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Carbohydrate-based adjuvants

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Carbohydrate adjuvants are safe and biocompatible compounds usable as sustained delivery systems and stimulants of ongoing humoral and cellular immune responses, being especially suitable for the development of vaccines against intracellular pathogens where alum is useless. The development of new adjuvants is difficult and expensive, however, in the last two years, seven new carbohydrate-based adjuvants have been patented, also there are twelve ongoing clinical trials of vaccines that contain carbohydrate-based adjuvants, as well as numerous publications on their mechanism of action and safety. More research is necessary to improve the existent adjuvants and develop innovative ones.

Introduction

Vaccination is a strategy to fight diseases that consists of the induction of a specific pathogen-immune response by the administration of an attenuated pathogen or its antigens which leads to protective immunity against the pathogen over time. These triggered defences against the target pathogen are: (1) antibodies produced by type B lymphocytes that bind to the specific exogenous molecule (humoral immunity); (2) the cytotoxic CD8+ type T lymphocytes that recognize and kill infected cells or secrete antiviral cytokines (cellular immunity); and (3) CD4+ T helper lymphocytes (Th) that produce cytokines and supports B (usually Th2) and T lymphocytes (mostly Th1) [1]. In this context, an immunological adjuvant is a compound that enhances and modulates the capacity of an antigen to generate an immune response by increasing the response and lengthens the memory of the immune response. Adjuvants make a great impact in public health enhancing immune coverage and adding to vaccine development since less intrinsic components from the pathogen are necessary [2].

The most used adjuvants are aluminium-based, and although there are many types of adjuvant molecules few are approved, due to the necessity to find the right quali/quantitative match between the antigen and the adjuvant. It is possible to select an adjuvant that stimulates a specific immune pathway or combine different adjuvants. Carbohydrate adjuvants can be delivery systems helping the uptake of the antigen by the antigen-presenting cells like some plant extracts [3]. They can be exogenous immunoactive microbial compounds such as TLR4 agonist [4,5] or other sources like chitosan [6]. Immunoactive carbohydrate adjuvants enhance the immune response as agonists of the Toll-like receptors (TLRs) [7–9], nucleotide-binding oligomerization domain-containing protein 2 (NOD2) [8], C-type lectins (CLR) [10,11] or the CD1d-dependent natural killer T (NKT) [12] which then stimulates the production of cytokines [12,13]. In addition, adjuvants can be both sustained delivery systems and immune response enhancers, like poloxamer 407-chitosan system [14]. These signalling cascades end up generating antigen-specific T- and B-cell responses which aluminium

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cannot produce [9], making them suitable for the development of vaccine-formulations against intracellular pathogens such as Chlamydia, Herpes Zoster, human immunodeficiency virus (HIV), human papillomavirus (HPV) and hepatitis B virus (HBV) [15] or other bacteria such as Group A and B Streptococcus [16,17].

Advantages of the use of carbohydrates as adjuvants include their capacity to play crucial roles in the immune system, as well as their safety and tolerability. Carbohydrates are not accumulated in the body like the alum, because they are easily metabolized and excreted, avoiding any negative effects of excessive or prolonged immune activation time because of the adjuvant [18]. In addition, carbohydrates have shown to have less side effects than alum, such as the development of allergies [19] and do not carry the negative public concerns of aluminium adjuvants [20].

Carbohydrates are ubiquitous and present in all life forms (including viruses), as they play a critical role in energy production and as structural and protection materials. But, also glycans can act as virulence factors or be involved in cellular recognition as antigens [21]. Nevertheless, the development of carbohydrate adjuvants is very challenging especially as aluminium is cheap, and its safety has already been established. Indeed, carbohydrate chemistry can be recalcitrant making the manufacturing scale-up complex and expensive; also, purification of natural carbohydrates is difficult, making hard to obtain homogeneous compounds.

This review intends to be an update on the current situation of carbohydrate adjuvants development by examining the patents, clinical trials and publications from the last two years.

Lipid A

The lipid A consists on a β-(1→6)-linked diglucosamine backbone with different patterns of acylation at the amino and hydroxyl (3 and 3′) functions and different levels of phosphorylation. It is the immunostimulant part of the lipopolysaccharide (LPS), the main component of the outer membrane external leaflet of Gram-negative bacteria. The lipid A stimulates the immune system by activation of the TLR4/MD-2 complex that mediates gene expression and pro-inflammatory cytokine secretion, working as exogenous immunoactive compound from microbial source. This activation is structure-dependent, determined by the phosphorylation and acylation patterns, and it is demonstrated that the phosphate is essential for the homodimerization of the TLR4/MD-2 complexes [22]. The bisphosphorylated hexa-acylated lipid A specie with a 4 + 2 distribution of the acyl chains (*E. coli* like) (Fig. 1) is the most toxic, its recognition by TLR4 and the following downstream cascade can lead to sepsis [22]. Bacteria can modify their lipid A, for example by removing fatty acid chains or adding decorations to neutralize the phosphates, changing the molecule from agonist to partial agonist or antagonist, which is seen in the lipid A from bacteria in the gut microbiota [7].

A combination of LPS, Pam3Cys (TLR1/TLR2 agonist) and poly(I:C) (TLR3 agonist) has been used as the adjuvant of the formalin-inactivated vaccine for respiratory syncytial virus in mice. The formulation containing LPS was superior as the presence of LPS inhibits the excessive inflammation in the lungs while allowing antibody production [23].

The O-deacylated lipooligosaccharide from *E. coli* J5 is a potent adjuvant used in combination with alum or liposomes. When administered with Japanese encephalitis vaccine, it induces a significant level of serum IgG and virus-neutralizing antibodies. In addition, antibody titers after immunization were better when using any kind of O-deacylated LPS combination, compared to alum-only adjuvated vaccine [24]. Some partially O-deacylated commercial LPS are: Monophosphoryl 3-Deacyl Lipid A (3D-PHAD®) and 3-Deacyl Monophosphoryl Hexa-acyl Lipid A (3D-(6-acyl) PHAD®) synthetic molecules. 3D-PHAD® and 3D-(6-acyl) PHAD® augment antibody production of HPV and HBV vaccines [15].

The Mono-Phosphoryl Lipid A (MPL) is an adjuvant originally made from the hepta-acylated lipopolysaccharide of *Salmonella minnesota* R595. Although there are currently numerous lipid A derived adjuvants that have been synthesized and commercialized, the most used is the mono phosphorylated and C14:0 and C14:0 (3-OH) penta-acylated lipid A (Fig. 1). The lack of the phosphate at position O-1 of the reducing glucosamine, in addition to the different acylation pattern from *E. coli* lipid A reduces the endotoxicity capacity. HIV-1 and HBV vaccine candidates containing MPL are in the advanced steps of clinical trials (CT) (NCT03961438; NCT03408262; CT04066881; EUCTR2016-004991-23-ES; Table 1). MPL is also a component of new adjuvant formulations, one of them has recently passed animal and Phase 1 CT (NCT01657929; Table 1) and another one was just patented (WO 2017068482; Table 1). MPL has also been combined to 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine in the H5-VLP pandemic influenza vaccine antigen [25].

Some adjuvant formulations that contain MPL have already been approved and are widely used such as AS01, AS02 and AS04. AS01 is a liposome containing MPL and QS-21 (*vide infra*) and seems to be a good candidate for vaccines against malaria (NCT03917654 and NCT03143218; Table 1). AS02 is a squalene-containing emulsion of MPL and QS-21, recently used for malaria [5], *Campylobacter jejuni* [5] and HIV (NCT03122223; Table 1) vaccines. AS04, a MPL and alum salts combination, is used for HPV Bivalent (Types 16, 18) Recombinant Vaccine (EUCTR2017-000416-42-Outside-EU/EEA; Table 1) and HBV vaccine.

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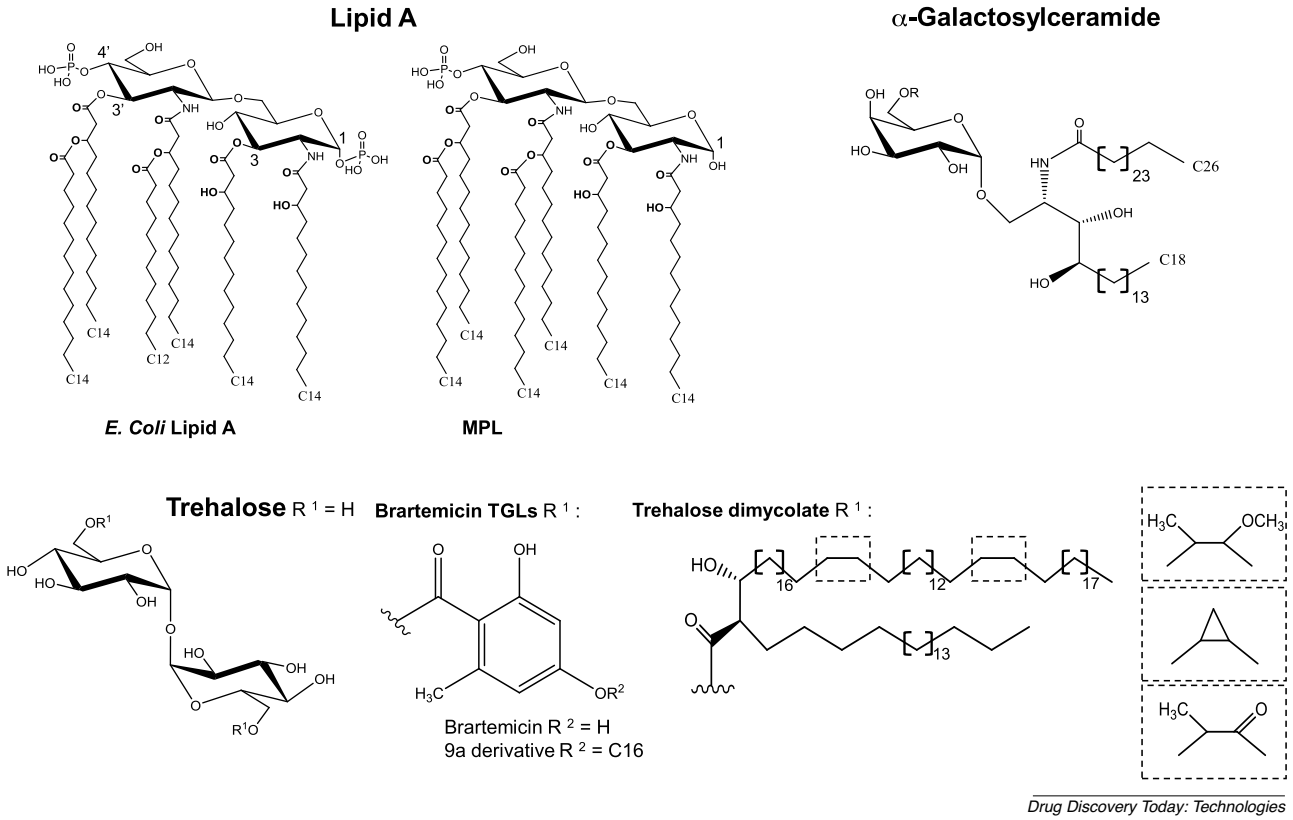

Fig. 1. Glycolipid-based adjuvants. *E. coli* lipid A (PubChem CID 9877306), Monophosphoryl lipid A (PubChem CID 151110) and Trehalose (PubChem CID 7427), and Galactosylceramide [12].

Lipid A and outer membrane vesicles

Bacterial outer membrane vesicles (OMV) are nanometric proteoliposomes mostly derived from the outer membranes of Gram-negative bacteria. They are ubiquitous structures secreted during the host infection (or *in vitro*), and they play key roles during host-pathogens interactions. These proteoliposomes are composed of LPS, peptidoglycan, phospholipids, and proteins. Due to the diverse library of different Pathogen-associated molecular patterns (PAMP) and specific bacterial antigens, OMV have been explored as antigen carriers triggering humoral and cellular immune responses [26]. In addition, OMVs can simultaneously induce the up-regulation of pro-inflammatory cytokines skewing a Th1 phenotype and triggering specific adaptive immune responses against the protein antigens contained in the OMV. These adjuvants-like properties are linked with the OMV LPS, making these structures a valid carrier for different antigens delivery [27].

Engineered OMV have been recently produced with a modified (truncated) LPS structure to attenuate their pro-inflammatory behaviour [26] for example, *E. coli* attenuated OMV have been designed as a carrier for H1N1 influenza antigens [28] and different bacterial OMV have been recently explored as a carrier for cancer antigens in preclinical studies [29] Table 2.

Other glycolipids

Trehalose glycolipids

Trehalose is α-D-glucopyranosyl-(1→1)-α-D-glucopyranoside (Fig. 1) with 6-OH groups which can be ester linked to the lipophilic chains producing trehalose glycolipids (TGLs). The 6,6′-trehalose diesters can bind and activate macrophage inducible C-type lectin (Mincle), leading to the induction of the Syk-Card9-Bcl1-Malt1 signalling pathway and a Th1 and Th17 immune responses [10]. Recently, it has been discovered that the diester trehalose dimycolate (Fig. 1) is toxic when presented in monolayer hydrophobic surface, but not when it occurs in micelles or on bacteria [10]. Moreover, within this study a row of different TGLs was tested, proving that physicochemical presentation of these lipids (micellar solutions, coated on plates, coated on beads or surfactant solubilized) influences the cytokine response by bone marrow derived macrophages. However, diverse presentation modes (micelles, plate, beads or solubilized) alter the activation of each synthetic TGL differently. It was also clear, that medium to long-chain TGLs, either coated on plates or surfactant solubilized, resulted in the highest activation of the macrophages and TGLs coated on beads had a smaller cytokine response [10].

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Table 1. Patents and clinical trials involving carbohydrate-based adjuvants (2017/2019).

Family	Carbohydrate-based molecule	Role in formulation	Product patented	Year	Organization	Patent number
Chitosan	9012-76-4P sulfated chitosan	Immunostimulant	Adjuvant	2018	Chinese Academy of Sciences, China	CN 107648603
Chitosan and inositol	9012-76-4 Chitosan 24939-03-5 Poly IC	Immunostimulant	Adjuvant	2017	China	WO 2017080098
Novel plant polysaccharide	β-D-Glucopyranosiduronic acid derivative	Immunostimulant	Cancer vaccine	2019	Imugene Limited, Australia	WO 2019153042
Glucan	9005-25-8 Starch 9005-82-7 Amylose	Immunostimulant	Adjuvant	2017	National Autonomous Uni., Mexico	MX 2016005434
Lipid A	1246298-63-4 MPL	Immunostimulant + Sustained rel.	Adjuvant	2017	Cadila Healthcare Limited, India	WO 2017068482
Peptidoglycan	Muramyl dipeptide derivative	Immunostimulant	Adjuvant	2017	Bharat Biotech Int., India	WO 2017098529
Saponins	Ophiopogonis Radix saponin (OP-D)	Immunostimulant	Adjuvant	2018	PLA Army Medical Uni., China	CN 108853493
Sucrose	9013-95-0 Levan (6-kestose)	Immunostimulant	Adjuvant	2017	The Institute of Food Research, UK	GB 20165287

Molecule	Aim of CT	CT Phase	Product studied	Completion year	Organization	Registration number
Dextran	Vaccine test	Phase II/III	BCG vaccine	Unknown	Serum Institute, India	CTRI/2017/03/008266
Dextran	Vaccine test	Phase III	BCG vaccine	2018	London School of Hygiene and Tropical Medicine, UK	ISRCTN11311670
MPL-DPPC	Adjuvant test: Immunostimulant + Sustained release	Phase I	H5-VLP for avian influenza virus	2018	Infectious Diseases Research Institute, USA	NCT01657929
MPL	Vaccine test	Phase I	ACTHIVE-001 against HIV-1	2021	Universiteit van Amsterdam (AMC-UvA), Netherlands	NCT03961438
MPL	Vaccine test	Phase I	Ad4HIV against HIV	2020	Imperial College London, UK	NCT03408262
MPL	Vaccine test	Phase III	Hepatitis B	2020	Instituto de Investigación Biomédica de Salamanca, Spain	EUCTR2016-004991-23-ES
MPL	Vaccine test	Phase III	DNA/AIDSVAX and DNA/CN54gp140 against HIV-1	2020	MRC/UVRI Uganda Research Unit on Aids, Uganda	NCT04066881
AS01	Vaccine test	Phase II	Pfs230D1M-EPA/AS01 or malaria	2020	National Institute of Allergy and Infectious Diseases (NIAID), USA	NCT03917654
AS01	Vaccine test	Phase IIIB	RTS,S/AS01 and SP/AQ for malaria	2020	London School of Hygiene and Tropical Medicine, UK	NCT03143218
AS02	Vaccine test	Phase I/IIa	ALVAC-HIV (vCP2438) and of MF59®- for HIV	2019	National Institute of Allergy and Infectious Diseases (NIAID), USA	NCT03122223
AS04	Vaccine test	Phase IIIb	HPV-16/18 L1 AS04 vaccine	Unknown	GlaxoSmithKline Biologicals, Belgium	EUCTR2017-000416-42-Outside-EU/EEA
AS04	Vaccine test	Phase IV	CERVARIX for HPV	2018	Federal University of Rio Grande do Norte, Brazil	Not registered

Clinical trials (CT); Phase I: Human pharmacology and safety; Phase II therapeutic exploratory; Phase III therapeutic confirmatory/Efficacy; Phase IV Therapeutic use; MPL: Monophosphoryl lipid A; DPPC: Dipalmitoylphosphatidylcholine.

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Table 2. Summary of findings.

	Structure	Novelties	Action pathway
Lipid A	β-(1→6)-linked 1-4′-bisphosphorylated diglucosamine backbone	Modified acylation and phosphorilation [23–25]	TLR4/MD-2 complex
Trehalose	α-D-glucopyranosyl-(1→1)-α-D-glucopyranoside	Trehalose glycolipids: lipophilic chains on OH [10]	Mincle, Th1 and Th17
Galactosylceramide	Galactose α-linked to a ceramide	α- or β-linked monoglycosylceramides [12]	CD1d-dependent Natural killer T
Peptidoglycan	GlcNAc β-1→4 MurNAc + peptide chain	Muramyldipeptide derivative VIII: N-acetyl-muramyl-L-alanine-D-isoglutamine branched [WO 2017098529]	NOD2 and TLR
Chitosan	Partial de-acetylation of chitin β-D-GlcN (1→4) GlcNAc	Sustained delivery systems [14,31] Intraocular [33] Buccal mucosa [34] Nasal [6] Aminated and aminated-thiolated chitosan polymers nanocarriers [6] Methylation: Trimethyl chitosan [16,32] Sulfation of the amino or hydroxyl functions (CN 107648603)	Cytokine binding Dectin-1 and TLR-2
Glucans	D-Glucose derived polysaccharides	Dextran with cyclic dinucleotide 3′3′-cGAMP [36] Zymosan for lung administration [38]	Interferons, Th1, B cells and memory T cells $CD8^+$ T into lung memory T cells
Inulin	Linear β-(2→1)-D-polyfructofuranosyl-α-D-glucose	Advax™, microcrystalline polysaccharide from δ-inulin [41]	Humoral and cellular responses
Mannans	Linear polymers of (1→4) mannose	Lipomannan: α-(1→6) mannose with phosphatidylinositol mannoside at reducing end, Regio- and stereo-controlled synthesis, antigens on amine at the reducing end [46]	TNF-α, IL-8, IL-12, apoptosis in macrophages and Th1 cell polarization
Alginate	β-D-ManA(1→4)-α-L-GulA(1→4)	Oral delivery of antigens [48]	Resist acid pH
Saponins	Sapogenin/aglycone + saccharide chains attached	Glabilox: isolated from *Glycyrrhiza glabra* [49]	Increase levels IgA, IgG and IgM and Th
Novel polysaccharide	Plants can be good sources of new polysaccharides; however non purified preparations can be toxic	Isolated from *Angelica sinensis*: delivery system [3] *Alhagi pseudalhagi* [52] Flaxseed hull polysaccharide (FP-1) [55]	Th1 and Th2 responses Lymphocyte proliferation, IgG levels, Th1 polarization TNF-α, nitric oxide, and IL-6 and IL-12

Brartemicin TGLs and derivatives containing long-chain lipids also open a promising path as adjuvants, as they are strong agonists of Mincle and recent results support that the aromatic groups play an important role on this interaction. The so called 9a derivative (Fig. 1) has superior *in vivo* adjuvant activity because of the Th1 response that generates [11].

Galactosylceramide

α-Galactosylceramide (α-GalCer) is composed of galactose α-linked to a ceramide comprised of a C26:0 acyl chain and 18-carbon phytosphingosine chain (Fig. 1). While the ceramide interacts with the glycoproteins that present lipid antigens to T cells CD1d, the α-galactose is exposed to interact with the receptor of the CD1d-dependent Natural killer T (NKT) cells, leading to the secretion of IFN-γ, IL-4, and IL-13 cytokines [12,13]. α-GalCer has already been used for immunization strategies such as intragastric immunization with whole-cell killed *H. pylori*. This leads to vigorous intestinal and systemic Th1 responses [13]. Also, when combined with B cells and early secretory antigenic target-6, α-GalCer is effective for the vaccine and treatment against *Mycobacterium kansasii* [30].

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Although α-GalCer is very active, the use of other derivatives should also be considered, like α- or β-linked monoglycosylceramides. The recognition greatly depends on the configuration, and normally β-linked monoglycosylceramides are not recognized by NKT even though with the β-mannosylceramide similar effects can be generated [12].

Peptidoglycan

Peptidoglycan (PG) is a protective layer that surrounds the cytoplasmic membrane of both Gram-positive and Gram-negative bacteria, although it is significantly thicker in Gram-positive bacteria. PG is composed by linear chains of a repeating N-acetyl-glucosamine β-1→4-linked to N-acetyl-muramic acid (MurNAc) disaccharide. MurNAc has a peptide chain usually formed by five aminoacids in the following order: L-Alanine-D-Isoglutamine-L-Lysine-(or meso-diaminopimelic acid-)-D-Alanine-D-Alanine. Generally, the third aminoacid is L-Lysine in Gram-positive and meso-diaminopimelic acid in Gram-negative bacteria. The L-Lysine is usually chemically linked with aminoacids from another peptidoglycan strand to form the PG 3-D network (Fig. 2) [8].

Muramyldipeptide is N-acetyl-muramyl-L-alanine-Disoglutamine (Fig. 2) and it is a natural component of the PG of mycobacteria. As a bacterial component, it activates NOD2 and TLR receptors, leading to potent activation of NF-κB and inflammatory cytokine production [8]. Although, many analogues have been developed to be used as vaccine adjuvants, their high toxicity has limited their usage. Recently a novel muramylpeptide derivative compound called VIII (Fig. 2) has been developed and seems promising (WO 2017098529; Table 1).

Chitin/chitosan

Chitin is a linear polymer of β-1→4 N-acetyl-D-glucosamine (Fig. 3) that is present in the exoskeletons of arthropods. The result of its partial de-acetylation is known as chitosan (Fig. 3). These polymers stimulate cytokine production, leucocyte recruitment, and alternative macrophage activation by binding TLR2 receptors producing high levels of IL-2 and IFNγ [6]. Chitosan is more commonly used than chitin as a vaccine adjuvant since its bioadhesive properties and solubility are more convenient for antigen delivery. Chitosan is used in many formulations with a novel formulation recently patented (WO 2017080098; Table 1).

In addition, chitosan slowly releases drugs, making it optimal for sustained delivery systems. For example, an intradermal composite microneedle has recently been developed which is composed of two parts: a sodium hyaluronate tip that rapidly dissolves within the skin releasing the encapsulated antigens; and a chitosan base that prolongs antigen

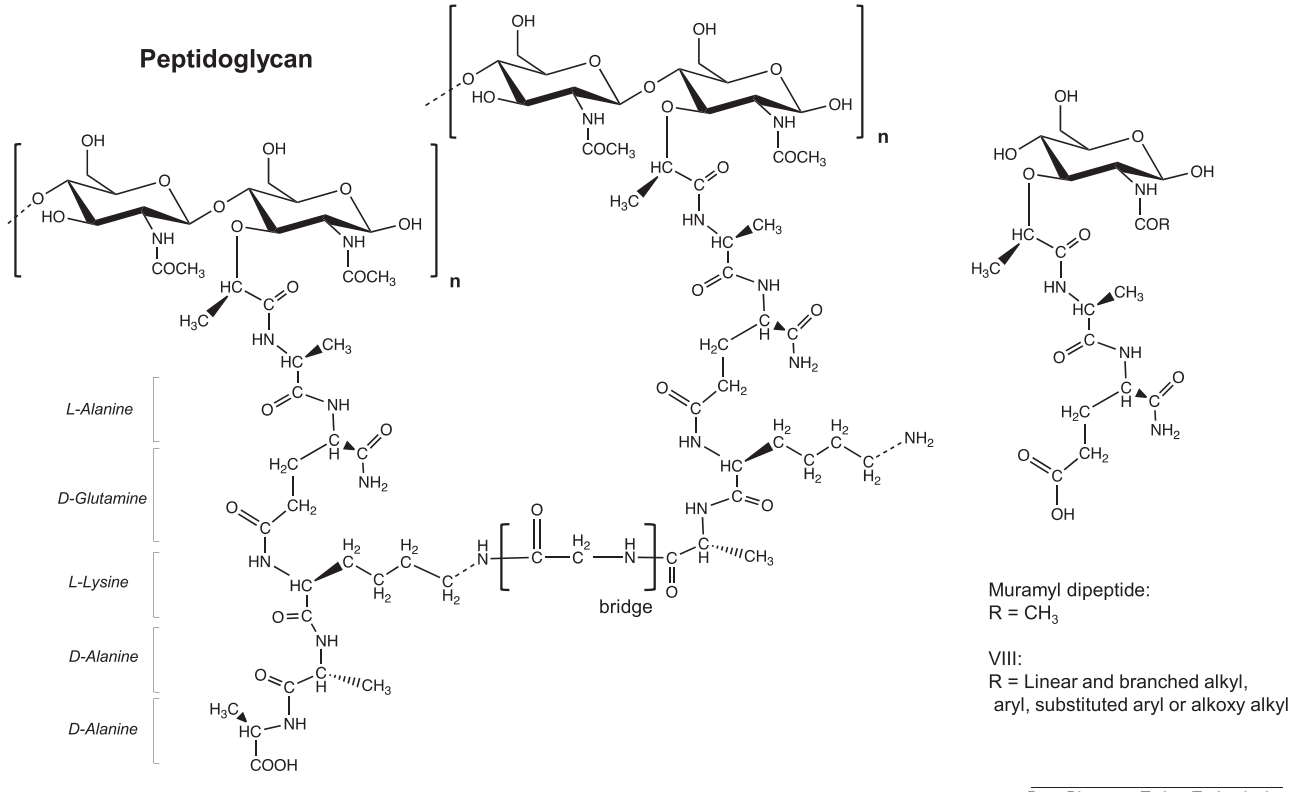

Fig. 2. Peptidoglycan (left) and peptidoglycan derived adjuvants structure (right) [8 and WO 2017098529].

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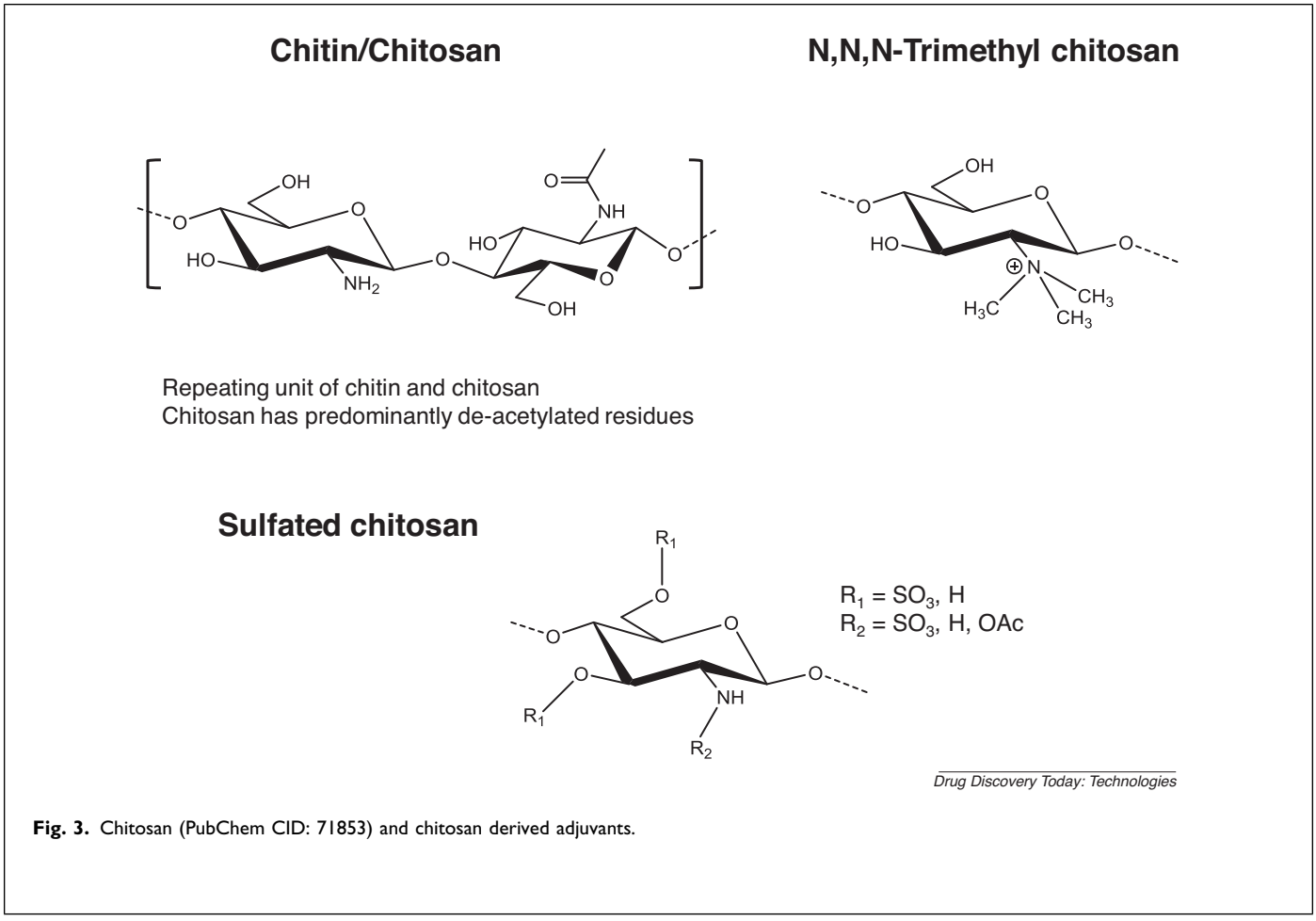

Fig. 3. Chitosan (PubChem CID: 71853) and chitosan derived adjuvants.

release for 4 weeks stimulating both Th1 and Th2 responses [31]. In a new thermoresponsive hydrogel, a copolymer of chitosan and Poloxamer 407 achieves a complete antigen release after 18 days inducing uninterrupted cellular and humoral responses [14].

Chitosan also have mucoadhesive qualities making it ideal for nasal vaccines. It can open the space between the nasal epithelial cells, facilitating the antigen intake, in addition to the previously mentioned immunostimulatory properties [6]. One advantage is that it is suitable for chemical modifications that improve its properties. For example, aminated and aminated plus thiolated chitosan polymer nanocarriers exhibit great potential for nasal application of vaccines [6]. Trimethyl chitosan (Fig. 3) increases the mucoadhesive properties and has been included in formulations for protection against group A streptococcus [16] and influenza virus vaccine [32], producing long lasting humoral and cellular immune responses in mice. Another modification is the sulfation of the amino or hydroxyl functions; indeed, 6-O-sulfated chitooligosaccharide and 2-N,6-O-sulfated chitooligosaccharide (Fig. 3) have recently been patented as potential vaccine adjuvants (CN 107648603; Table 1).

Chitosan can be used as adjuvant for intraocular administration. Protection against *Mycoplasma gallisepticum* respiratory disease in poultry has shown to be more effective the intraocularly than the intramuscular vaccine [33]. In addition, chitosan is used for administration through the buccal mucosa for successful caries prevention [34].

Glucans

Glucans are D-glucose derived polysaccharides widely extended in nature. Variations on the chemical structure depend on the organism from which it is isolated, and some organisms produce a blend of different glucans. They can be α-configured such as dextran (α-1,6-glucan), glycogen (α-1,4- and α-1,6-glucan), pullulan (α-1,4- and α-1,6-glucan) and starch (α-1,4- and α-1,6-glucan) or β-configured like cellulose (β-1,4-glucan), curdlan (β-1,3-glucan), laminarin (β-1,3- and β-1,6-glucan), chrysolaminarin (β-1,3-glucan), lentinan (purified β-1,6:β-1,3-glucan from *Lentinus edodes*), lichenin (β-1,3- and β-1,4-glucan), pleuran (β-1,3- and β-1,6-glucan isolated from *Pleurotus ostreatus*) and zymosan (β-1,3-glucan from *Saccharomyces*). Glucans bind to lectins activating humoral and cellular immunity, in particular, β-glucans are recognized by TLR2 receptors and dectin-1 of macrophages and dendritic cells leading to Th1/Th17 differentiation [35], although the literature is still discussing this phenomena due to contamination during the isolation of the polysaccharide. Moreover,

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nanoparticles with aminated β-glucans act as carrier and immunopotentiators without toxicity [9].

Dextran is one of the most used glucan adjuvants. In a new intramuscular vaccine platform, cyclic dinucleotide 3′,3′-cGAMP (stimulator of interferon genes agonist and adjuvant of interest) was encapsulated in acid-sensitive acetalated dextran polymeric microparticles. This formulation was superior to any other as it managed to intracellularly deliver the stimulator of interferon genes agonist, enhanced type-I interferon responses nearly 1000-fold *in vitro* and 50-fold *in vivo*. It also increased Th1-associated responses, and leaded to robust expansion of germinal centre B cells and memory T cells, against influenza virus infections [36]. In addition, there are currently two ongoing CT in advanced phases on the already commercialized BCG vaccine for the prevention of tuberculosis recurrence and the possibility of adding killed *Mycobacterium leprae* to the BCG vaccine to protect against leprosy (CTRI/2017/03/008266 and ISRCTN11311670; Table 1).

New adjuvant patent on raw starch microparticles to be used in mucosal or systemically was also deposited (MX 2016005434; Table 1).

Curdlan particles alone or in combination with chitosan could be used as adjuvant for vaccines against the HBV as it is capable to generate a specific cytokine-mediated immunity with Th1, Th2, Th17, Th22, and T-regulatory immune responses [35]. Curdlan can also activate dendritic cells through dectin 1 and TLR4. Curdlan sulfate is a soluble derivative of curdlan but its negative charge limits its adsorption. For this reason, curdlan sulfate-O-linked-(2-Hydroxyl) propyl-3-trimethyl ammonium chitosan chloride was synthesized enabling proteins or peptides delivery and showing specially promising results as nasal mucosal immunoadjuvant [37].

Zymosan shows $CD8^+$ T cells differentiation into tissue-resident memory T cells in the lung, presumably because it creates an anti-inflammatory environment likely to support Trm development. This provides a frontline defence against respiratory pathogens and has potential to be another ideal adjuvant for intranasal vaccines [38].

Inulin

δ-inulin is a polymer consisting of linear β-(2→1) linked D-fructosyl residues with a α-D-glucose end group that can vigorously stimulate humoral and cellular immune responses and can be conjugated with antigens to increase the vaccine protective effect [39]. It can also be decorated with mannan to create microparticles to work as carriers and immunostimulants for immunization against foot-and-mouth disease [40].

Advax™, a novel microcrystalline polysaccharide particle engineered from δ-inulin, provides robust adjuvant potency together with tolerability and safety. It has recently been evaluated as mucosal adjuvant for whole inactivated influenza vaccine administered in liquid or dry powder formulations. Advax-adjuvanted inactivated influenza vaccine generated memory B cell responses and increased lung localization factors [41].

Mannans

Mannans are linear polymers of (1→4) linked mannose residues. They activate the immune system by binding to mannan-binding lectin and other C-type lectins of the mannose receptor family [42]. They can be used as adjuvants in natural or oxidized forms. Mannan derivatives can be covalently linked to antigens in order to adjuvate humoral and cellular immunity as in the new vaccine against Leishmaniosis [43] or linked to muramyl peptide derivatives [44].

The β-1→2-mannan oligosaccharides are components of *Candida albicans* cell wall, that when covalently linked to immunologic stimulants, like tetanus toxoid, induce humoral responses and antibodies production. Recently, it has been postulated that the β-1→2-mannan and N-terminal peptide epitopes of *Candida albicans* could be used as antigens with self-adjuvant properties to develop a vaccine against candidiasis [45].

Lipomannan is a glycolipid with a repeating unit of α-(1→6) mannose, present in the surface of *Mycobacterium tuberculosis,* which is connected to a phosphatidylinositol at the reducing end. This group of mannan can stimulate TNF-α, IL-8, IL-12 production, apoptosis in macrophages and Th1 cell differentiation and their derivatives can be now synthesized in a regio- and stereo-controlled way [46].

Alginate

Alginic acid (E400) is a polysaccharide composed by a β-D-ManA(1→4)-α-L-GulA(1→4) repetitive disaccharide unit and is present in cell wall of brown algae. Alginate is hydrophilic and can be used to encapsulate antigens, for example it has shown excellent results for vaccines against mycobacterial infections [47].

In addition, alginates resist acid pH, making them excellent candidates as carrier for oral delivery of protein antigens since they can protect from the premature release of the antigens and drugs in the stomach [48].

Saponins

Saponins are amphipathic glycosides, composed by a sapogenin/aglycone to which saccharide chains are attached in varying number and length. Saponins, and in particular those obtained from *Quillaja saponaria* the QS saponins, have been used as vaccine adjuvants for long time. Due to the amphipathic qualities of saponins, they can bind to lipids and glycoprotein antigens forming virus-like nanostructures and consequently improving the presentation of the antigen [49].

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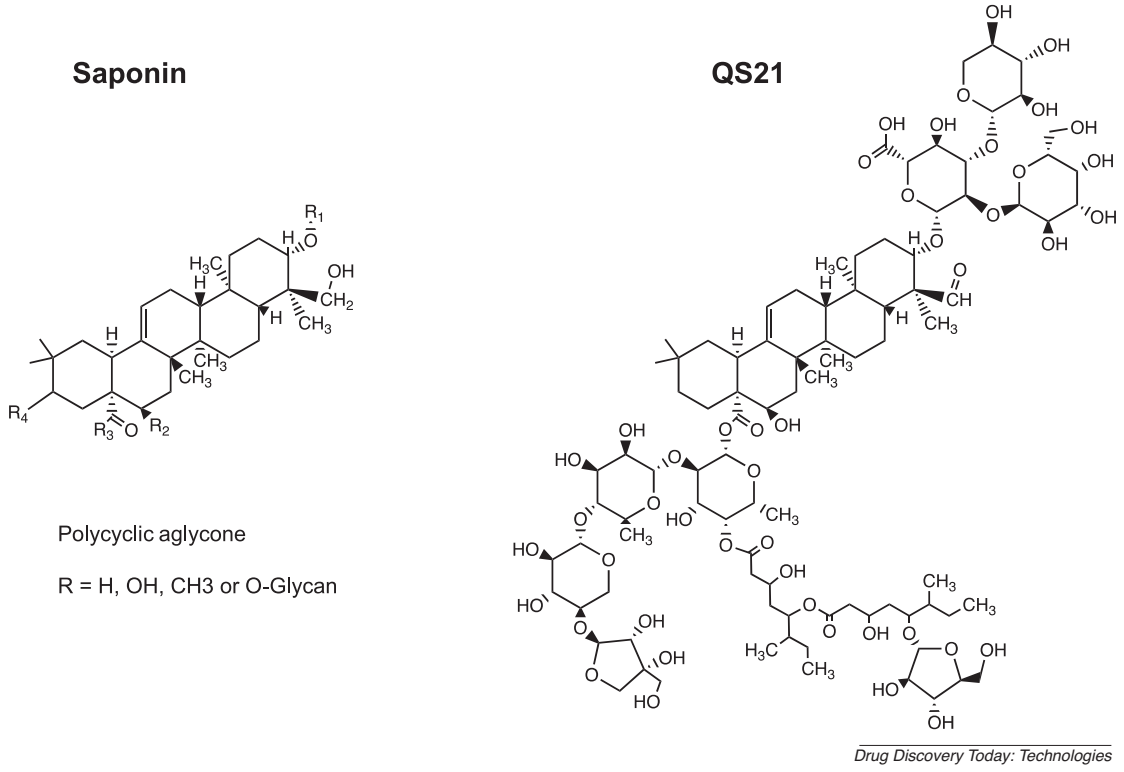

Fig. 4. Structure of a general saponin consisting on a polycyclic aglycone with different glycan substituents (left) and structure of the commercial adjuvant QS21(right, PubChem CID: 72809956).

QS21 saponin (Fig. 4) is one of the most used saponin adjuvants, thanks to its capacity to stimulate the Th2 humoral and Th1 cell immune responses. It is present in vaccine formulations against Herpes Zoster, HIV, malaria and cancer [50]. QS21 has a unique synergy mechanism with MPL (Monophosphoryl Lipid A), and usually can be found together formulated in a liposome (AS01) or emulsion (AS02); see Lipid A section [5,50].

AbISCO 100 is a commercial mix of highly purified saponins containing Matrix A (QS7) and C (contains QS21). AbISCO 100 has recently been used in a vaccine against Group B *Streptococcus*, obtaining for the first time significant cellular immunity associated with the surface Immunogenic Protein antigen of Streptococcus [17].

Recently a new saponin mix, Glabilox, was isolated from the roots of *Glycyrrhiza glabra*. Glabilox in combination with lipids and glycoproteins of H7N1 influenza virus produced high titres of IgA, IgG and IgM, and additionally the response of T helper lymphocytes without toxic effects [49].

Astragaloside VII, a immunostimulant triterpenoid saponin isolated from *Astragalus trojanus*, has been used in a newly developed nanocarrier system on seasonal influenza A (H3N2) vaccine showing Th1 and Th2 response and production of IFN-γ, IL-17A and IgG2a [51].

Novel plant polysaccharides

Plants are good sources of new polysaccharides, however non purified preparations can be toxic due to their high content of alkaloids, flavonoids, tannins, and others.

A new polysaccharide has recently been isolated from *Angelica sinensis* which consists of a (1→3)-Gal*p*-(1→6)-Gal*p*OMe→ backbone repeating unit, with the latter monosaccharide in turn linked at *O*-3 to Glc*p*A or Ara*f* monosaccharides. This polysaccharide was encapsulated into poly (lactic-co-glycolic acid) nanoparticles to build a new delivery system which produces strong and sustained Th1 and Th2 responses [3].

A polysaccharide obtained from the secreted fluid of the leaves of *Alhagi pseudalhagi*, also appears to be a good candidate as an adjuvant, although it is unstable over time and difficult to obtain in appropriate amounts. Its structure consists of a heteropolysaccharide based on rhamnose, mannose and galactose [52].

The polysaccharide mix from Purslane plants (POL-P3b), enhances the effectivity of the foot-and-mouth disease vaccine when administered orally with food before the immunization. It has been hypothesized that it acts by promoting intestinal dendritic cell maturation [53].

A polysaccharide derived from *Poria cocos* (PCP-I) comprising fucose, mannose, glucose and galactose enhances the

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immunogenicity and protection of an anthrax vaccine formulation amplifying the production of specific antibodies and memory B cells, proliferation of specific splenocytes, stimulation of the secretion of IL-4 and the activation of dendritic cells [54].

A novel hetero-polysaccharide from *Linum usitatissimum* (FP-1), with a backbone of 2-α-L-Rha*p*, 4-α-D-Gal*p*A, 4-β-D-Xyl*p*, 3,5-α-L-Ara*f* and 2-α-D-Xyl, induces expression of TNF-α, nitric oxide, and IL-6 and IL-12 in murine macrophages; FP-1 also inhibits hepatitis B virus growth and potentially can be considered as an immunostimulant vaccine adjuvant [55].

Vegetables are also a huge potential area for the discovery of new carbohydrate adjuvants. New formulations for vaccines against *Helicobacter pylori* include the epithelium polysaccharide of *Trollius chinensis* polysaccharide, rhizome polysaccharide of *Siberian solomonseal* and polysaccharides of Astragalus plants [56]. As well, a new formulation for Newcastle disease vaccine contains a flower polysaccharide of *Paulownia tomentosa* [57] and the Astragalus polysaccharides can be used for adjuvancy on *Vibrio harveyi* [58] and *Edwardsiella ictaluri* vaccines [59].

Concluding remarks and future perspectives

Carbohydrate-based molecules are potent and safe adjuvants used alone or in combination. They show advantages over alum such as reduced side effects and the activation of both humoral and cellular immune responses, being capable to adjuvate an immune response against intracellular pathogens.

There are numerous ongoing investigations and clinical trials as well as new patents related to carbohydrate-based adjuvants. Nonetheless, current research teams continue to produce innovative modifications of the bacterial molecules Lipid A and Peptidoglycan changing their adjutancy power. Likewise, significant research is being carried out on chitosan and alginates, paving the way for the development of innovative delivery systems that permit intraocular, buccal, nasal and sustained oral administration of antigens. Besides, glucans and mannans present extraordinary libraries of vaccine adjuvants. Derivatizations on dextran with cyclic dinucleotides have given good results as well as the use of zymosan for lung administration. In addition, the trehalose glycolipids and galactosylceramide are also in consideration for the development of new adjuvants.

Moreover, there are many positive prospects on the novel formulations Advax™ and Glabilox. Both Advax™; a microcrystalline polysaccharide particle engineered from δ-inulin, and Glabilox; a new saponin mix, show a positive stimulation of the immune system.

The data included in this review demonstrates that carbohydrate-based adjuvants are excellent candidates for vaccine development, however there is still much research to be done on the already known carbohydrate-based adjuvants, to test them with new antigens and in new formulations. In addition, the exploration for new carbohydrates should continue; perhaps plants and other natural products can be a very interesting source for new polysaccharides, with new and promising activities.

Conflict of interest

None declared.

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