

# Biopolymers and Osmolytes – A Focus towards the Prospects of Stability and Adjuvanticity of Vaccines

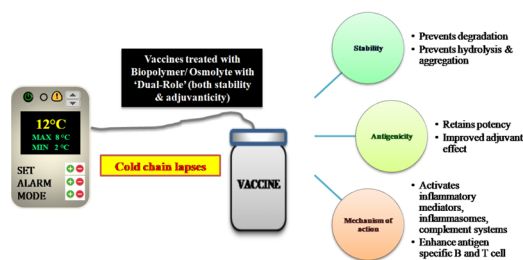
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**Abstract:** ‘New-Gen Vaccines’ are grabbing the attention of scientists as they are much suitable for an immune-compromised group of individuals as well as infants. The major drawbacks of these vaccines are lower immunogenicity and instability. The need for a convenient and safe adjuvant is still under exploration. On the other hand, thermal instability leads to the inactivation of the vaccine and becomes detrimental in many cases. Thus, there is a need to incorporate new kinds of excipients into vaccine formulation to enhance the potency/immunogenicity of vaccine antigens and also act as stabilizers. A limited or single excipient in providing the required dual-activity is vital to break the stereotypical usage of the well-entrenched adverse ingredients. In the proposed review, the efficiency of naturally occurring biocompatible carbohydrate polymers and osmolytes and their ‘dual-role’ is briefed. In addition, the information on the possible mechanisms of action of carbohydrate polymers in vaccines as adjuvants and stabilizers are also discussed.



**Keywords:** bio-molecules, carbohydrate polymers, osmolytes, mechanism of action, new-gen vaccines, vaccine excipients.

## 1. Introduction

Infectious diseases are becoming the major cause of death worldwide (WHO).<sup>1</sup> Vaccination remains one of the most efficient ways to protect against a wide range of infectious diseases. Substantial efforts have been undertaken by researchers to develop effective vaccines against various emerging and re-emerging diseases globally. The new generation vaccines/“new-gen vaccines” including sub-unit vaccines, DNA vaccines, and synthetic protein vaccines, are particularly useful for the vaccination of immuno-compromised individuals and more distinct compositions that suffer lower immunogenicity due to the absence of pathogen-associated molecular patterns (PAMP).<sup>2</sup> Adjuvants (compounds that can enhance as well as modify the intrinsic immunogenicity of an antigen) are therefore required to aid these vaccines to induce effective and stable immune responses.<sup>3</sup>

The traditionally used aluminum salts predominantly induce antibody responses, while the new-gen vaccines often require the induction of strong cell-mediated responses, including T helper (Th) cells and sometimes cytotoxic T lymphocytes (CTLs), in addition to antibodies.<sup>4</sup> Apart from the immunological aspect, such vaccines also face instability issues (related to efficacy and safety). The poor stability of vaccines creates a barrier to their

development where an increase in the storage temperature or freezing might be detrimental in many cases. Several other (non-potency indicating) factors like pH, antigen content, adjuvant content, microbial contamination or sterility, changes in physicochemical parameters also affect vaccine stability. Hence, there is a need to incorporate new kinds of excipients into vaccine formulation to enhance the potency/ immunogenicity of vaccine antigens that can also act as a stabilizer, which will be further discussed in this review.

## 2. Inter-relation between stability and potency of vaccines

Identifying the process of degradation responsible for each component’s instability is a critical difficulty in vaccine formulation development. The stability of vaccines can be disturbed in two major ways, namely physical and chemical degradation. The physical method includes aggregation and denaturation. Aggregation is not only associated with proteins, but also with other components of vaccine formulation like immunostimulators (lipid-based), inorganic adjuvants, emulsion-based adjuvants, or whole microbe in its attenuated form. Degradation occurs due to changes in temperature, pressure, or chemicals. Temperature-based degradation plays a major role in determining the potency of vaccines, as they are irreversible. Apart from these two factors, adsorption of vaccine components to the surface of the containers leads to instability. On the other hand, the chemical destabilization method includes deamidation, oxidation, disulfide exchange, *etc.* that targets various bio-

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molecules (like proteins, polysaccharides, phospholipids, and nucleic acids) involved in vaccine production.<sup>5</sup> The rate of the potency of vaccines largely relies on their stability and is influenced in a temperature-dependent manner. Hence most of the vaccines are maintained at cold temperature (mostly 2–8 °C) until used and require a cold-chain facility to maintain the stability. The potency of vaccine can be lost at any stage of the vaccine supply chain, *i.e.* long time storage, transit, or shipping for distribution, the time before immunization, and most importantly during and post-re-constitution of (lyophilized) vaccines. Not only do higher temperatures inactivate vaccines, but also the freezing temperatures influence the vaccine potential. Additionally, multiple freeze-thaw cycles largely affect the vaccine's stability and potency.

### 3. Monitoring vaccine stability and potency

The basic tools available to monitor the stability of vaccines (mainly the temperature excursions) is to use temperature monitoring devices like electronic shipping monitors, vaccine cold chain monitors, vaccine vial monitors, irreversible freeze indicators, temperature data loggers (mainly the 30-day electronic refrigerator temperature logger), *etc.*, recommended by the WHO.<sup>6</sup> However, these tools do not directly reciprocate the potency of the vaccine. Evaluating the stability and potency of vaccines largely relies on analytical tests called the 'potency tests'. The potency test includes biological assays like *in-vitro* antibody assays or cell-based assays, and *in-vivo* immunogenicity tests using animals. Generally, multiple vials of the vaccines are tested randomly at some time point and a mean potency is reported, which end up giving uncertain vaccine profiles. This may not be performed continuously during the handling and storage process where the stability and potency are largely influenced. Also, these tests lack consistency during clinical development, for instance, the early stage of clinical trials investigates the potency of the vaccine while, the later stage focuses more on safety, shelf-life, and efficacy. Thus this method lacks real-time stability testing, which becomes mandatory for the regulatory approvals.

Hence, for real-time stability profiling, accelerated stability studies or forced degradation studies<sup>6</sup> are to be performed during the vaccine development process, for which, the choice of vaccine excipients that can be compatible with the particular antigen is really important. Whatever the nature of an antigen or the excipient, extensive stability studies are always required both under accelerated conditions (like temperature, pH, freeze/thaw cycles, ionic strength, mechanical strength, *etc.*) and under the actual storage environment to ascertain the shelf-life and expiration of every vaccine. Complexity in vaccine formulation due to the addition of more excipients delays the evaluation of the antigen's behavior with each component during the vaccine formulation development. Hence, choosing an appropriate vaccine excipient that can act as an adjuvant and as a stabilizer becomes the 'need of the hour'. It also becomes easier to study the compatibility and mechanism of action of that particular excipient with the antigen of choice in an extensive manner for stability as well as adjuvanticity, the key factors for better vaccine development.

### 4. The classic vaccines and challenges

Live vaccines (usually the wild strains of bacteria or viruses) are weakened/attenuated and immunized, where the attenuated strain replicates in the host to stimulate an immune response. Oral polio vaccine, rotavirus, chickenpox, Bacille Calmette-Guérin vaccine (BCG), measles, mumps, and rubella (MMR), and yellow fever vaccines are categorized as live attenuated vaccines by the WHO. Though these vaccines cause no or mild disease after immunization and have an excellent immune response (thus they do not need adjuvant), there are some safety concerns. Live attenuated vaccines strictly require cold chain facilities to be active and failure to proper storage has resulted in the loss of vaccine efficacy. The need for the stabilization of these vaccines began in the early 1950s. In 1953, Pollard suggested that inactivation of viral-based infections prevailed due to an increase in temperature and this was because of the denaturation of some protein components in the virus.<sup>7</sup> To overcome the instability issues, freeze-drying or lyophilization procedures (removing water content under controlled temperature and pressure) are performed. The lyophilized vaccines are reconstituted in recommended diluents (like saline or appropriate buffers) before vaccination, while the reconstituted vaccine cannot be stored for a longer period. The freeze-drying procedure encounters stresses like the formation of ice crystals, reduced temperature, alterations in pH and ionic strength, phase separation, *etc.* Hence additional excipients like cryoprotectants are required to maintain the stability and potency of the vaccines, and preservatives are also required to maintain sterility. Amidst improving the shelf-life of vaccines and remaining a successful stabilization method, most of these lyophilized vaccines still need to be stored at 4–8 °C or below to maintain long-term stability, for which a cold chain is again required.

Inactivated (or non-replicating) whole-cell vaccines are made from pathogenic microorganisms that have been destroyed by physical or chemical methods without damaging their antigenicity. They are available in liquid formulations where neither of these killed species causes disease and hence these vaccines are considered to be safe and highly stable when compared to live attenuated vaccines. These vaccines are not capable of stimulating a complete or long-lasting immune response, thus require adjuvants to elicit the required immunogenicity, which is sensitive to freezing. When exposed to freezing temperatures, adjuvants like aluminum salts form ice crystals and lead to agglomeration or coagulation, thereby reducing vaccine potency. Thus temperature protecting agents (like polyethylene or propylene glycol, glycine, *etc.*) for such adjuvants are to be added additionally, which increases the vaccine development cost. Non-compatible interactions between antigen and adjuvants can also influence the potency of these vaccines (adsorptive coefficient). When the adsorption of antigen to the adjuvant (mainly aluminum salts) is very strong, the processing and presentation of antigens to the antigen-presenting cells (APCs) decrease, thus affecting the immunity, and it has also been reported that such adsorptions influence the stability of the antigen.<sup>8</sup> Also, the large size of enveloped viruses and bacteria affects vaccine stability due to osmotic sensitivity and complex cell components, thereby

limiting their potency.

## 5. The new-gen vaccines

Though early vaccinations discussed above expressed excellent immune response, some of these vaccines have historically experienced reactogenicity with reversal issues, incomplete inactivation, and reduced stability. In the 1970s, the outbreak of whole-cell pertussis associated with the accidental emergence of encephalitis in children ruined the people's trust towards vaccination<sup>9</sup>, which urged the researchers to focus on purified antigens (the subunit vaccines). This was the period when some novel techniques including recombinant DNA technology, multivalent vaccines, and other delivery techniques of the kind took scientists to a new horizon in vaccine research. When compared to whole-cell and live attenuated vaccines, the sub-unit or acellular vaccines containing the purified antigens remained to be safe but were less immunogenic and less stable. Current vaccine research focuses more on the safety aspect of individuals with a weakened immune system including infants/children for making vaccines with no or marginal potential risks or side effects. Hence sub-unit or acellular vaccines produced using recombinant technology containing only the microbial components (where the potential antigenic parts of the pathogen are alone used to provide the necessary immune response) are under the limelight. These recombinant vaccines can further be categorized into protein-based subunit vaccines (hepatitis B and acellular pertussis), polysaccharide vaccines (against meningococcal and pneumococcal diseases), conjugate sub-unit vaccines (*Haemophilus influenzae* type B and pneumococcal conjugate vaccines), toxoid vaccines (tetanus toxoid and diphtheria toxoid), nucleic acid vaccines (DNA and RNA vaccines), and viral vectored vaccines [replicating Ebola vaccine – Ervebo (rVSV-ZEBOV) and non-replicating COVID 19 vaccine - ChAdOx1], and are collectively termed as new generation or new-gen vaccines.<sup>10</sup>

As mentioned earlier, pure antigens weakly possess the ability to elicit an immune response, thus subunit vaccines desperately require vehicles for antigen delivery or typically adjuvants.<sup>11</sup> Also, these current vaccines express diverse behaviors where some require freezing and some don't, some express excellent stability, while certain vaccines are highly sensitive to temperature changes, leading to loss of potency and causing issues in vaccine safety. Due to this diverse behavior, formulating a compatible vaccine becomes challenging, and requires the addition of various compounds or excipients to maintain the stability and potency of the vaccine. Hence, developing a successful vaccine formulation requires careful customization of pH, ionic strength, selection of appropriate buffer and excipients, storage, administration route, *etc.*, which the scientific community is effectively working out for years.

## 6. The rationale behind the vaccine excipients to possess 'dual-role'

In general, new-gen vaccines are delivered not only through conventional parenteral injection methods, but also through

different delivery systems including microneedle jet injectors, intradermal needles as nanocarriers for specific antigens (for diseases like AIDS, cancer, swine flu, *etc.*), and other needle-free systems. Among various routes, intranasal drug delivery plays distinct parts in delivering the antigens efficiently that are not appropriate for parenteral adjuvants such as alum. Currently, nucleic acid vaccines are trending as they are capable of inducing both humoral and cellular adaptive immune responses. In the case of the severe acute respiratory syndrome corona virus (SARS-CoV-2) vaccines and other nucleic acid vaccines, the chances for genome instability (both DNA and mRNA) during the delivery to the immune cells become the major challenge to be addressed.<sup>12,13</sup> Concerning vaccine stabilization, as alternatives to the cold chain, there are promising methods like freezing and drying. Yet, their applicability is limited as these methods require sophisticated types of equipment and skilled technicians for sample preparation.

The licensed adjuvants used in human vaccines include mineral salts (like aluminum phosphate or hydroxide in rabies, Japanese encephalitis, and human papilloma viral vaccines), oil-in-water (O/W) emulsions like MF59 (citrate buffer suspension with squalene, polysorbate 80 and span 85), AS03 ( $\alpha$ -tocopherol, squalene and polysorbate 80), and AF03 (squalene, montane 80 and eumulgin b1 ph) in influenza vaccines, alum-adsorbed Toll-like Receptor-4 (TLR4) agonist (like AS04 in hepatitis B (Hep A) and human papilloma vaccines) and liposomes (like virosomes in Hepatitis (A and B) and influenza vaccines); while gelatin and albumins are the widely used stabilizers.<sup>14,15</sup> The broadly used aluminum adjuvants show excellent immunity and safety records for years (almost 70 years), yet their different physical properties (like particle size, surface charge, morphology, antigen binding, *etc.*) influence their interaction with the vaccine antigen. For instance, the positive charge, as well as the large size of the traditional aluminum adjuvant, inhibits their entry into the lymph node. As the APCs are largely present inside the lymph node when compared to the peripheral zone, their recruitment at the injection site is low, thus the immune activation becomes restricted.<sup>16,17</sup> The widely used Freund's adjuvants are non-degradable due to the presence of paraffin oil, and the emulsion particle size of the O/W adjuvants suffers a major setback in pharmaceutical applications.<sup>18</sup> Besides, health issues have also been raised about aluminum<sup>19</sup> as an adjuvant and gelatin<sup>20</sup> as a stabilizer, making it important that every new excipient has good human health and tolerability data.

These major challenges could be addressed by the use of simple and new excipients with dual-role *i.e.* to enhance the potency/immunogenicity of vaccine antigens that can also act as a thermal stabilizer. Apart from this perspective, an excipient with a dual role is expected to have added advantages such as a very low additive concentration, inter-batch reproducibility, and a rate-limiting step during manufacture and storage. Here, we will be discussing the excipients belonging to polymer and osmolyte groups that can potentially exert the dual-role and help in reducing the complexity and the cost of vaccine production.

## 7. Overview of carbohydrate polymers with dual-role

Polysaccharides are a group of carbohydrate compounds that are generally regarded as safe (GRAS) with limited risk of toxic metabolites or long-term deposits of tissues and are biocompatible. As summarized in Table 1,<sup>21-24</sup> due to their size, molecular weight, and chemical structure, many carbohydrate polymers play important signaling roles within the immune system by targeting various immune cells and are also capable of stabilizing biomolecules against thermal degradation and agitation. Some naturally occurring polymers such as chitosan, glucan (like dextran), inulin, and other forms of polymers including non-ionic block copolymers that are anticipated to express the proposed dual role are detailed below.

### 7.1. Chitosan

Chitosan (CS) or chitins are cationic  $\beta$ -1-4-linked polymers of D-glucosamine and N-acetyl-D-glucosamine, which allows the development of polyelectrolyte complexes with negatively charged biomolecules and cell membrane interaction.<sup>25</sup> CS has been used successfully as a drug conjugate, biodegradable release system, polyelectrolyte complex, and hydrogels in delivering drugs, proteins, peptides,<sup>26</sup> and viral vaccines.<sup>27</sup> Acid-soluble chitosan when used as an adjuvant is capable of eliciting strong cellular immunity as it is taken up by the dendritic cells. CS is readily dissolved in the lysosomal acid environment *via* the lysozyme escape pathway, facilitating the release of antigen by the proton effect or by evoking the CTL response promoted by a cluster of differentiation 8 (CD8+) through the major histocompatibility complex (MHC) I pathway upon intranasal delivery.<sup>28</sup> In one of the recent animal experiments<sup>29</sup>, CS nanoparticles

containing inactivated antigen of the Rift Valley Fever Virus was capable of inducing innate and adaptive immunity by the up-regulation of important cytokines like Interleukin (IL)-2, interferon (IFN)- $\gamma$ , and IL-4.

Generally, the intrinsically soluble polymers are capable of enhancing the stability of the vaccines (mainly DNA vaccines) when used as carriers, while chitosan's protonated form is poorly soluble at physiological pH.

To overcome this, chemical modification of the polymer backbone can be performed to increase hydrophilicity. This can be achieved by the addition of large hydrophilic groups (like polymeric methacrylates or *N*-[(2-hydroxy-3-trimethylammonium) propyl] chloride), or deacetylation, or trimethylation.<sup>24</sup> A modified form of CS developed by quaternization of the amino group has been experimented overcoming chitosan's insolubility issues. This strategy has also presented significant influence over the cationic character without affecting its pH independence and mucoadhesive property which is advantageous to facilitate ionic complex stability and gene delivery.<sup>30</sup> In a recent study, the quaternary chitosan nanoparticles combined with fucoidan and anthrax vaccine exhibited excellent Immunoglobulin G (IgG) titers and the challenge studies with mice proved a good survival rate in the chitosan-based anthrax vaccine.<sup>31</sup> Apart from quaternization, another modified form (sulfating) of CS called 6-O-sulfated chitooligosaccharide and 2-N, 6-O-sulfated chitooligosaccharide have been patented (patent number: CN 107648603) as potential adjuvants for a variety of new-gen vaccines.

Ghenodan *et al.* (2009)<sup>32</sup> reported that the soluble form of CS expressed excellent humoral and cellular immunity when combined with the inactivated monovalent and trivalent vaccine against human influenza viruses (both types A and B). A high

**Table 1.** Role of natural carbohydrate polysaccharides in vaccine formulation

S. No	Polysaccharide	Advantages	Disadvantages	Formulation methods	Mode of action	References
1	Chitosan	Mucoadhesion (good for oral delivery); Good sustained release and control; Cost-effective; Low allergenicity; Efflux pump inhibitor	Poor solubility, Weak targeting, Lower immunogenicity, Low transfection	Emulsification, Nano-precipitation, Solvent evaporation, Ionic gelation	Production of inflammatory cytokines; Receptor-mediated endocytosis (NLRs, dectin-1, leukotriene B4, TLRs, and mannose receptors); APC uptake by charged interactions	21, 23, 24
2	Modified Chitosan: Quaternized, Carboxylated, Sulfated, Aminated, Thiolated, Methylated	Enhanced water solubility; Improved cationic nature; More adsorption/encapsulation of antigen; Boosts cellular internalization; Increased antigen retention time; Low toxicity	Residues of organic solvents or heavy metals retain			
3	Dextran	Improve immunogenicity; Regulate immune system; Low toxicity; Anticoagulant; Antithrombotic; Easy to chemically modify	Side-effects of non-steroidal anti-inflammatory drugs	Solvent evaporation, Emulsification (Electrospray)	Promote antigen cross-presentation on both MHC (I & II), thereby activating interferons, Th1, B cells, and memory T cells	22-24
4	Acetal dextran	Low toxicity; Tunable degradation rate; Acid degradation (promising for nucleic acid delivery)	Degradation product (methanol) is toxic			
5	Inulin	Good tolerability and safety; Lower reactogenicity	Less immunogenic in soluble form; Stimulation of <i>in-vitro</i> dendritic cell maturation is poor	Emulsification, Ionic gelation	Alternative complement pathway (unexplored), TNF signaling, Priming APCs and increase antigen-specific humoral and cellular immunity	21, 22

antibody titer was found to be retained even after six months, with minimal antigen, upon intramuscular delivery. The study has also shown that CS did not induce any allergic reactions (based on IgE expression). In another study, oral administration of tetanus toxoid (TT) vaccine loaded with glucomannosylated chitosan was capable of eliciting strong humoral, mucosal, and cellular immune response with high mechanical strength and stability compared to commercial TT vaccine.<sup>33</sup> The cellular uptake of this vaccine was due to mannose and glucose receptor-mediated endocytosis, and this glucomannosylated chitosan additionally enhanced the conformational stability by encapsulation, suitable for oral delivery of the vaccine. A recent study has investigated the stability and immunogenicity of Chitosan-modified poly(lactic-co-glycolic acid) (PLGA) nanoparticles against *Escherichia coli* K1 neonatal meningitis.<sup>34</sup> These chitosan-based nanoparticles were shown to improve the stability of the recombinant OmpAVac protein, an artificial form of the outer membrane protein A (OmpA) of *E. coli* K1 (potential candidate), by the freeze-drying method. The chitosan-nanoparticle not only increased the stability but also affords immunoprotection in mice even after 140 days of storage at 4 °C. These findings have expressed the potential dual-role of chitosan in vaccine formulation.

### 7.1.1. Trimethyl chitosan

Among various anionic polysaccharides, the modified form of chitosan called the trimethyl chitosan (TMC) expresses higher immune-stimulating efficacy, due to its hydrophilic and mucoadhesive characteristics.<sup>35</sup> Also, the TMC aids in DNA condensation by a non-covalent electrostatic interaction with the negatively charged phosphate group of the DNA backbone. DNA condensation plays a major role in maintaining the integrity (or stability) and targeted delivery of DNA vaccines, facilitating easy cellular uptake.<sup>24</sup> A DNA vaccine constructed by a research group against SARS-CoV nucleocapsid protein with plasmid DNA (pDNA) loaded with chitosan and anti-DEC-205 antibody showed enhanced immunogenicity upon nasal delivery. They reported that the cellular uptake of the designed DNA vaccines was through the mannose-binding receptor DEC-205 in the surface of dendritic cells (DCs).<sup>36</sup> In another similar study, chitosan nanoparticles loaded with pDNA vaccine successfully induced humoral and cellular immunity against hepatitis B (after two weeks) upon intranasal delivery when compared to alum. The study also showed that chitosan was capable of protecting the pDNA against DNase I digestion.<sup>37</sup> This stabilization property of chitosan against nuclease digestion favors the maintenance of DNA vaccines under physiological conditions such as mucosal membrane surfaces with more nucleases. Recently, TMC prepared by ionic gelation technique was capable of effectively stabilizing tetanus vaccine at 4 °C and 37 °C and is reported to be a good preservative and antibacterial excipient in replacing thimerosal.<sup>38</sup> Further, TMC coated inactivated polio vaccine prepared as pH-sensitive microneedle gave a promising antigen dose with significant antibody response, showing its suitability for dermal vaccination.<sup>39</sup> Studies on many new-gen vaccine administration routes (including nasal and intradermal vaccines) in combination with different forms of CS<sup>40-44</sup> have effec-

tively highlighted its ability to act as a promising stabilizer as well as an adjuvant.

### 7.2. Dextran

The Food and Drug Administration (FDA) approved polysaccharide Dextran is a branched, microbial, natural homo-polysaccharide containing  $\alpha$ -1, 6-glucan with  $\alpha$ -1, 3-branches. It is water-soluble irrespective of pH, biodegradable, with a wide range of molecular weights making it suitable for therapeutic purposes. The dextran-based polycationic polymers like diethylaminoethyl (DEAE)-dextran and dextran-spermine have been effective in delivering nucleic acids<sup>45</sup> and have been explored as potent delivery vehicles in veterinary vaccines like foot-and-mouth disease and Venezuelan equine encephalomyelitis.<sup>46</sup> Self-assembled virus-like particle (VLP) enclosing CHIKV (Chikungunya V) maintained its globule structure (without aggregation) even at an elevated temperature at neutral pH when the polyanion dextran-sulfate was utilized as a stabilizer,<sup>47</sup> suitable for the parenteral route. The key mechanism for most of the protein stabilizing polysaccharides is macromolecular crowding. Another study conducted by Caterina Alfano (2017) on frataxin yeast protein using synthetic crowder dextran 40 showed an increase in circular dichroism signal and melting point, representing the increase in crowding concentration, parallelly signifying an increase in the folded state.<sup>48</sup> Another study has reported that dextran nanoparticles combined with Zidovudine-stearic acid (drug against HIV) by double emulsion solvent evaporation method exhibited controlled release with efficient cellular uptake when compared to the free drug which suffers an inability to cross the biological barriers and solubility issues. The study emphasizes that nanoformulation has improved stability efficiently.<sup>49</sup> Similar stability and biocompatibility have been reported when albumin-derived iron oxide (Fe<sub>3</sub>O<sub>4</sub>) nanoparticles were combined with dextran. The dextran not only enhanced the stability but also improved blood circulation half-life (to limit the easy elimination of the drug by phagocytosis), thereby enhancing drug targeting and delivery.<sup>50</sup> Cross-linked dextran microspheres have been shown to enhance the immune response (mainly IgG and secretory IgA) when encapsulated with tetanus toxoid in rabbits through the nasal route. They have been a potential excipient in overcoming the mucosal barrier by improving trans-epithelial absorption of antigens.<sup>51,52</sup>

Acetalated dextran (Ac-DEX) produced by acetal formation using 2-methoxypropene, a modified form of dextran, has become one of the widely studied derivatives of dextran for vaccine/ drug delivery. Drug encapsulation in both micro and nanostructures is the key mechanism of Ac-DEX facilitated by precipitation and emulsion procedures (mainly nanoprecipitation, electrospinning, and single/double emulsion). Hydrolysis of the non-water-soluble acetals occurs in the acidic environment and becomes water-soluble dextran (the parent structure) to break the hydrophobic microparticles. These derivatives when included in vaccine formulations were capable of stabilizing horseradish peroxidase at room temperature and temperature even above 45 °C.<sup>53</sup> When compared to conventional vaccine formulations, acetalated dextran formulations have significantly increased

antigen presentation by both MHC I and II responses.<sup>54</sup> In a recent study, the potential malaria vaccine antigen Merozoite surface protein 2 (MSP2) along with the acetalated dextran (Ac-DEX microparticles) was capable of eliciting both antibody-mediated (IgG response) and cellular immune response (Th1 response) when compared to alum adjuvant.<sup>55</sup> Similarly, Ac-DEX microparticles that are stable at pH 7 were capable of enhancing the cellular uptake (by cross-presentation) of the melioidosis subunit antigen through phagocytosis in APCs (where the pH is 5), as they are degradable in an acid environment. The microparticle has been reported to elicit a strong antibody response against *Burkholderia pseudomallei* by both Th1 and Th2 immune pathways<sup>56</sup> when administered to mice through the intraperitoneal route. Though dextran derivatives have established considerable stability and immune stimulation properties, it is yet to achieve effectiveness as human adjuvants, for which an extensive focus towards their synthesis, characterization, reproducibility, toxicity profile, *etc.* is needed.

### 7.3. Inulin

The FDA-approved Inulin is a fructan ( $\beta$ -D-(2-1) poly(fructofuranosyl)  $\beta$ -D-glucose) based natural carbohydrate polysaccharide. This flexible, water-soluble fiber has widely been used as a stabilizing agent, cryoprotectant, and drug carrier and it is the first polysaccharide to have immunological effects (mainly the activation of alternative complement pathway). Inulin has been proved to be an excellent stabilizer of the influenza subunit vaccine in different forms *viz.* freeze-dried, spray-dried, and spray-freeze-dried.<sup>57</sup> In an interesting experiment, using various carbohydrates like trehalose and inulin, four separate protein models were lyophilized. It was found that trehalose provided the best temperature stabilization at 60 °C but in a humid environment, inulin was a stronger protein stabilizer compared to the other carbohydrates.<sup>58</sup> In one of the studies, the pulmonary administration of the spray-freeze-dried influenza subunit vaccine was not only found to be stable but was also capable of inducing systemic (serum IgG titer) and mucosal (nasal IgG and IgA antibodies) humoral immune response, as well as cell-mediated (IL-4 and IFN- $\gamma$ ) immune responses in mice when inulin was used as the major excipient, without adjuvant.<sup>59</sup> Thus inulin can be a promising excipient for alternative vaccine administrations (*i.e.* inhalation) also. Another study has reported inulin microparticles to encompass the proposed dual-role when combined with ovalbumin and become a supportive study.<sup>60</sup> The study has demonstrated the potential of soluble inulin in generating robust antibody titers (Th2 immunity) compared to alum adjuvanted antigens, by enhancing the efficient uptake of antigen by APCs. This was the first study to evaluate the adjuvanticity of inulin. Inulin along with sugar excipients (trehalose/dextran) for stabilizing the HBV vaccine in the powdered form has also been reported.<sup>61</sup> This was achieved by the spray-drying method, where the HBV vaccine was thermally stabilized by inulin at 20 °C and 60 °C. The inulin formulation was capable of withstanding almost ten freeze-thaw cycles, maintaining the antigenicity, and was also found to be stable at room temperature for more than three months. This becomes

evident in proving the dual-role exerted by inulin.

An isomer of inulin called delta inulin ( $\delta$ -inulin), produced by precipitation from water at higher temperatures has been used as an adjuvant in Deltin™/Advax™ to enhance the immunogenicity of the hepatitis B antigen by activation of complement pathway followed by monocyte binding.<sup>62</sup> The study shows that delta inulin is not only an effective humoral adjuvant, but it also induces antigen-specific CD4 and CD8 T-cell responses, suggesting that it could be used in T-cell vaccines where neutralizing antibodies alone are inadequate. Regarding stability, delta isoforms (being highly stable in aqueous suspension at higher temperatures) were found to be safe and non-toxic upon intramuscular administration. A recent study on the intrapulmonary vaccination of delta inulin adjuvanted CysVac2 fusion protein (a vaccine candidate for tuberculosis) has shown to potentiate innate cell recruitment to the lungs and lymph nodes thereby, increasing chemotactic signaling. Thus delta-inulin's potential as an adjuvant for lung vaccinations is again supported by this preclinical study.<sup>63</sup> Human clinical trials using delta inulin as adjuvant against hepatitis B<sup>64</sup> and influenza vaccine<sup>65</sup> have also been reported to be successful in providing safety, immunogenicity, and tolerability for even infants and children between ages 6 months and 9 years, supporting the objective of this review. Apart from delta inulin, another crystalline type of inulin called the gamma inulin ( $\gamma$ -inulin) has also shown adjuvant activity.<sup>66</sup>

### 7.4. Non-ionic block copolymers

The pH and temperature sensitivity, amphiphilic block copolymers like poloxamers and poloxamines formed by chains of ethylene oxide block (EO) and propylene oxide (PO) have been reported for efficient vaccine delivery. Among these, the poloxamers have more than 50 copolymers that are neutrally charged, making it convenient for them to condense and encapsulate into micelles. It has been proposed that these copolymers can destabilize the plasma membrane of APCs enabling antigen to channelize into the MHC class I pathway which is important for cell-mediated immunity.<sup>67</sup> It is shown that the polymeric adjuvant CRL1005, a commonly studied poloxamer, improves the *in vivo* antigen expression. Another pentablock copolymer (poloxamer 407) exhibits promise as a vaccine adjuvant that can sustainably co-deliver protein and DNA.<sup>68,69</sup> An interesting study on tetanus toxoid delivery using poloxamer (poloxamer 188) encapsulation has shown improved compatibility *i.e.* prevented the inactivation of the toxoid and showed higher neutralizing antibody production compared to aluminum phosphate adsorbed toxoid, establishing its dual-role.<sup>70</sup> Similarly, poloxamines are also proposed to have the same properties, but their adjuvant potential has not been widely evaluated.

### 7.5. Possible mechanisms of action (MOA) of carbohydrates to the dual-role

The polysaccharides stand out promising amongst carbohydrates by preventing the degradation and aggregation of proteins or antigens by various stabilization methods (Figure 1)

and also boost immune responses by activating inflammatory mediators, inflammasomes, complement systems, thereby enhancing the antigen-specific B and T cell memory. Generally, the vaccine immunogenicity is enhanced by carbohydrate polysaccharides as they activate innate immune receptors like TLR, NOD-like receptors (NLR) upon binding to them, thereby activating the nuclear factor kappa B (NF- $\kappa$ B) signaling pathway resulting in the production of inflammatory cytokines (mainly IL-1 and TNF- $\alpha$ ).<sup>71</sup> Complement activation and generation of chemotaxis are also associated with the adjuvant action of carbohydrate polysaccharides. These cytokines and chemokines further facilitate priming, expansion, and polarization of the immune system. Apart from these pathways that produce excess inflammatory signals, the polysaccharide inulin (mainly  $\delta$ -inulin) is capable of eliciting immune action by alternative pathways (unexplored) preventing reactogenicity. It has also been proposed that the multimeric carbohydrate structure of  $\delta$ -inulin solubilizes and renders immune response readily and is also immunologically inert in its solubilized form.<sup>62</sup> The possible roles of carbohydrate polysaccharides in activating immune responses are given in the figure (Figure 2).

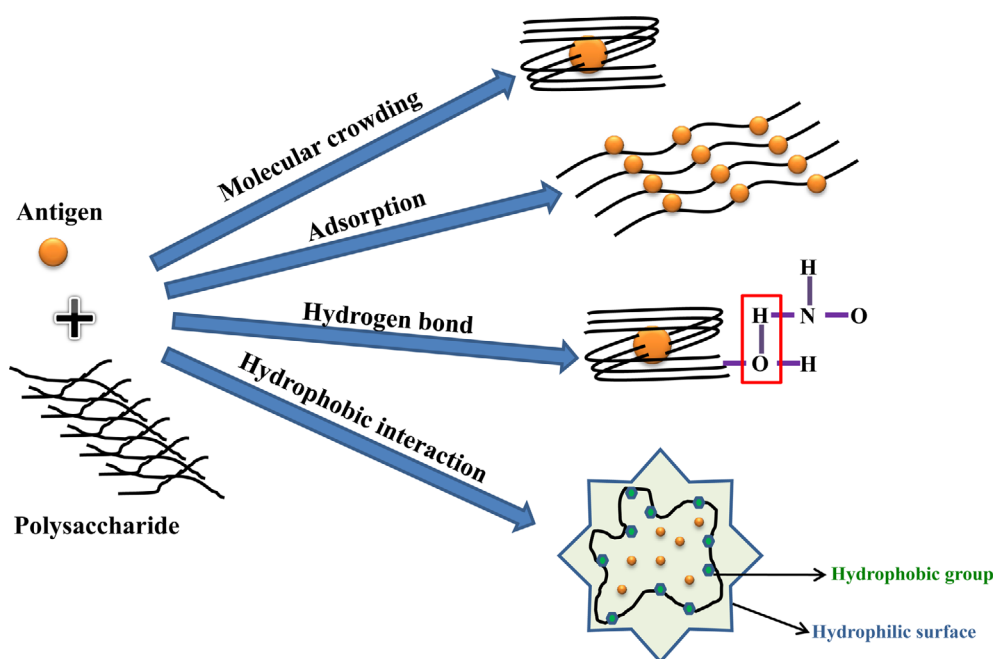
## 8. Overview of osmolytes with dual-role

Osmolytes are organic compounds with low molecular mass. The sub-groups of osmolytes like polyhydric alcohols (*e.g.* glycerol, sorbitol), sugars (trehalose), anionic polyols (diglycerol phosphate), and cyclitols (Myo-inositol) are categorized as carbohydrate osmolytes. The next sub-group includes amino acids (*e.g.* glycine, proline), their derivatives (*e.g.* taurine), and methylammonium derivatives (including trimethylamine N-oxide (TMAO) and sarcosine). These biomolecules have been proposed to stabilize protein from aggregation and prevent them from misfolding<sup>72</sup> *i.e.* protecting the structural and functional

integrity of macromolecules under varying environmental conditions including pH, denaturation, temperature, *etc.* Osmolytes actively perform various key roles like osmophobicity, excluded volume effects, preferential exclusion or hydration from surfaces (a solvophobic mechanism), and surface tension and increase the free energy to drive the folding symmetry to re-folding the proteins to their native conformation. Apart from stabilization, osmolytes have been identified to render important immunological functions including antigen presentation, immune cell proliferation, antigen-antibody interaction, inflammatory reaction, and many more.<sup>73</sup>

### 8.1. Trehalose

Trehalose is a kosmotrophic, disaccharide compound with two glucose in alpha, alpha-1, 1 linkage, produced by various organisms. Trehalose is used in major sectors including food and pharmaceuticals mainly as an energy source and freeze protectant. Trehalose has expressed the proposed dual role in many instances that includes the following research experiments. Unlike the regular injectable form, in 2009, innovative 'microneedle vaccination' came into existence. Vaccination of inactivated influenza virus as a microneedle on the skin was attempted by Fu-Shi Quan *et al.* to induce protective immunity.<sup>74</sup> As this method of vaccination suffered vaccine instability issues due to drying, an efficient stabilizing formulation was prepared that contained trehalose as the major stabilizer. These vaccine formulations were investigated both *in vitro* and *in vivo* and the stabilized influenza vaccine was found superior to the unstabilized vaccine in regulating the replication of viruses as well as promptly inducing immune reactions after challenge. In another study, researchers have utilized simple excipients like trehalose, sucrose, glycine, and polysorbate 80 along with phosphate buffer to stabilize the influenza subunit H1NI antigen.<sup>75</sup> This thermally sta-



**Figure 1.** Mechanisms of stabilization by carbohydrate polysaccharides.

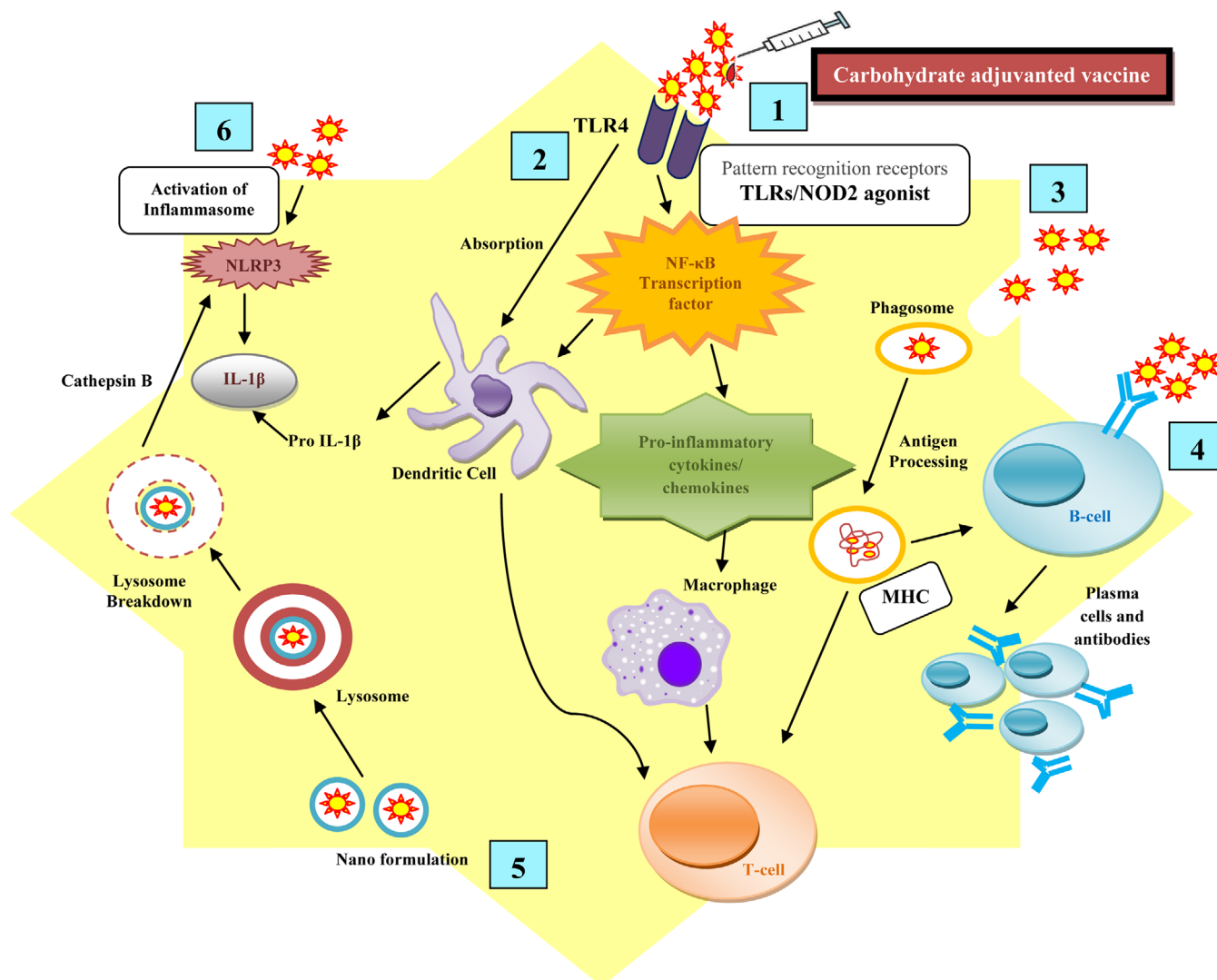


Figure 2. Mechanisms of immunogenicity by carbohydrate polysaccharides.

ble dry formulation of the antigen prepared by freeze-drying technology proved to be immunogenic. The single-radial-immunodiffusion (SRID) assay along with the *in vitro* immunogenicity test revealed no loss of activity of antigen after 40 months at the temperature of 25 °C & 37 °C.

The glass-forming excipient trehalose along with histidine, alum, glucopyranoside lipid A (GLA) was able to protect human papillomavirus (HPV 16L1) in lyophilized formulation for 12 weeks at 50 °C.<sup>76</sup> This formulation had retained tertiary and quaternary structures and conformational epitopes. In another study, around 15% of trehalose has stabilized virus-like particles containing HIV-1 Pr 55gag antigen and retained their original appearance over 12 months periods at -70 °C.<sup>77</sup> Similarly, another study has proved the efficacy of pullulan and trehalose (low-cost sugar film) in stabilizing the inactivated influenza A vaccine at 40 °C for at least 3 months by the *in-vivo* study.<sup>78</sup> In a recent study, a natural deep eutectic system (NADES) consisting of trehalose and glycerol against the storage of influenza hemagglutinin (HA)-displaying virus-like particles (VLPs) has shown physical integrity as well as promising activity at 50 °C for four hours. The same formulation has also shown a notable

stabilization potential at room temperature for more than a month.<sup>79</sup> Apart from stabilization, trehalose-6-6-dimycolate has been established as a potent inducer of inflammatory cytokines (mainly TNF- $\alpha$ ) and has been proposed to increase cellular and humoral immune response along with vaccine antigens.<sup>80</sup> The dual role of trehalose has been postulated in a study where trehalose-coated microneedle patches of inactivated influenza virus exhibited improved thermal stability (even at a range between 4-37 °C) as well as immunogenicity (by inducing systemic and functional antibodies).<sup>81</sup>

### 8.2. Taurine

Taurine (2-aminoethanesulfonic acid) is a conditionally essential amino sulfonic acid present in the body.<sup>82</sup> There is the following evidence to recommend the use of taurine as a vaccine adjuvant and stabilizer. Taurine has been proposed to potentiate adjuvant effects upon oral administration of rubella and influenza viral vaccines<sup>83</sup> and hepatitis B viral vaccines<sup>84</sup> by increasing the antibody response (inducing IL-1) thus, enhancing the proliferation and differentiation of B cells. It has also



been patented for its stabilization effect against the measles antigen in a dry solid form suitable for microneedle coating (Patent number: US 10,736,840. 2020). Though the above-mentioned research works provide hints on taurine expressing the proposed dual-role separately, to date no efficient study has been undertaken that describes the role of taurine in the aspect of stabilization and immunogenicity for new-gen vaccines, which is highly recommended.

## 9. Conclusion and future view

There are issues of safety, efficacy, and lack of long-term studies on vaccine excipients that the medical community must look up. It is difficult for the antigen alone to express the required therapeutic effect. Adjuvants can modulate the body's response to antigens in a variety of ways, including fastening the antibody production, extending protection time, and reducing side effects. It is highly required to choose an adjuvant that helps the antigen to elicit an early, high, and long-lasting immune response with fewer antigens and also stabilizes the vaccine at the same time, thus saving on vaccine production costs. Extensive studies and trials of new compatible excipients must be encouraged to break the stereotypical usage of the well-entrenched adverse ingredients.

Carbohydrate polysaccharide's stability, biocompatibility, abundant raw material availability, and high immunological efficacy make it a highly attractive new vaccination adjuvant. There are carbohydrate compounds (like lipopolysaccharides) that can cause reactogenicity and toxicity due to their complex molecular structure. But the biodegradable carbohydrate polysaccharides discussed here (mainly inulin and dextran) can be easily purified; as a result, issues with complex polyvalent mixes can be avoided. They are safe, well-tolerated, and can be built into specified semi-crystalline forms capable of improving antigen presentation and co-stimulation. Several types of research discussed in this review express their potential activity in antigen delivery and also display remarkable features related to the stabilization or protection of various vaccine antigens to their target site. But the activation of the NF- $\kappa$ B pathway by carbohydrate polysaccharides that produces more inflammatory cytokines (a critical mediator of inflammation) becomes a notable issue related to the toxicity. Carbohydrates can be examined in combination with other adjuvants, or in chemically modified forms to overcome these shortcomings. Osmolytes are equally shown to increase the shelf-life of proteins and are also capable of stabilizing protein antigens from aggregation and degradation. Osmolytes actively aid the re-folding of protein, thereby preventing several diseases associated with protein misfolding. Yet, osmolytes amidst being economical have been shown to express distinct behavior with different protein moieties, hinting at the importance of proper testing of its behavior in each formulation. The regulatory approval and clinical trials of carbohydrate polysaccharides and osmolytes for vaccine application focusing more on their dose optimization and long-term safety need to be addressed. The safety evaluation of every excipient by screening its hemolytic potential, protein-binding studies to check tolerability, in case of subcutaneous or intra-

muscular vaccination, the plasma concentration of creatinine kinase must be determined. Polysaccharide and osmolytes' scope of application in the vaccine will gradually broaden in the future, with them primarily targeting stability and adjuvanticity. Thus we hope that this review may be the driving force required for bringing carbohydrate polysaccharides and osmolytes for the development of a stabilized biocompatible vaccine formulation that can effectively provide the required immunogenicity.

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