, leading to the induction of both humoral and cellular immune responses, as well as a robust memory response [51].

Two approved liposomal vaccine formulations, Inflexal® V (influenza vaccine) and Epaxal® (hepatitis A vaccine), utilize virosome-based technology, wherein viral proteins are affixed to the surface of a liposome carrier [52]. Various methods have been explored to enhance the stability of liposome formulations during storage, including freeze-drying, spray-drying, supercritical fluid technology, and lyophilization [52]. These approaches hold significant potential for advancing the field of liposomal vaccines and their successful application in future immunization strategies.

##### Carbohydrate-based adjuvants

The presence of carbohydrates on bacterial cell surfaces serves multiple purposes, including adhesion to human tissue, protection against desiccation, prevention of complement deposition, and

evasion of innate defense mechanisms. Carbohydrates possess inherent immunomodulatory properties and can act as natural and relatively safe vaccine adjuvants or immune stimulators because they are recognized by receptors present on the surface of APCs. The immunomodulatory properties, biocompatibility, biodegradability, and low toxicity of carbohydrates have led to an intensified investigation of their potential as adjuvants and delivery systems [53, 54].

### Mannose

Mannose, a common component on the surfaces of bacteria, fungi, and viruses, is a recognized target for various immune receptors [55]. Among these receptors are different C-type lectin receptors (CLRs), such as mannose-binding lectin, mannose receptors, Mincle, and DC-SIGN receptors, as well as TLR4, which specifically binds to O-linked mannosylated ligands [56]. Recognition of mannose by these receptors can elicit complement activation and phagocytosis and subsequently trigger innate immune responses [57].

Moreover, the stimulation of mannose receptors can lead to receptor-mediated endocytosis and influence TLR signaling pathways, thereby activating the adaptive immune system [58]. For instance, the mannose receptors CD206 and CD209, which are expressed on macrophages and DCs, play a crucial role in recognizing mannosylated antigen-bearing constructs and facilitating the delivery of antigens to MHC I and MHC II receptors. This recognition by T cells ultimately initiates adaptive immunity [59]. Activation of mannose receptors has been shown to enhance CD4<sup>+</sup> and CD8<sup>+</sup>T-cell responses, leading to the induction of both Th1- and Th2-type immunity, subsequent IgG production, and establishment of long-lasting immunity [60]. Furthermore, the activation of mannose receptors in tumor-associated macrophages has been demonstrated to enhance both innate and adaptive antitumor immunity [61].

Given the significant role of mannose in immune activation, it is frequently employed in vaccine design to augment the immunogenicity of co-administered antigens. For instance, anticancer vaccines were developed by linking two mannosyl glycolic acids to the amino groups of the N-terminal lysine of a peptide derived from mucin 1 protein, an antigen overexpressed in several types of cancer [62]. This strategy harnesses the potential of mannose to enhance immune responses and holds promise for the development of effective vaccines against various diseases.

### Glucan

Glucan, a polymerized form of glucose and a natural constituent of yeast and certain bacterial cell walls, possesses notable immunostimulatory properties [63]. This polysaccharide comprises various glycosidic bonds, including  $\alpha$ -,  $\beta$ -1,3 and 1,4 glycoside bonds. Additionally, glucan can be obtained as hollow spherical particles upon isolation from *Saccharomyces cerevisiae* [64]. Immune cells, including neutrophils, macrophages, and DCs, recognize all forms of glucose homopolymers [65]. Glucan engages several PRRs, such as dectin-1, TLR2, TLR6, and TLR9, thereby promoting phagocytosis and endocytosis of antigens. This leads to CD4<sup>+</sup> and CD8<sup>+</sup> cell proliferation, Th1 and Th17 differentiation, upregulation of IL-4 and IL-3 cytokines, and generation of substantial levels of IgG and IgA antibodies [66].

Moreover, glucan is non-toxic and possesses anticoagulant, antithrombotic, and antioxidant properties [67]. Given these characteristics, glucans and related GPs have been explored for their potential in vaccine delivery. For instance,  $\beta$ -glucan was conjugated to three types of hollow silica particles: (a) *Escherichia coli* particles (rod-shaped, 900 nm  $\times$  1.2–3.2  $\mu$ m), (b) *Staphylococcus aureus* particles (spherical, 900 nm), and (c) polystyrene particles (spherical, 220 nm) [68]. These particles were subsequently loaded with the OVA antigen. All glucan-conjugated particles were effectively internalized by dectin-1, complement receptors, and TLR-2 on APCs, resulting in successful APC maturation, upregulation of MHC II, and robust IgG antibody responses compared with plain particles. Furthermore, all glucan particles induced both Th1 and Th2 responses, with minor differences observed in Th1/Th2 specificity among the particles. These findings highlight the potential

of glucan-based vaccine formulations to elicit robust immune responses with balanced Th1/Th2 polarization [68].

### Lipid A and its derivatives

Lipid A, a constituent of bacterial endotoxins, represents a lipid component that exhibits slight structural variations among different Gram-negative bacterial species, including *Acinetobacter baumannii*, *Burkholderia pseudomallei*, *Campylobacter jejuni*, *Escherichia coli*, *Helicobacter pylori*, *Klebsiella pneumoniae*, *Neisseria gonorrhoeae*, and *Salmonella Minnesota* R595 [69]. Typically, lipid A consists of a disaccharide (comprising two glucosamine residues), two phosphate groups, and five or six fatty acids, with chain lengths ranging from 12 to 16 carbon atoms. Notably, lipid A exhibits potent adjuvant properties, sourced naturally, although its toxicity remains a concern [70]. Fortunately, a derivative of lipid A, monophosphoryl lipid A, offers an advantageous alternative. MPLA lacks one phosphate group, resulting in reduced toxicity while retaining robust immunostimulatory capabilities [70].

MPLA-adjuvanted vaccines have undergone extensive evaluation in the context of various diseases, including influenza [71], hepatitis B [72], rabies [73], and parasitic infections [74], among others. Notably, the inclusion of MPLA in the rabies vaccine formulation resulted in the promotion of robust cell-mediated immune responses characterized by increased production of IL-4 and IFN- $\gamma$ , and activation of CD4<sup>+</sup>/CD8<sup>+</sup>T cells compared to formulations lacking MPLA [73]. Furthermore, when *Toxoplasma* GRA2 and GRA6 antigens were combined with MPLA, a pronounced Th1 response was induced, accompanied by a significant increase in the expression of both IFN- $\gamma$  and IL-2 in mice [74].

### CAF01

CAF01 is a liposomal formulation featuring a cationic liposome carrier (dimethyl octadecyl ammonium) along with a glycolipid immune stimulator, trehalose-6, 6-dibehenate [75]. Unlike MPLA, TDB engages with CLR receptors rather than TLR4, with the sugar moiety playing a pivotal role in its immune recognition [76]. The major hydrophobic pocket of the mincle receptor, a specific type of CLR, is proposed as the recognition site for the lipid chains of TDB, underscoring the significance of the lipid components in the formulation's immune-stimulating activity [76]. The presence of two fatty acids, ranging from 5 to 14 carbon atoms, is essential for maximizing TDB potency [77].

Several vaccine formulations that utilize CAF01 have been examined in various studies. In one study, mice were subcutaneously immunized with a vaccine formulation consisting of epitopes from HIV-1 proteins (Vif, Gag, Env, Pol, and Vpu), the universal T-helper PADRE, and CAF01 [78]. This immunization strategy elicited cellular immune responses targeting HIV-1 CTL epitopes, comparable to those elicited by incomplete Freund's adjuvant. Notably, CAF01 was employed as an adjuvant in tuberculosis vaccine Hybrid 1 (H1), which carried the hybrid protein of Early Secretory Antigenic Target (ESAT) and Antigen 85B. In this context, CAF01 demonstrated a dose-dependent effect, inducing a robust and persistent CD4<sup>+</sup>T-cell response lasting up to three years [75].

### Saponin (QS-21)

Saponins, which are intricate natural liposaccharides, exhibit notable immunostimulatory properties and can be sourced from various herbs, such as *Saponaria officinalis*, *Quillaja saponaria*, and *Gynostemma pentaphyllum* [79]. The structure of saponins comprises four domains: branched trisaccharide, quillie acid triterpene, binding linear tetrasaccharide, and pseudodimeric acyl chain [80]. The immunostimulatory activity of saponins has been acknowledged for nearly a century [81]. The fucosyl residue present in saponins is known to bind to lectin DC-SIGN, contributing to its immunostimulatory capacity [82]. The adjuvanticity of saponins is influenced by the number, nature, and connectivity patterns of the glycosyl groups in the sugar chains [83]. For instance, reducing the number of sugars at the C-3 position or increasing them at the C-28 position enhances the adjuvant activity of saponins [84]. Numerous studies have evaluated the immunostimulatory effects of QS-21. In one study, SPf66, an early malaria vaccine candidate composed of a

synthetic 45 amino acid peptide derived from four *Plasmodium falciparum* proteins [85], exhibited limited efficacy when tested with alum as an adjuvant [86].

#### **$\alpha$ -Galactosylceramide**

$\alpha$ -Galactosylceramide ( $\alpha$ -GalCer) is a glycolipidic adjuvant derived from marine sponges and has shown remarkable immunostimulatory properties. It serves as an antigen recognized by natural killer T cells (NKT). The interaction between  $\alpha$ -GalCer and NKT cells occurs through the presentation of  $\alpha$ -GalCer by APCs via the MHC I-like molecule CD1, resulting in the activation of pro-inflammatory and immunomodulatory cytokine responses [87]. In turn, activated NKT cells can stimulate dendritic cells and enhance the responses of antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup>T-cells. Consequently,  $\alpha$ -GalCer has been employed as an adjuvant in the development of antiviral and antitumor vaccines [88]. The immunogenicity of  $\alpha$ -GalCer is influenced by the conformation and structure of the sugar moiety as well as the length of the fatty acyl chains of glycosylceramide [89].

The immunostimulatory potential of  $\alpha$ -GalCer was evaluated in conjunction with HIV CTL epitopes derived from the gp120 envelope protein [90]. The vaccine formulation was administered to mice via the intranasal and oral routes. Notably, both immunization routes elicited robust IFN- $\gamma$  production in the spleen and mucosal tissues, indicating the induction of potent immune responses. This highlights the efficacy of  $\alpha$ -GalCer as an adjuvant for stimulating both systemic and mucosal immunity. Additionally,  $\alpha$ -GalCer demonstrated the capability to elicit humoral immune responses, as evidenced by a significant increase in IgG titers (including both IgG1 and IgG2a) following intranasal administration of  $\alpha$ -GalCer and OVA. These findings suggest that  $\alpha$ -GalCer can promote a balanced Th1/Th2 immune response profile in mice [91].

#### **Muramyl dipeptide**

Carbohydrate-peptide conjugates have demonstrated potential as adjuvants in vaccine development. Among these, the peptidoglycan N-acetyl-muramyl-l-alanyl-d-isoglutamine (muramyl dipeptide, MDP, Gerbu adjuvant) is derived from both Gram-positive and Gram-negative bacterial cell walls and can also be synthesized. MDP acts as a ligand for PRRs, particularly NLRs, which are present on APCs [92]. Upon binding to NLRs, MDP trigger the production of pro-inflammatory cytokines (e.g., TNF- $\alpha$  and IL-1) and co-stimulatory molecules (e.g., IL-6 and IL-12) by APCs, thereby activating both humoral and cellular immune responses [93]. Although its pyrogenic effects render it unsuitable for human vaccines, MDP has the potential for use in animal vaccines [94, 95]. An example of MDP's effectiveness in animal vaccines can be observed in a study in which MDP was combined with inactivated porcine epidemic diarrhea virus (PEDV) and administered subcutaneously to mice. This formulation induced the production of PEDV-specific IgG antibodies and cytokines while also activating CD3<sup>+</sup> and CD4<sup>+</sup> cells. Notably, the inclusion of MDP significantly enhances the levels of PEDV-specific IgA antibodies following intranasal immunization [96].

#### **Polymer-based adjuvants**

Recently, there has been a growing interest in exploring the adjuvanticity and antigen-delivery potential of polymers. These versatile compounds can serve as immunostimulants and delivery systems in vaccine formulations. Polymers possessing immunostimulatory properties interact with specific receptors found on immune cells, thereby directing the vaccine to specific sites of antigen uptake and activating distinct immune pathways [97]. Immunostimulants are typically administered alongside antigens either as a physical mixture or through chemical conjugation with the aim of eliciting targeted and desired immune responses [98, 99]. The polymer-based adjuvants are discussed below.

#### **Oligo-and polysaccharides of mannose**

The oligo-and polymerized forms of mannose have undergone extensive evaluation and have been found to be readily recognized by PRRs located on the surface of various human immune cells,

including dendritic cells, macrophages, epithelial cells, and endothelial cells [100]. Mannan, the polymerized form of mannose, is recognized by a broad spectrum of receptors, such as mannose receptors, dectin-2, dectin-3, Mincle, DC-SIGN, galectin-3, Fc $\gamma$ R, TLR2, TLR4, and TLR6 [53]. Consequently, mannan holds promise as an APC targeting agent, effectively enhancing the uptake and processing of co-administered antigens within APCs. Although mannan shares a similar immune recognition mechanism with mannose [101], it displays heightened adjuvanticity. This can be attributed to its ability to bind more effectively to receptors comprising multiple carbohydrate-recognizing domains owing to the presence of multiple ligands (mannose moieties) [102]. Structurally, mannan consists of linear and branched polymers of mannose sugars linked through  $\alpha$ -1,2,  $\alpha$ -1,3,  $\alpha$ -1,4,  $\alpha$ -1,6, and  $\beta$ -1,2 glycosidic bonds [103]. The interaction between ligands and receptors, as well as the subsequent stimulation of immunity, relies on the conformation of mannan (and other polysaccharides), the specific types of glycosidic bonds within the molecule, and factors such as the degree of branching, charge, and molecular weight [104].

The strategy employed for the conjugation of mannan with antigens significantly influences receptor recognition. An investigation involving reductive coupling between mannan and the antigen elicited robust humoral immunity but no cellular response. Mice immunized with oxidized mannan coupled with the antigen demonstrated protection against tumor challenge with mucin-1 3T3 tumor cells, whereas mice treated with the reduced mannan-antigen conjugate did not exhibit inhibited tumor growth. The observed effect of oxidizing conditions was attributed to the formation of Schiff bases between mannan aldehyde groups, antigens, and APCs, which consequently induced potent antitumor immunity by targeting the antigen to the intracellular processing pathway for presentation with MHC I molecules. However, contradictory findings from another study have indicated high antibody responses against vaccines bearing oxidized mannan [105].

#### **Chitosan and its derivatives**

Chitosan, a biodegradable and biocompatible polymer, exhibits notable properties such as mucoadhesion and cationic characteristics because of its high proportion of free amine groups ( $\leq 95\%$ ) that form salts under acidic conditions [106]. The hydroxyl groups at the C-2, C-3, and C-6 positions of chitosan can be utilized for the modification or attachment of peptides or protein antigens [107]. The immunostimulatory effects of chitosan include enhancement of both humoral and cellular immune responses [108]. This polymer and its derivatives interact with various receptors on APCs, including dectin-1, TLR-2, leukotriene B<sub>4</sub>, and mannose receptors [109].

Chitosan serves as an excellent adjuvant for mucosal administration because of its mucoadhesive properties and ability to facilitate the opening of tight junctions, thereby enabling the paracellular transport of vaccine antigens [110]. The cationic charge of chitosan and its derivatives facilitates increased cellular interactions with negatively charged epithelial cells, thereby prolonging the residence time of antigens in the nasal cavity [111]. Charge-mediated interactions have been employed to incorporate various protein and peptide vaccine epitopes into chitosan particles [109]. For instance, co-administration of matrix protein 1 (M1) of influenza A virus (100  $\mu$ g) with chitosan via intranasal administration induced higher levels of IgG and IgA antibody titers against H9N2 virus in mice than intraperitoneal administration [112].

#### **Alginate**

Alginate, a bioadhesive polysaccharide polymer, is widely recognized for its anionic nature and has been extensively employed in drug delivery systems, primarily because of its ability to shrink in the stomach and release cargo into the intestine. Recent advancements have expanded the use of alginate in vaccine delivery. Derived from the cell walls of algae, alginate is a copolymer comprising (1-4)-linked  $\beta$ -D-mannuronate and  $\alpha$ -L-guluronate residues. Although alginate is insoluble in water, its salt form, sodium alginate, is commonly used in biomedical applications. Studies have shown that alginate possesses adjuvant properties and

stimulates monocytes/macrophages [113]. Notably, alginate has a significant utility in site-specific vaccine antigen delivery to mucosal tissues. Its incorporation into formulations enhances phagocytosis and promotes increased adhesion of the formulation to dendritic cells [114].

Alginate has emerged as a promising candidate for subunit vaccine delivery, with various formulations, including conjugates, nanogels, and microparticles (MPs). A notable example involves the conjugation of peptide antigens derived from *Pseudomonas aeruginosa*, namely peptide294 (ERRANAVRDVLVNEY) and peptide176 (AGLGVGFNFGGSKAA), with alginate [114]. Subcutaneous administration of the peptide294-alginate conjugate emulsified with incomplete Freund's adjuvant in mice resulted in the generation of robust titers of protective and opsonophagocytic antibodies. In contrast, peptide294 emulsified with IFA alone, without the incorporation of the polymer, failed to elicit a significant antibody response [114].

#### Hyaluronic acid

Hyaluronic acid (HA), also known as hyaluronan, is a linear mucopolysaccharide composed of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine linked through alternating  $\beta$ -1,4 and  $\beta$ -1,3 glycosidic bonds [115]. Notably, HA exhibits remarkable hydrophilicity, making it the most hydrophilic polymer in nature [116]. One of the unique features of HA is its non-antigenic and non-immunogenic nature, attributed to its highly conserved structure across species. This natural polymer is abundantly present in both prokaryotes and eukaryotes and is widely distributed within the extracellular and pericellular matrices, as well as intracellularly.

HA has primarily been employed in transdermal immunization because of its ability to hydrate the skin tissue, facilitate absorption from the skin surface, and traverse the stratum corneum and underlying skin layers. When combined with antigenic peptides, HA enables the delivery of these peptides to the deep layers of the skin owing to its hygroscopic and skin-penetrating properties [117]. HA interacts with dermal dendritic cells and epidermal Langerhans cells (LCs) through HA receptors and TLRs present on the immune cells. Remarkably, low-molecular-weight (MW) HA serves as an endogenous danger signal by activating the transduction pathway mediated by TLR2 and TLR4 [118]. Additionally, it possesses immunostimulatory properties, prompting the production of chemokines and cytokines. Activation of TLR2 and TLR4 pathways by low-MW HA enhances the skin's self-defense mechanisms, leading to the production of  $\beta$ -defensin 2 [119]. In a study focusing on transdermal immunotherapy for Duchenne muscular dystrophy (DMD), antigenic peptides derived from the myostatin fragment (MstnF), namely MstnF (VFLQKYPHTLHVHQA) and scrMstnF (TFHQVLQHKVAPYLH), were conjugated to HA [117]. Transdermal immunization of mdx mice with the HA-MstnF conjugate resulted in a significant increase in antibody titers against myostatin, along with notable improvements in the biochemical and pathological conditions of skeletal musculature, as well as functional behaviors [117].

#### Dextran

Dextran, a complex branched polysaccharide, is composed of a linear  $\alpha$ -1,6-linked d-glucopyranosyl backbone with branches formed through  $\alpha$ -1,3 linkages. This polysaccharide is synthesized by specialized lactic acid bacteria using sucrose as the substrate. Dextran exhibits high water solubility, and controlled degradation leads to the production of dextran with a diverse range of molecular weights. The adjuvant properties of numerous dextran derivatives have been investigated. Dextran sulfate, in particular, has shown great promise as a matrix material for the controlled release of pharmaceuticals. The high charge density of dextran sulfate, resulting from the high ratio of negatively charged sulfate to glucosyl residues, enhances the loading of positively charged molecules [120].

Conjugation of dextran with bovine serum albumin (BSA) has proven to be highly effective in stimulating a robust and persistent antibody response in mice, even in the absence of additional adjuvants. Remarkably, even at a relatively low dose of 10  $\mu$ g, detectable antibody titers were achieved, and a dose-dependent increase in titers was observed at higher doses. The molecular weight of dextran plays a

critical role in the generation of these antibody titers, as dextran within the range of 500-2000 kDa was found to be indispensable, while the use of 70 kDa dextran failed to induce detectable antibody production [121]. Furthermore, dextran served as an effective platform for the conjugation of the CpG oligodeoxynucleotide (CpG-ODN) adjuvant and TLR7 agonist 1V209, resulting in enhanced targeting capabilities and improved immunostimulatory profiles for these adjuvants [122]. This highlights the versatile nature of dextran in augmenting the functionality of adjuvants, further expanding its potential applications [121].

#### Carrageenan

Carrageenan, derived from red seaweed, has emerged as a promising adjuvant for peptide vaccines, garnering significant attention in recent research. The anionic nature of carrageenan arises from the presence of hemisulfate ester groups, which contribute to its unique properties. Structurally, the carrageenan backbone is composed of a polymer chain comprising hemisulfated galactose and 3-6 anhydrogalactose residues, interconnected through alternating  $\alpha$ -1,3 and  $\beta$ -1,4 glycosidic bonds. Based on the distribution and location of ester sulfate groups along the repeating galactose units, carrageenans can be categorized into three primary types: kappa ( $\kappa$ -), iota ( $\iota$ -), and lambda ( $\lambda$ -) carrageenans [123].

The use of carrageenan as an adjuvant was prompted by its remarkable capacity to elicit immune responses specific to antigens and exert antitumor effects. In a study utilizing mice vaccinated with a physical combination of carrageenan and a peptide derived from the E7 protein of human papillomavirus type 16 (HPV-16), carrageenan was observed to significantly augment immune responses specific to the E7 antigen through activation of the TLR4 pathway [124]. Importantly, the intensified immune response induced by carrageenan was comparable to that triggered by other TLR4 ligands, including monophosphoryl lipid A as well as structurally related compounds such as dextran [124]. This finding highlights the promising adjuvant properties of carrageenan, thus substantiating its potential to effectively enhance immune responses in a manner akin to established immunostimulants.

#### Poly( $\epsilon$ -caprolactone)

Poly( $\epsilon$ -caprolactone) (PCL) is a semi-crystalline polyester synthesized through the ring-opening polymerization of  $\epsilon$ -caprolactone using a suitable catalyst [125]. This polymer exhibits inherent biodegradability as its ester linkages undergo hydrolysis under physiological conditions. Notably, the degradation rate of PCL is slower than that of polylactide polymers. One advantageous characteristic of PCL is its ability to avoid the generation of an acidic environment upon dissolution, a property not shared by other polyesters, such as PLGA. This absence of acidity is particularly beneficial, as it prevents any potential negative impact on the antigenicity of loaded proteins or peptides. PCL has been recognized as an FDA-approved polymer for long-term implantable devices owing to its biodegradability and safety. Furthermore, PCL possesses additional merits, including its hydrophobic nature, biocompatibility, and cost-effectiveness [126].

PCL is frequently employed as a viable option for sustained-release delivery of antigens, obviating the necessity for a prime-boost regimen because of its inherently sluggish biodegradability *in vivo*. This characteristic affords the opportunity for a two-fold effect, wherein the initial release of antigen from the surface of PCL microspheres acts as a priming dose, while the subsequent delayed release of antigen over an extended duration, arising from either diffusion or the breakdown of MPs, serves as a boost. In a specific study, the immunogenic properties of PCL MPs (23  $\mu$ m in size) loaded with ovalbumin (OVA) were assessed to evaluate their ability to elicit both humoral and cell-mediated immune responses [127]. Immunization with these MPs demonstrated heightened levels of IgG responses compared to OVA alone, albeit significantly lower than OVA administered alongside complete Freund's adjuvant.

#### Poly(lactic-co-glycolic acid)

Poly(lactic-co-glycolic acid) (PLGA) is a copolymer in which consecutive monomeric units of glycolic acid or lactic acid are

interconnected via ester linkages. The utilization of PLGA as a vaccine delivery system holds significant promise, which is primarily attributable to several key factors. First, the gradual degradation rate of PLGA particles prior to internalization into antigen-presenting cells enhances their desirability. Additionally, the nontoxic nature of this system, coupled with its ability to facilitate the controlled release of encapsulated payloads [128], further contributes to its appeal.

PLGA degradation occurs through bulk erosion, which results in the ingress of water into the polymeric matrix. This event prompts the hydrolysis of ester linkages, leading to the reconstitution of the original monomers, lactic acid, and glycolic acid. Notably, these monomers are byproducts of various metabolic pathways in the body and do not give rise to substantial toxic effects. Concurrently, the degradation process engenders an increase in matrix porosity, facilitating the sustained release of the entrapped antigen as the degradation progresses [129].

In an experimental setting, self-encapsulating PLGA microspheres loaded with the adjuvanting calcium phosphate gel ( $\text{CaHPO}_4$ ) were employed for the encapsulation of ovalbumin antigen. Upon nasal immunization, these microspheres, characterized by a size of 7  $\mu\text{m}$  and a surface charge of -22 mV, predominantly elicited IgG1 titers in both the serum and local mucosa. These IgG1 titers signify a Th2 immune response and are comparable to the titers induced by administering OVA alongside cholera toxin subunit B (CTB) [130]. Consequently, the PLGA formulation alone exhibited adjuvant-like properties similar to those of the established mucosal adjuvant CTB [130].

#### Polyglutamic acid

Polyglutamic acid (PGA) is a biodegradable, biocompatible, and non-toxic anionic polymer composed of repetitive units of glutamic acid. There are two distinct forms of PGA:  $\alpha$ -PGA and  $\gamma$ -PGA, wherein the glutamic acid units are linked through  $\alpha$ - or  $\gamma$ -carboxylic acids, respectively.  $\alpha$ -PGA is typically synthesized chemically, while  $\gamma$ -PGA is biosynthetically produced by bacteria, predominantly *Bacillus* species. The application of high-molecular-weight  $\gamma$ -PGA spans various domains, including its utilization as a metal chelator, a carrier for cisplatin and paclitaxel, a bio-adhesive, and in tissue engineering [131].

However, it is noteworthy that only modified PGA nanoparticles (NPs) exhibit exceptional capabilities as vaccine carriers, enabling efficient delivery of antigenic proteins to antigen-presenting cells and eliciting potent immune responses. In one study, the  $\gamma$ -PGA polymer was grafted with the hydrophobic compound L-phenylalanine ethyl ester (L-PAE) to form NPs with a size of 200 nm [132]. These  $\gamma$ -PGA-L-PAE NPs efficiently encapsulated ovalbumin and demonstrated effective uptake by immature dendritic cells, subsequently inducing maturation [133]. Comparatively, OVA-loaded  $\gamma$ -PGA-L-PAE NPs exhibited enhanced efficiency in inducing cellular CTL responses compared to OVA alone while maintaining equivalent efficiency to OVA combined with complete Freund's adjuvant. Moreover, OVA/ $\gamma$ -PGA-L-PAE NPs elicited an antigen-specific IgG response similar to that elicited by OVA/CFA, significantly surpassing the response induced by OVA alone. Furthermore, in a separate study, mice immunized with  $\gamma$ -PGA-L-PAE NPs coated with a CD8<sup>+</sup>T cell epitope listerolysin (LLO) peptide (VAYGRQVYLKLS) displayed a remarkable survival period of 11 d post-challenge, in stark contrast to mice receiving either phosphate-buffered saline (PBS) or LLO alone, which succumbed to infection [134].

#### Polyacrylates

Poly(methyl methacrylate) (PMMA), also referred to as poly(methyl 2-methyl propanoate), is a homopolymer synthesized from methyl methacrylate monomers. It is an extensively investigated biomedical polymer owing to its exceptional biocompatibility. Although inherently hydrophobic, PMMA exhibits slightly increased hydrophilicity upon contact with water. Its biocompatibility and well-established safety profile in biomedical applications render it

generally regarded as non-toxic. PMMA can be used as an implant material in total hip replacements and in mandibular and dental corrections [135].

The potential of PMMA as a nanoparticulate vaccine adjuvant was first demonstrated by Speiser *et al.*, who reported that PMMA facilitated stronger immune responses when utilized in conjunction with the inactivated influenza virus [136]. Furthermore, it has been observed that PMMA microspheres are capable of being absorbed by Peyer's patches within the gut-associated lymphoid tissue following oral administration [137]. Despite its nonbiodegradability, PMMA has been used for vaccine delivery. For instance, core-shell nanoparticles composed of an anionic core and a shell derived from Eudragit were prepared by emulsion polymerization, with HIV Tat protein adsorbed onto these NPs (220 nm in size) [138]. The administration of these NPs resulted in the induction of significant anti-Tat IgG titers, although they did not surpass those achieved by the Tat protein alone. Intramuscular vaccination of mice with these NPs elicited higher IFN- $\gamma$  and IL-2 responses along with lower IL-4 levels, thereby indicating the prevalence of a Th1 immune response [135].

#### Nanoparticle-based adjuvants

The use of nanoparticles (NPs) as adjuvants in vaccine formulations has garnered considerable attention in recent years. Incorporating NPs into vaccine formulations offers several advantages, including enhanced antigen stability [139], targeted antigen delivery [140], prolonged antigen release [141], and obviating the need for booster shots [142]. Various types of NPs have been extensively investigated for their potential to effectively deliver antigens and augment immune responses against vaccine antigens [143]. The different nanoparticle-based vaccine adjuvants are discussed in this section and summarized in table 3.

#### Natural nanoparticles

##### Bacterial spore

Spores are quiescent cells that can be generated by specific bacterial species, including Gram-positive *Bacilli* and *Clostridia* [144]. Spore formation serves as a survival strategy that enables bacteria to withstand adverse environmental conditions. These mature spores typically exhibit a size range of 800-1200 nm and possess spherical or ellipsoidal morphology [145]. Notably, spores have a remarkable ability to self-assemble into functional structures, thereby serving as effective carriers for vaccines. This property allows spores to shield surface-bound antigens from degradation and stimulates an immune response [146]. *Bacillus subtilis* spores, in particular, offer several advantages, including high stability, low production costs, ease of construction, and a well-established safety profile, leading to their designation as Generally Recognized as Safe (GRAS) [147]. Furthermore, their oral administration route allows spores to protect antigens against degradation by gastric acid, facilitating their delivery to the immune cells within the small intestine [148].

In the development of an oral influenza vaccine, researchers have utilized *Bacillus subtilis* spores, incorporating the sport coat protein of *B. subtilis* PY79 fused with three copies of the conserved matrix protein (M2e). M2e represents the ectodomain of the M2 protein, a proton channel present in the influenza virus, and is highly conserved across all human influenza virus A strains. Thus, it is a prominent target for universal influenza vaccine strategies [146]. The successful display of M2e on the spore surface leading to the generation of a recombinant spore (RSM2e3), which exhibited notable immunogenicity in mice. Upon repeated immunization, M2e-specific IgG responses were elicited with an impressive titer of 1:12,800 at week 17 post-1st immunization, alongside robust cellular immune responses. Following immunization, when mice were subsequently challenged with the A/PR/8/34 (H1N1) influenza virus, lung specimens from the vaccinated group demonstrated substantially reduced virus titers compared to those of the control group, indicating the effectiveness of the vaccine. Moreover, vaccinated mice exhibit a remarkable survival rate of 100% [146].

**Table 3: Approved and clinically tested vaccines using nanocarrier-based adjuvants and delivery systems [142]**

Product	Application	Adjuvants used	Approval year, company, status of research
<b>Viral vectored vaccines</b>			
ACAM2000	Smallpox	MVA-BN	2007, Sanofi Pasteur Biologics Co., Cambridge, MA, USA
Chimpanzee adenovirus vector (ChAdOx1)	Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), Coronavirus disease (COVID-19)	Chimpanzee Adenoviral vector	2020, University of Oxford in collaboration with AstraZeneca, Cambridge, UK
Sputnik V (Gam-Covid-Vac)	SARS-CoV-2, COVID-19	Replication-deficient Ad types 5 and 26 vectors	2020, Gamaleya Research Institute, Acellena Contract Drug Research and Development, Moscow, Russia
COVISHIELD™ (ChAdOx1)	SARS-CoV-2, COVID-19	Chimpanzee Adenoviral vector	2020, Serum Institute of India Pvt. Ltd., Pune, Maharashtra, India
Convidicea (Ad5nCoV)	SARS-CoV-2, COVID-19	Recombinant Adenoviral vector, Ad5	2020, CanSino Biologics, Tianjin China (approved for use in Mexico, China)
Janssen COVID-19 Vaccine (Ad26)	SARS-CoV-2, COVID-19	Adenoviral vector, Ad 26	2021, Janssen Biotech, Inc., Horsham, PA, USA (Emergency use authorization by US FDA)
<b>Virus like particles</b>			
Recombivax HB®	Hepatitis B Virus (HBV)	Amorphous aluminum hydroxyphosphate sulfate	1986, Merck and Co. Inc., Kenilworth, NJ, USA
Engerix-B	HBV	Aluminum hydroxide	1989, Glaxo Smithkline (GSK), Middlesex, UK
Gardasil®	Human papillomavirus (HPV), cervical cancer and genital warts	Hydroxyphosphate sulphate	2006, Merck and Co. Inc., Kenilworth, NJ, USA
Cervarix	HPV	AS04 (aluminum hydroxide and MPLA)	2009, Glaxo Smithkline Biologicals SA, Rixensart, Belgium
Hecolin	Hepatitis E Virus (HEV)	Aluminum hydroxide	2011, Xiamen Innovax Biotech, Xiamen, Fujian, China
Gardasil-9®	HPV	Hydroxyphosphate sulphate	2014, Merck and Co. Inc., Kenilworth, NJ, USA
Heplisav-B	HBV	1018 ISS CpG ODN	2017, Dynavax Technologies Corporation, Emeryville, CA, USA
Sci-B-Vac®	HBV	Aluminum hydroxide	2020 (under regulatory approval process) VBI Vaccines Inc., Cambridge, MA, USA
Mosquirixs	Malaria and HBV	AS01 (MPL and Quilajja saponaria 21 (QS21))	2015, GlaxoSmithKline Biologicals S. A., Rixensart, Belgium
<b>Virosome-based vaccine</b>			
Epaxal™	Hepatitis A virus (HAV)	IRIV	1994, Berna Biotech Ltd., Berne, Switzerland
Inflexal®V	Influenza vaccine	IRIV	1997, Berna Biotech Ltd., Berne, Switzerland
Invivac®	Influenza vaccine	IRIV	2004, Solvay Pharmaceuticals B. V., DA Weesp, The Netherlands
NasalFlu®	Influenza vaccine	IRIV	2001, Berna Biotech Ltd., Berne, Switzerland
Epaxal Junior™	Novel pandemic A influenza virus (H1N1)	IRIV	1994, Berna Biotech Ltd., Berne, Switzerland.
<b>Non-viral vectored vaccines</b>			
Celtura®	H1N1	MF59	2009, Novartis AG, Basel, Switzerland
Fluad®	Seasonal influenza in infants and young children	MF59	1997, Novartis AG, Basel, Switzerland Phase III Trials Completed 2010-11
Aflunov®	Pre-pandemic influenza (H5N1)	MF59	2010, Seqirus S. R. L., Monteriggioni, SI, Italy
Montanide	Malaria, HIV, cancer	MF59	Under clinical trial
FENDRIX	HBV	Aluminum phosphate and MPLA	2005, GlaxoSmithKline Biologicals., Rixensart, Belgium
Stimuvax®	Lung, breast, prostate and colorectal cancer	Liposome, MPLA	Merck KGaA, Darmstadt, Germany, Phase III Clinical Trial Completed
mRNA-1273	COVID-19	Liposome	2020, Moderna, Cambridge, MA, USA
BNT162b2	COVID-19	Liposome	2020, Pfizer, New York, NY, USA and BioNTech, Mainz, Rhineland-Palatinate, Germany
Prevnar®	Invasive Pneumococcal disease	Aluminum phosphate	2000, Wyeth Pharmaceuticals, Madison, NJ, USA
Menactra®	Meningococcal disease	Aluminum	2005, Sanofi Pasteur, Lyon, France

### Virus-like particles

Virus-like particles (VLPs) are self-assembling and non-replicating entities that lack infectious genetic material [149]. VLPs can be derived from diverse host cells, including bacteria, yeast, insects, and animal cell lines. Their application in vaccine development is two-fold, serving as particulate carriers and immunopotentiators owing to their immunogenic properties, such as resembling the size of the original pathogen, presenting repetitive surface geometry, and eliciting innate and adaptive immune responses [150]. The fundamental advantage of VLP-based vaccines lies in the ability of the host immune system to recognize VLPs in a manner analogous to an authentic virus, thereby provoking a robust immune response [151]. These vaccines are primarily engineered to activate B cells and trigger potent antibody responses through activation of T helper cells [152]. Several prophylactic VLP-based vaccines have obtained regulatory approval for human use, such as Cervarix®, Gardasil®, and Gardasil9®, targeting human papillomavirus (HPV), and the

third-generation Sci-B-Vac™ vaccine developed against hepatitis B virus (HBV) [153]. Furthermore, VLP-based strategies hold promise for the pursuit of a universal influenza vaccine [153].

### Bacteriophage VLPs

Phage VLPs offer a safe alternative for vaccine development, as they are non-pathogenic and do not elicit pre-existing immunity in humans [154]. These VLPs use phage capsid proteins to present peptides or proteins on the phage surface. The cargo capacity of phage VLPs varies depending on the phage type. For instance, capsid proteins 10A and 10 B of bacteriophage T7 can accommodate approximately 400 copies of peptides or proteins, ranging from 50 to 1200 amino acids. On the other hand, the gene VIII protein of the Ff phage allows for a higher display valency of up to 8000 copies on its filamentous structure, but it is limited to displaying peptides and small proteins. The gene III protein of the Ff phage, although having a lower copy number, exhibits greater tolerance for larger displays [155].

## Synthetic nanoparticles

### Virosomes

Virosomes, as lipid vesicles, have gained considerable attention in vaccine development owing to their unique composition. These vesicles incorporate virus-derived proteins and are devoid of the viral genome and internal proteins [156]. Membrane proteins incorporated into virosomes can be obtained using recombinant technology or purified from the corresponding viruses. Purification involves solubilizing the virus membrane using mild detergents without denaturation, followed by the removal of the nucleocapsid and other viral components through ultracentrifugation [157].

Virosomes, which are biodegradable and non-toxic, have emerged as promising entities in the field of vaccine development [158]. These vesicles offer several advantages over liposomes, particularly in terms of their ability to protect active substances from proteolytic degradation within the acidic endosomal environment, thereby enhancing their cytoplasmic delivery [159]. Moreover, virosomes demonstrate remarkable potential as adjuvants, exhibiting the ability to specifically target antigen-presenting cells and effectively stimulate both B- and T-cell responses against associated antigens, including surface hemagglutinin (HA) proteins [160]. One notable example of a virosome-based vaccine that exemplifies these characteristics is Inflflexal® V. This trivalent virosome subunit vaccine is commercially available and is suitable for administration across all age groups, highlighting the versatility and applicability of virosome technology in vaccine formulations [161].

### Immune-stimulating complexes

Immune-stimulating complexes (ISCOMs) have emerged as particulate adjuvant systems that hold great promise for vaccine development [162]. These complex structures consist of antigens, cholesterol, phospholipids, and saponins, forming hollow cage-like particles with a diameter of approximately 40 nm [163]. ISCOMs offer a unique advantage by combining the characteristics of a particulate carrier system with the presence of an inherent immunopotentiator known as Quil A. This intrinsic immunopotentiator attribute contributes to the enhanced immunogenicity exhibited by ISCOMs, surpassing that of liposomes [24].

Furthermore, ISCOMs have been shown to require lower quantities of antigen and other adjuvants to elicit a robust immune response in the host than simple mixtures of free antigen and saponins [164]. Standardized procedures are crucial for the formulation of ISCOM-based vaccines to ensure the production of high-quality vaccines with consistent batch-to-batch performances. One such important step involves separation and purification of a heterogeneous mixture of ISCOM components. Reversed-phase high-performance liquid chromatography (HPLC) has been successfully employed to achieve this, effectively eliminating potentially toxic fractions within the vaccine preparation [165].

The utilization of ISCOMs incorporating influenza viral proteins has demonstrated notable improvements in CD8<sup>+</sup> immune responses in both murine and human models [166]. Matrix M, a third-generation ISCOM adjuvant, has exhibited promising results in various studies. In a phase II clinical trial, Matrix M was employed as an adjuvant in combination with an H7N9 VLP vaccine, leading to significantly higher seroconversion rates compared to the non-adjuvanted VLP vaccine [167].

### Inorganic nanoparticles

The use of inorganic nanoparticles as adjuvants in vaccine development has garnered increasing interest [168]. Among inorganic NPs, gold nanoparticles (AuNPs) have gained attention because of their unique properties that enable the conjugation of target antigens or adjuvants onto their surfaces at high densities. Importantly, synthetic AuNPs composed of a natural element do not induce carrier-specific immunity upon immunization [169].

In a notable study, an AuNP-based vaccine candidate was developed by immobilizing the conserved M2e of the influenza virus onto AuNPs, along with cytosine phosphoguanine-oligodeoxynucleotides

(CpG-ODNs) as an immunopotentiator [170, 171]. This formulation demonstrated remarkable immunogenicity, inducing strong M2e-specific antibody responses and providing 100% survival in mice that were lethally challenged with the influenza A/PR/8/34 (H1N1) virus [170].

Further research demonstrated that the immunogenicity of AuNPs can be enhanced by co-delivery with flagellin, a bacterial component, as an immunopotentiator [171]. When gold nanoparticles coupled with the HA protein A/Aichi/2/68(H3N2) and flagellin (FliC) were co-delivered, stronger cellular immune responses were observed. Additionally, the addition of AuNPs-FliC improved mucosal B-cell responses, as evidenced by elevated levels of influenza-specific IgA and IgG in the nasal, tracheal, and lung washes. Furthermore, the AuNP-HA/AuNP-FliC formulation stimulated the proliferation of antigen-specific IFN- $\gamma$ -secreting CD4<sup>+</sup> cells [172].

## CONCLUSION

In this review, the different adjuvants employed in the development of subunit vaccines are comprehensively examined. The use of adjuvants has proven to be instrumental in enhancing the immunogenicity and efficacy of subunit vaccines, ultimately contributing to improved protection against infectious diseases and malignancies. Our examination focused on four major categories of adjuvants, namely liposome-based, carbohydrate-based, polymer-based, and nanoparticle-based adjuvants, while also providing an overview of vaccine adjuvants in general and exploring their molecular mechanisms of action.

Adjuvant development for subunit vaccines holds tremendous promise. The integration of advanced technologies such as nanotechnology and synthetic biology presents exciting opportunities for the design of next-generation adjuvants. The field of adjuvant research for subunit vaccines continues to evolve, offering new possibilities for enhancing vaccine efficacy and broadening immunization strategies. The utilization of liposome-based, carbohydrate-based, polymer-based, and nanoparticle-based adjuvants has demonstrated remarkable potential for improving immune responses. While challenges and research gaps remain, ongoing efforts in adjuvant development coupled with interdisciplinary collaborations and cutting-edge technologies hold the promise of revolutionizing vaccine design and improving global public health outcomes.

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## AUTHORS CONTRIBUTIONS

Fredmoore Orosco: Conception and design of the study, wrote the first draft of the manuscript, critically revised the manuscript, funding acquisition.

Llewelyn Moron-Espiritu: Wrote the first draft of the manuscript, revised the manuscript

## CONFLICT OF INTERESTS

The authors declare no conflicts of interest.

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