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Research review paper

Nanoengineering of vaccines using natural polysaccharides

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ABSTRACT

Currently, there are over 70 licensed vaccines, which prevent the pathogenesis of around 30 viruses and bacteria. Nevertheless, there are still important challenges in this area, which include the development of more active, non-invasive, and thermo-resistant vaccines. Important biotechnological advances have led to safer subunit antigens, such as proteins, peptides, and nucleic acids. However, their limited immunogenicity has demanded potent adjuvants that can strengthen the immune response. Particulate nanocarriers hold a high potential as adjuvants in vaccination. Due to their pathogen-like size and structure, they can enhance immune responses by mimicking the natural infection process. Additionally, they can be tailored for non-invasive mucosal administration (needle-free vaccination), and control the delivery of the associated antigens to a specific location and for prolonged times, opening room for single-dose vaccination. Moreover, they allow co-association of immunostimulatory molecules to improve the overall adjuvant capacity.

The natural and ubiquitous character of polysaccharides, together with their intrinsic immunomodulating properties, their biocompatibility, and biodegradability, justify their interest in the engineering of nanovaccines. In this review, we aim to provide a state-of-the-art overview regarding the application of nanotechnology in vaccine delivery, with a focus on the most recent advances in the development and application of polysaccharide-based antigen nanocarriers.

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1. Challenges and advances in vaccine development

Throughout the last decades, vaccination has played a fundamental role in the prevention of severe infectious diseases, and even in the eradication of some of them. Despite the advances achieved to date, significant challenges still need to be faced in order to gradually increase vaccine coverage. These include not only the development of new vaccines against certain pathogens such as human immunodeficiency virus (HIV), malaria and tuberculosis, among others, but also the development of single-dose and needle-free vaccines intended to improve patient compliance and reduce associated costs. Lastly, the production of formulations that can avoid the cold chain of transport represents a keystone to improve vaccination worldwide. Progress in both antigen and adjuvant development has led to the recognition of the value of nanotechnology to deal with the above indicated challenges. For the preparation of nanovaccines, different immunomodulating biomaterials have been proposed, including polysaccharides. This innovative approach is the main focus of this review and is summarized in Fig. 1.

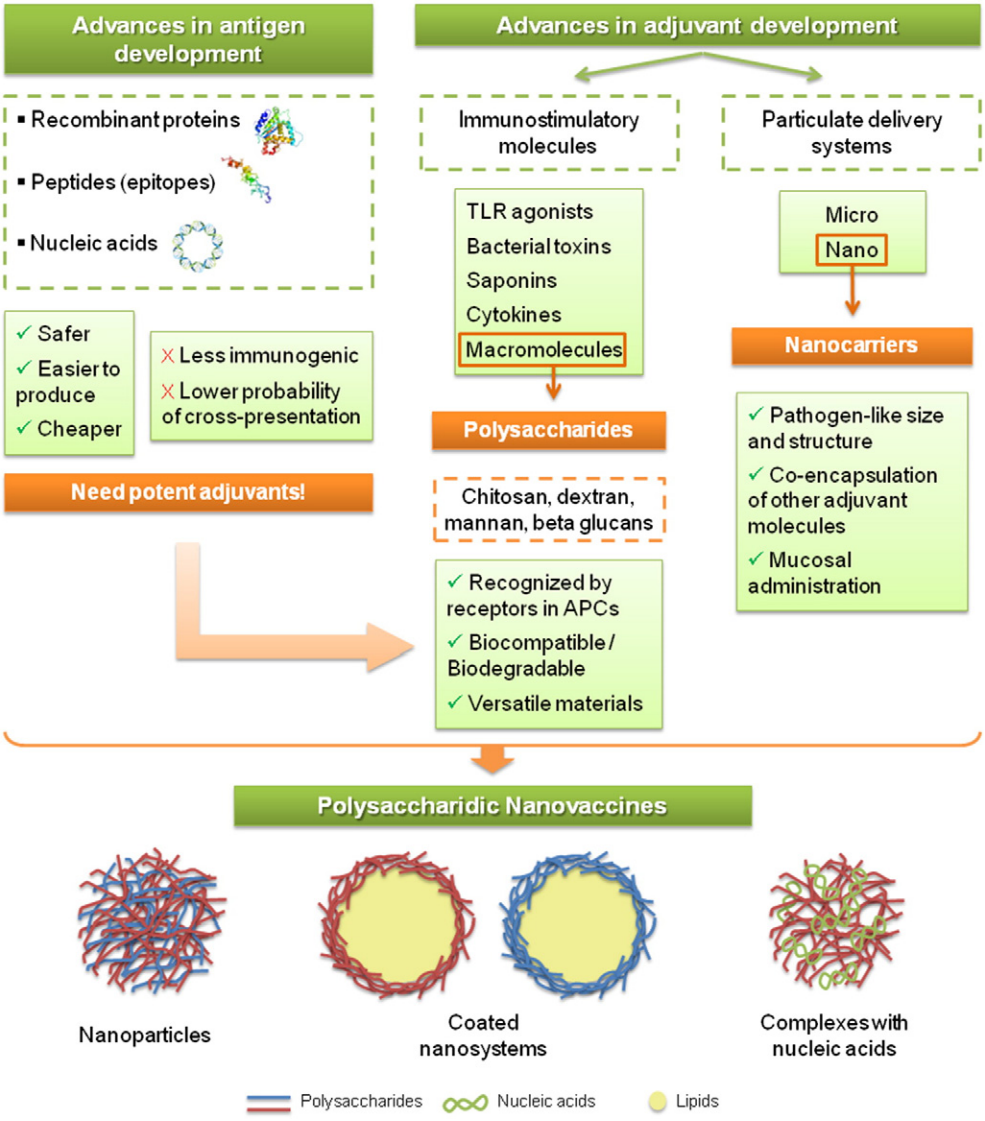

Fig. 1. Advances in biological and microbiological technologies have increased the knowledge of pathogens and led to the development of newer and safer subunit antigens. Nevertheless, these antigens are less effective in inducing protective immune responses and therefore require a parallel development of potent adjuvants such as immunomodulating molecules and particulate delivery systems. Among these, polysaccharide-based nanosystems have demonstrated potential to be successfully used in vaccine formulations.

1.1. Biotechnology and antigen development

The first commercialised vaccines against rabies, poliomyelitis, tetanus and childhood tuberculosis, among others, were based on the attenuation of pathogens and toxins. However, the potential toxicity and the difficulty in carrying out this process with complex pathogens, such as HIV and hepatitis C virus (HCV), has led to the search for optimised antigens (Delany et al., 2014). Advances in biotechnology and an increased knowledge of the pathogen characteristics have led to the development of newer and safer subunit antigens, in particular proteins, peptides and nucleic acids (Nabel, 2013).

1.1.1. Recombinant proteins

Recombinant DNA technology has allowed the production of several proteins with antigenic activity, using expression vectors such as bacteria or yeast. A well-known example of this application is the production of the hepatitis B surface antigen (rHBsAg) in *Escherichia coli*, which has led to the first recombinant protein-based vaccines reaching the market using alum as adjuvant (Engerix-B® from GlaxoSmithKline Biologicals and Recombivax HB® from Merck & Co., Inc.). Similarly, antigens of the human papillomavirus (HPV), expressed in *Saccharomyces cerevisae* and *Trichoplusia ni*, are commercialised as Gardasil® (Merck & Co., Inc.) and Cervarix® (GlaxoSmithKline Biologicals), with alum and AS04® (a combination of alum and monophosphoryl lipid A (MPLA)) as adjuvants, respectively. Other pathogens for which recombinant protein antigens have been identified and studied include hepatitis C and E and rotavirus (Ohtake and Arakawa, 2013).

The concept of "reverse vaccinology", focussed on the scan of the whole genome of the pathogen for identification of antigenic protein candidates, represented an important advance towards the development of new vaccines. This strategy has led to the development, for example, of a new meningococcal vaccine, commercialised in Europe under the brand name of Bexsero® (by Novartis Vaccines). Vaccines against pathogens such as *Streptococcus pneumoniae* and *Leishmania infantum*, among others, have also been investigated using this approach (Donati and Rappuoli, 2013).

1.1.2. Peptides

The above indicated antigenic proteins still have important limitations such as complex production processes, difficult purification steps, and instability in a liquid form. More importantly, protein-based vaccines may induce autoimmunity and allergic reactions, as reported, for example, in the development of a vaccine against group A *Streptococcus* (GAS) (Batzloff et al., 2004; Skwarczynski and Toth, 2011). For these reasons, efforts are currently focussed on the production of small peptides as antigens. These antigenic peptides can be identified through the analysis of specific antibody-inducing regions within larger proteins (epitopes), and are generally obtained through simple synthesis with high purity, in large scale, and at a lower cost (Gori et al., 2013; Purcell et al., 2007; Rosendahl Huber et al., 2014; Skwarczynski and Toth, 2011). Several peptide-based vaccine formulations have already reached the clinical development phase, as it is the case for some preventive HIV vaccines (Girard et al., 2006) as well as some therapeutic anticancer vaccines (Mohit et al., 2014; Ott et al., 2014).

Overall, it can be stated that proteins and peptides have improved the vaccine safety profile in comparison with live attenuated pathogens, however, their poor immunogenicity is definitely hindering their development and success (Gori et al., 2013; Purcell et al., 2007). For this reason, it is essential to advance in the design of novel adjuvants that may help providing these vaccines with a robust immune response (Fox et al., 2013).

1.1.3. Genetic vaccination

In the last decades, nucleic acid-based vaccines have gained increasing attention (Deering et al., 2014; Xiang et al., 2010). Essentially, nucleic acids such as plasmid DNA (pDNA) and messenger RNA (mRNA) allow *in situ* production of the antigen by the cellular machinery of the host. This strategy mimics the natural infection by intracellular pathogens as it leads to the local formation of the antigenic molecule. Moreover, nucleic acids can be tailored to express antigens that are chemically or structurally different from their native form, with the intention of improving their immunogenicity (Alpar et al., 2005; Deering et al., 2014; Kramps and Probst, 2013). The main limitation of this approach is the low level of gene expression achieved upon administration, a limitation that can be overcome by designing effective viral and non-viral transfection vectors (Alpar et al., 2005; Mazid et al., 2013).

Plasmid DNA vaccination, based on the administration of selected antigen-encoding DNA through a plasmid vector, has been applied to prevent diseases such as malaria and HIV and also as a therapeutic approach in cancer immunotherapy. Some of these formulations have already reached the clinical development phase, as recently reviewed by Mazid et al. (Liu, 2003; Mazid et al., 2013). Nevertheless, the risk of genome integration and long-term effects of these vaccines are yet to be clarified (Schalk et al., 2006). As an alternative, messenger RNA has raised particular attention, with preclinical proofs-of-concept described for prophylactic influenza vaccination and some formulations in clinical development for anticancer immunotherapy (Deering et al., 2014; Kramps and Probst, 2013).

1.2. Vaccine adjuvants and antigen nanoengineering

Alum has been the traditional adjuvant of choice for vaccines, though its mechanism of action is not yet completely understood. It also has specific limitations such as the necessity to be stored at low temperature, a limited efficacy for peptide antigens, and inability to generate Th1 (cellular) immune responses (Azmi et al., 2014; Bomford, 1989; Lindblad, 2004). The need to overcome these limitations has stimulated the search for new adjuvants. As a consequence, there is currently a large variety of adjuvants, which for the purpose of this review we have classified in two groups, molecular adjuvants or immunostimulatory molecules and antigen delivery systems produced by the nanoengineering of antigens.

1.2.1. Molecular adjuvants

Small molecules, targeted at specific receptors present on immune cells (pattern recognition receptors (PRRs)), such as Toll-like receptors (TLRs), have the ability to trigger stronger immune responses (Demento et al., 2011). The most studied molecules are agonists for TLRs, as for example CpG oligonucleotides (TLR 9), poly(I:C) (TLR 3) or imiquimod (TLR 7/8), which have already been evaluated for their adjuvant properties in vaccines against malaria, hepatitis B, influenza, as well as in different therapeutic anticancer vaccines (Steinhagen et al., 2011).

Other molecules such as bacterial toxins (cholera toxin, *E. coli* heat-labile toxin and others), saponins (Quil-A or QS-21) and cytokines, are also used as immunostimulants in vaccine formulations. In particular bacterial toxins are known to enhance the immune response by targeting the antigen to the M cells in the intestinal tract, thereby boosting a strong humoral response at a mucosal level. An example of these toxins is cholera toxin (recombinant B subunit), which is used as an adjuvant for a commercialized oral cholera vaccine (Hill et al., 2006). Another example refers to a transdermal patch containing heat-labile *E. coli* enterotoxin for the enhancement of the immune response against pathogens such as *E. coli* and influenza (Behrens et al., 2014; Frech et al., 2008; Glenn et al., 2009). Nevertheless, a major limitation of these toxins is related to the immune response that can be generated against themselves rather than against the associated antigen (Mallapragada and Narasimhan, 2008; Reed et al., 2009; Wilson-Welder et al., 2009).

In the case of saponins, which are plant-derived triterpene glycosides, a detoxified derivative (QS-21) of Quil-A (from *Quillaja saponaria*) has been successfully included in particulate formulations such as

AS01™ (liposomes based on monophosphoryl lipid A (MPLA)) and AS02™ (squalene oil-in-water emulsion also containing MPLA), currently under clinical development for malaria vaccines (Kester et al., 2014; The RTS.S Clinical Trials Partnership, 2014). Also, the inclusion of saponins in immunostimulating complexes (ISCOMs) has been evaluated in vaccination against influenza, toxoplasmosis or Epstein–Barr virus-induced tumours, among others, achieving protective immunity in clinical studies (Barr et al., 1998; Sun et al., 2009).

With respect to cytokines, IL-2, IL-12 and IFN-γ are some of the molecules that have been studied for immune response modulation, both at preclinical and clinical levels, though some toxic effects have been observed with high doses in human studies (Hedlund et al., 2002; Hughes, 1998; Lynch et al., 2003).

1.2.2. Nanoengineering of antigens: antigen delivery systems

Macromolecules such as polymers, among them polysaccharides (as reviewed in Section 3), lipids such as MPLA and squalene, as well as several phospholipids, have also been used in some cases for the nanoengineering of antigens, leading to the formation of nanocarriers or antigen delivery systems (Fox, 2009; Perrin-Cocon et al., 2006). Specific moieties, such as pathogen-associated molecular patterns (PAMPs) that can be recognized by PRRs, are in some occasions naturally present in these macromolecules, or can be synthetically included in their structures to potentiate their function (Demento et al., 2011; Mora-Solano and Collier, 2014). In the case of MPLA and squalene, their recognized immunomodulation features have led to their inclusion in marketed vaccines, or as components of approved adjuvants such as MF59, AS03™ (in the case of squalene) and AS04™ (in the case of MPLA), as well as in other formulations still in preclinical and clinical development (Rappuoli et al., 2011).

Nanoengineering approaches can be used to associate antigens to delivery carriers made of specific biomaterials, normally recognized for their adjuvant properties. These delivery carriers are able to transport and control the release of antigens to the cells where they should exert a biological activity. Polymeric nanoparticles, ISCOMs, liposomes and lipid nanoparticles, among others, are included in this category (Azmi et al., 2014; Correia-Pinto et al., 2013; González-Aramundiz et al., 2012; Sahdev et al., 2014).

In general, it is recognized that the depot effect generated by the majority of antigen delivery systems after subcutaneous injection, allowing their uptake by the antigen presenting cells, is an attractive feature of this type of adjuvants (Aguilar and Rodríguez, 2007; Bachmann and Jennings, 2010). Moreover, they are able to mimic the particulate nature of pathogens, therefore increasing the possibilities of an effective immune response (De Temmerman et al., 2011). In this context, nanotechnology is expected to have a significant impact, as will be discussed in the following section.

2. The potential of nanotechnology for vaccine delivery

The use of technologies and biomaterials at nanometric scale in therapeutics and diagnostics is a growing research field since the second half of the 20th century (Duncan and Gaspar, 2011). A large variety of nanoparticulate systems has been developed throughout the past decades to improve the delivery, targeting and efficacy of drugs, biomolecules, nucleic acids and antigens (Etheridge et al., 2013). Depending on the components and methodology chosen, it is possible to develop a wide range of nanostructures, as for example (i) polymeric nanoparticles, based on a matrix-type entanglement of selected polymers, (ii) oil-in-water (O/W) nanoemulsions, consisting in oil nanodrops stabilized by adequate surfactants, (iii) nanocapsules, which are polymer-coated nanoemulsions, forming a core–shell nanostructure, and (iv) lipid-based nanosystems, such as liposomes and solid lipid nanoparticles, among others.

In the area of vaccination, nanotechnology has led to the development of nanostructures holding specific advantages for antigen delivery. First of all, as mentioned before, due to their particulate structure and nanometric size, similar to the ones of virus and bacteria, nanoparticles can mimic the natural infection process and be taken-up by the antigen presenting cells (APCs), thereby leading to enhanced immune responses (Bachmann and Jennings, 2010; Reddy et al., 2006b; Storni et al., 2005; Zolnik et al., 2010). A number of authors have reported that particles in the nanometric range are particularly suitable for their interaction with the immune system (Fifis et al., 2004; Joshi et al., 2013; Reddy et al., 2007). In addition, in our lab, working with PLA–PEG micro and nanoparticles, we have also observed that 200 nm-nanoparticles can enhance the transport of the antigen through the nasal mucosa more efficiently than microparticles, (either 1 or 5 μM) after intranasal administration (Vila et al., 2005). However, there is still some controversy regarding this issue, with other works supporting the idea that microparticles can elicit stronger immune responses (Gutierro et al., 2002; Kanchan and Panda, 2007). This disagreement may come from the difficulty to compare different studies, as many variables account for the total outcome of the immune response, such as the constituting biomaterials, the nature and doses of antigen, and the route of administration (Oyewumi et al., 2010). It has also been hypothesized that using nanoparticles may favour cellular immune responses through optimal interactions with $CD8^+$ dendritic cell (DC) subsets (Bachmann and Jennings, 2010). For example, using model carboxilated polystyrene micro (2 μm) and nanoparticles (40 nm), loaded with ovalbumin (OVA) (Fifis et al., 2004), it was shown that nanoparticles were able to elicit significantly higher IgG and T-cell responses. Finally, it has been reported that a size below 100 nm is desirable if the purpose is to facilitate the transport of nanoparticles from the subcutaneous tissue up to the lymph nodes, where the antigens will be presented to mature immune cells for an adaptive immune response (Reddy et al., 2006a, 2007).

Nanostructures have also shown an interesting potential for mucosal antigen delivery, due to their ability to interact and get across mucosal barriers (Csaba et al., 2009; des Rieux et al., 2006). This property is mainly related to the particle size (Desai et al., 1996; Jani et al., 1990), and also to the composition, being favoured when the systems include mucoadhesive materials such as chitosan (Grabovac et al., 2005). Moreover, our group was pioneer in demonstrating that the modification of nanocarriers with polyethylene glycol (PEG) units, in different degrees, was also responsible for improved transport of those systems across the nasal mucosa (Tobío et al., 1998). Overall, the increased interaction of these systems with the mucosae boosts the antigen presentation in those areas, where the natural entrance of several pathogens usually happens, therefore mimicking the natural infection process. Mucosal antigen delivery, or needle-free vaccination, allows overcoming important limitations associated to parenteral immunization such as the high cost of preparation, the need for specific administration materials (needles and syringes) and specialized technical staff, and is also more likely to be well accepted by patients, altogether resulting in an improved vaccine coverage (Chadwick et al., 2010; Giudice and Campbell, 2006).

Finally, another important advantage of nanosystems in vaccine delivery is the possibility to co-encapsulate additional immunostimulatory molecules, such as the ones described in Section 1.2.1, with the purpose of increasing the overall adjuvant capacity. As an example, TLR agonists such as CpG, poly(I:C) (Peine et al., 2013) or imiquimod (Vicente et al., 2013b), have been associated to dextran nanoparticles and chitosan nanocapsules, respectively. These molecules promote specific receptor-based recognition of the nanovaccines and the consequent cell activation, strengthening the elicited immune response.

To highlight the potential of nanoparticles in vaccination, it is worth mentioning the formulations that have already reached the market and others in advanced clinical stages of development. Nanoemulsions, such as AS03™ (an oil-in-water nanoemulsion containing squalene, DL-α-tocopherol and Tween® 80) (Roman et al., 2010) or MF59™ (also squalene oil-in-water nanoemulsion stabilized with Tween® 80 and Span®

85) (Esposito et al., 2011), are commercialized for influenza vaccines, namely Pandemrix™ (from GlaxoSmithKline Biologicals), and Fluad™ and Focetria™ (from Novartis), respectively. Virosomes, composed of a phospholipid membrane incorporating viral glycoproteins, are present in commercialized Hepatitis A and influenza vaccines, Epaxal™ and Inflexal™ (both from Crucell), respectively (Usonis et al., 2003). Other nanometric formulations such as AS01™ (liposomes based on MPLA) (Leroux-Roels et al., 2010), AS02™ (squalene oil-in-water emulsion also containing MPLA) (Aide et al., 2011), and one polymeric carrier based on PLGA, among others, are currently under clinical development for different vaccines, as described in Table 1. Considering this, it is clear that nanometric delivery systems are in the spotlight for their potential in vaccination.

3. Nanoengineering of vaccines using polysaccharides

In the late 70s, the work of Kreuter and Speiser opened the way for the specific use of polymers, such as polymethylmethacrylate, as materials for the engineering of antigen nanocarriers (Kreuter and Speiser, 1976). Since then, a significant number of studies have put in evidence the potential of nanoparticles to enhance the immune response against different antigens in a sustained and prolonged way (Correia-Pinto et al., 2013; González-Aramundiz et al., 2012; Reddy et al., 2006b; Rice-Ficht et al., 2010; Vicente et al., 2010). Our group, being particularly active in the field, pioneered the encapsulation of model proteins and antigens within poly(lactic-co-glycolic acid) (PLGA) (Blanco and Alonso, 1997) and polylactic acid–polyethylene glycol (PLA–PEG) nanoparticles (Tobío et al., 1998). Interestingly, this work was followed by a great number of authors (Danhier et al., 2012), whose contributions have led to the clinical development of PLGA-based nanovaccines (www.clinicaltrials.gov). All along this engineering trajectory, it became clear that a major inconvenience of this biomaterial was the degradation of the antigen encapsulated in the course of the polymer degradation (Alonso et al., 1994; Tobío and Alonso, 1998). Although specific formulation strategies were found to significantly reduce this effect over the encapsulated antigens (Sánchez et al., 1999; Schwendeman et al., 1996), overall the results achieved using PLGA-based nanoengineering persuaded us and others to search for new biomaterials which might have a mild interaction with antigens.

Naturally occurring polymers, in particular polysaccharides attracted our attention in the mid 90s as biomaterials for antigen nanoengineering. With this goal in mind, we reported for the first time the production of nanoparticles consisting of assemblies of proteins and chitosan (Calvo et al., 1997a), as described in the following section. Following this, other authors have proposed the use of polysaccharides, i.e. dextran, mannan and beta glucans for the nanoengineering of vaccines (Petrovsky and Cooper, 2011). These latter biomaterials are found in the cell walls of several pathogens such as bacteria or yeast, a characteristic that provides them with intrinsic targeting abilities to APCs (acting as PAMPs on the PRRs present in these cells) and, consequently, a natural capacity to enhance the immune response against the associated antigens (Demento et al., 2011; Dykstra et al., 2011; Mora-Solano and Collier, 2014; Petrovsky and Cooper, 2011). Other important features such as high biocompatibility and low toxicity make polysaccharides particularly interesting for pharmaceutical development purposes.

Another specific advantage associated to the use of polysaccharide-based antigen nanocarriers is related to the technologies used to produce them. These technologies rely on physicochemical processes such as ionic gelation (Calvo et al., 1997c), complexation (Kean et al., 2005) and solvent displacement (Calvo et al., 1997b), among others. These are generally simple and mild techniques, which minimize the use of solvents and high-energy sources, easy to scale-up, and importantly, suitable for the association of labile biomolecules (Vauthier and Bouchemal, 2011). Apart from selecting an appropriate technology, other relevant technical aspects for the development of nanovaccines, i.e. the stability of the antigen, in terms of biological activity, and the stability of the formulation during storage, are to be considered at early stages of development (Amorij et al., 2012; Chen and Zehrung, 2013). Selection of raw materials with pharmaceutical quality, i.e. produced according to specific criteria that assure their high purity and adequate characteristics for use in humans and with good inter-batch reproducibility, are also important topics to take into consideration in the process of nanovaccine design and manufacturing.

Table 1
Nanoengineered antigen formulations in clinical development.

Delivery System	Antigen	Phase	End	Identifier
MF59	Influenza (H5N1 inactivated virus)	n.d.	2014	NCT01578317
	Influenza (H7N9 inactivated virus)	I	Ongoing	NCT02251288
		II	2014	NCT01938742
		II	Ongoing	NCT02213354
	Influenza (killed virus, trivalent subunit vaccine)	I	Ongoing	NCT02126761
		II	2013	NCT01879540
	RSV (RSV F protein)	I	Ongoing	NCT02298179
	CMV (gp B)	II	2013	NCT00133497
MF59 & AS03	Influenza (H7N9 inactivated virus)	II	2015	NCT01942265
AS03	Influenza (H5N1 inactivated virus)	I/II	2012	NCT01353534
		I	2014	NCT01573312
		II	Ongoing	NCT01910519
AS03 & AS01	Dengue (inactivated virus)	I	Ongoing	NCT01702857
		I	Ongoing	NCT01666652
AS01	Malaria (FMP012; FMP2.1; RTS,S proteins)	I	Ongoing	NCT02174978
		I/II	2014	NCT02044198
		I/II	2014	NCT01883609
		II/III	Ongoing	NCT00872963
AS02	Malaria (FMP1; RTS,S proteins)	I/II	2014	NCT01556945
ISCOM	HSV (GEN-003 protein)	II	Ongoing	NCT02114060
		II	Ongoing	NCT02300142
	Malaria (viral vector with ME-TRAP protein)	I	2014	NCT01669512
	Influenza (H7N9 VLP)	I/II	Ongoing	NCT02078674
Iscomatrix	Cancer (tumour cell lysates)	I/II	Ongoing	NCT02054104
Hydrogel	Influenza (Act-HIB® vaccine)	I/II	2012	NCT01578070
Virosomes	Hepatitis A (inactivated virus)	II	2013	NCT01405677
	Candidiasis (n.d.)	I	2012	NCT01067131
PLGA microspheres	Cancer (HER-2/Neu peptide)	I	2012	NCT00005023

Abbreviations: RSV, respiratory syncytial virus; CMV, cytomegalovirus; gp B, glycoprotein B; HSV, herpes simplex virus; VLP, virus-like particle; n.d., not disclosed.

Nevertheless, the potential of these biomaterials in this field deserves a deeper analysis of the published material concerning polysaccharide-based nanosystems in vaccination. For this reason, the characteristics of the main polysaccharides with described adjuvant properties, as well as their application in the development of nanovaccines, are detailed as following.

3.1. Chitosan as a biomaterial for antigen nanoengineering

Chitosan (CS) is a naturally occurring polymer composed of a linear backbone of D-glucosamine and *N*-acetyl-D-glucosamine monomers connected through β-(1,4) bonds. This polysaccharide is mainly obtained from the deacetylation of chitin, present in the exoskeleton of crustaceans and squids, though other sources such as fungi have also been reported (García-Fuentes and Alonso, 2012; Gomes et al., 2014). One of the most relevant characteristics of chitosan is the possibility to modulate its degree of acetylation and therefore the number of amino groups (Gomes et al., 2014). Its cationic character allows electrostatic interactions with antigens that are negatively charged in physiological conditions, and is also responsible for its ability to interact with mucosal surfaces (Islam et al., 2012; Sogias et al., 2008).

Some authors have indicated potential chitosan immunomodulatory properties based on some *in vitro* and *in vivo* studies performed using chitosan in solution. More precisely, Peluso et al. found that chitosan solutions can activate peritoneal rat macrophages through the enhancement of nitric oxide secretion *in vitro* (Peluso et al., 1994). On the other hand, Porporatto et al. have reported the ability of chitosan to trigger local and systemic immune responses, evidenced by the enhancement of cytokine production upon oral administration of a single 3 mg dose of the polysaccharide in solution to rats (Porporatto et al., 2005). Finally, Zaharoff et al. evaluated the adjuvant potential of a chitosan solution (1 mg dose), given subcutaneously, in comparison with phosphate buffered saline solution (PBS) and with common adjuvants such as alum and Incomplete Freund's Adjuvant (IFA), using a model protein antigen, β-galactosidase (Zaharoff et al., 2007). Results have shown the ability of chitosan to induce humoral and cellular responses, which were superior to the non-adjuvanted formulation and the alum-adjuvanted one and equivalent to the ones achieved using IFA. Nevertheless, these immunomodulatory properties are still under question and could be related to the uncontrolled precipitation of chitosan at physiological pH, and also to the source and quality of chitosan used (García-Fuentes and Alonso, 2012).

With respect to the biocompatibility and biodegradability of chitosan, it is worth mentioning that chitosan has a "generally recognized as safe" (GRAS) status granted by the United States Food and Drug Administration (FDA). Chitosan has been used for a long time as a dietary supplement for the prevention of fat absorption, and as a component of wound dressings (Boateng et al., 2008). On the other hand, chitosan is present in new nasal vaccine formulations against meningitis and Norovirus, which are under clinical development (Atmar et al., 2011; Huo et al., 2005). In addition, it is present in a vaccine hydrogel formulation against influenza, for intramuscular injection (Neimert-Andersson et al., 2014). Overall, chitosan is therefore considered as one of the most advanced polymers in the regulatory path for the indication of vaccination. Taking this into account, and also preclinical evidence of the potential of chitosan-based nanocarriers as adjuvants for mucosal and parenteral immunization (Table 2), it could be expected that chitosan nanoformulations could enter clinical trials in the oncoming years.

3.1.1. Protein nanovaccines

3.1.1.1. Parenteral vaccination with chitosan-based nanovaccines. As indicated, chitosan nanoparticles, developed for the first time in the 90s (Calvo et al., 1997c), have been widely studied for the delivery of proteins and antigens (Arca et al., 2009; García-Fuentes and Alonso, 2012). For example, chitosan nanoparticles associating rHBsAg exhibited an adjuvant effect that was higher than that of alum, after intramuscular administration to mice (Prego et al., 2010). Other studies have been addressed to co-associate immunostimulant molecules, i.e. CpG, and rHBsAg in chitosan nanoparticles. This vaccine formulation increased the IgG titres in comparison with the antigen in solution, upon subcutaneous administration to mice (Borges et al., 2008). Chitosan nanoparticles have been additionally reported for their potential in a single-dose vaccination strategy for rHBsAg (Lugade et al., 2013). The results of this study indicated that after a single intraperitoneal, intramuscular or subcutaneous injection of the nanoparticles, the response achieved was stronger and lasted longer than the one elicited by a single dose of Recombivax HB® (alum-associated rHBsAg), given intraperitoneally.

A different type of chitosan-based nanocarrier, i.e. chitosan nanocapsules, originally disclosed by our group (Prego et al., 2006) was similarly proposed for a single-dose immunization schedule with rHBsAg (Vicente et al., 2013a). Additionally, we developed a freeze-dried formulation in order to improve the preservation of the vaccine. The results observed upon a single intramuscular administration to mice indicate that the IgG levels were comparable to those obtained after vaccination with the alum-adsorbed antigen in a prime-boost scheme (weeks 0 and 4). We could also prove that these nanocapsules were adequate for the co-encapsulation of the TLR 7/8-agonist imiquimod (Vicente et al., 2013b), a formulation approach for nasal immunization, further discussed in the next section. More recently, we conducted experiments to assess the biodistribution of these nanocapsules after subcutaneous injection (Vicente et al., 2014). The results evidenced the formation of a depot, followed by a slow drainage of the nanocapsules towards the lymph nodes, where they accumulate (for illustration see Fig. 2). These results allowed us to conclude that this biodistribution profile was responsible for the long-lasting adjuvant effect observed for chitosan nanocapsules.

3.1.1.2. Mucosal vaccination with chitosan-based nanovaccines. Apart from the promising results obtained with parenteral immunization using chitosan nanovaccines, a great deal of effort has been addressed to the development of chitosan-based nanovaccines intended for mucosal vaccination. Our group pioneered the development of the first chitosan-based nanovaccine as a needle-free vaccination strategy (Vila et al., 2004). Our results showed that after intranasal administration to mice, chitosan-based tetanus toxoid (TT) nanovaccine led to high and long-lasting IgG levels, which were comparable to those elicited by the alum-adsorbed vaccine administered intramuscularly (Vila et al., 2004). More recently, chitosan nanoparticles were studied for the association of the hemagglutinin protein of H1N1 influenza virus. After intranasal administration of hemagglutinin-loaded chitosan nanoparticles to mice, both the systemic and mucosal antibody levels (IgG and IgA) were significantly enhanced with respect to the controls (antigen in solution), and a T cell response was also reported. Importantly, after being challenged through the same route with the virus, the animals receiving the nanovaccine presented higher survival rates (Sawaengsak et al., 2014). In another study, chitosan nanoparticles were loaded with *Streptococcus equi* bacterial proteins and administered intranasally to mice. The results showed enhanced IgG and IgA responses as compared to those elicited by the antigen-loaded liposomes and the corresponding empty nanoparticles and liposomes (Figueiredo et al., 2012).

Our group has also explored the potential of co-encapsulation of rHBsAg and the immunostimulant imiquimod into chitosan nanocapsules, for enhancing the immune response following intranasal vaccination (Vicente et al., 2013b). The results obtained in mice evidenced an enhanced, specific and Th1/Th2 balanced immune response, which was significantly higher than the one observed for rHBsAg-loaded chitosan nanocapsules (without imiquimod) and the control rHBsAg-loaded nanoemulsion (Fig. 3). These results highlight the positive effect of co-

Table 2
In vivo evaluation of chitosan-based nanovaccines.

Nanosystem	Antigen	Administration route	*In vivo* efficacy results	Reference
Nanoparticles	OVA	IM, IN	After IM immunization (single dose), OVA-loaded TMC nanoparticles and TMC–OVA nanoconjugates provided higher IgG titres than the controls, and increased DC uptake and activation. IN immunization (2 doses) elicited strong and balanced IgG and IgA levels.	Slütter et al. (2010b,c)
			Higher IgG levels were achieved with TMC nanoparticles in comparison with PLGA nanoparticles (coated or not with TMC), irrespective of the administration route (3 doses).	Slütter et al. (2010a)
		Intraduodenal	TMC or chitosan nanoparticles (2 doses), increased the IgG levels and induced DC maturation in comparison with OVA in solution.	Slütter et al. (2009)
		IN, ID	TMC nanoparticles co-encapsulating additional adjuvant molecules (2 doses) were compared. In terms of IgG and IgA levels, LPS was best in both routes, followed by MDP for IN route and CpG for ID route.	Bal et al. (2012)
		IN, TD	Covalently-linked TMC:HA nanoparticles elicited higher IgG levels than free OVA and conventional TMC:HA nanoparticles based on electrostatic interactions (2 doses).	Verheul et al. (2011)
	rHBsAg	IM	Significantly higher IgG levels in comparison with alum-adsorbed antigen (2 doses).	Prego et al. (2010)
		IP	Stronger and longer-lasting IgG levels elicited in a single-dose schedule in comparison with commercial vaccine. Results with the nanovaccine were comparable irrespective of IP, IM or SC administration route.	Lugade et al. (2013)
		SC	Significantly higher IgG levels co-encapsulating CpG (2 doses) than with the antigen in solution. Coating the antigen-loaded nanoparticles with alginate and co-administering a CpG solution shifted the response towards Th1/Th2 balance and increased IFN-γ levels (cellular response).	Borges et al. (2008)
	pRc/CMV-HBs (plasmid)	IN	Protective and Th1-biased IgG levels, as well as high IgA levels in nasal, salivary and vaginal secretions, elicited after 2 immunizations.	Khatri et al. (2008b)
	TT		IgG levels upon 3 doses were higher than those reported with the antigen in solution, and comparable to IM alum-adsorbed vaccine.	Vila et al. (2004)
Nanoparticles	TT	IN	TMC nanoparticles (2 doses) elicited similar response than chitosan nanoparticles, which were significantly higher than the antigen in solution.	Sayın et al. (2008)
	Hemagglutinin		Two doses of the nanovaccine elicited high IgG and IgA levels, induction of IFN-γ production by spleen cells (cellular response) and an increased survival of challenged animals up to 100%.	Sawaengsak et al. (2014)
	Streptococcus equi		Higher IgA levels, increased mucosal uptake and Th1/Th2 balanced responses in comparison with cationic liposomes (2 doses).	Figueiredo et al. (2012)
	Antigen-encoding plasmids	IM	Plasmid encoding *Chlamydia trachomatis* proteins. High local (mice thigh muscles) and systemic (mice spleens) protein expression levels after a single-dose administration.	Cambridge et al. (2013)
			Plasmid encoding 3 T-cell epitopes of Esat-6 (*Mycobacterium tuberculosis* antigen). Strong Th1 and CTL responses as well as protection against challenge upon 3 immunizations.	Feng et al. (2013)
		SC/IN	Plasmid encoding antigen 85B (*M. tuberculosis*). Co-encapsulation of another plasmid encoding an autophagy-inducing factor (myc-mTOR). Strong IgG and cytokine (IL-4 and IFN-γ) levels after SC prime and two IN boosts.	Meerak et al. (2013)
		IN	Plasmid encoding pHSP65pep (*M. tuberculosis*). Strong antibody and T-cell responses and increased protection against challenge after 4 immunizations.	Ai et al. (2013)
			Plasmid encoding the SARS-CoV nucleocapsid protein. Particles functionalized with a protein vector for DC targeting (bfFP) and a DC maturation stimulus (aCD40) (2 doses) showed better targeting to DCs and increased mucosal response.	Raghuwanshi et al. (2012)
		Oral	Plasmid encoding Der p 2 (house dust mites allergen). Antibody (IgG2 and IgE) and cytokine (IFN-γ and IL-4) levels correlated with the minimization of the allergic process (2 doses).	Li et al. (2009)
			Plasmid encoding Rho1-GTPase (*Schistosoma mansoni* antigen). Significantly reduced liver granulomatosis and worm burden (after challenge) in comparison with controls (3 doses).	Oliveira et al. (2012)
Nanocapsules	rHBsAg	IM, IN	A single IM dose of the vaccine prototype elicited similar IgG levels as two IM doses of alum-adsorbed antigen. Including imiquimod (TLR-7/8 agonist) enhanced a specific Th1-biased immune response through IN route.	Vicente et al. (2013a,b)
Liposomes	Antigen-encoding plasmids	IN	Plasmid encoding HBsAg. Glycol chitosan-coated liposomes (2 doses) elicited seroprotection and increased IgA levels in nasal, vaginal and salivary secretions in comparison with controls.	Khatri et al. (2008a)
			Plasmid encoding *Streptococcus mutans* surface antigen. Chitosan-coated liposomes (2 doses) selectively released DNA at pH 7.4 (cellular cytoplasm), increased nasal residence time and enhanced IgA levels, in comparison with DNA-loaded chitosan nanoparticles.	Chen et al. (2013)

Abbreviations: rHBsAg, recombinant hepatitis B surface antigen; OVA, ovalbumin; TT, tetanus toxoid; IM, intramuscular; IP, intraperitoneal; SC, subcutaneous; IN, intranasal; ID, intradermal; TD, transdermal; IgG, immunoglobulin G; IFN-γ, interferon gamma; IgA, immunoglobulin A; TMC, trimethylchitosan; DC, dendritic cells; PLGA, poly(lactic-co-glycolic acid); MDP, muramyl dipeptide; LPS, lipopolysaccharide; HA, hyaluronic acid; MCC, mono-N-carboxymethyl chitosan; CTL, cytotoxic T lymphocyte; IL-4, interleukin 4; SARS-CoV, severe acute respiratory syndrome coronavirus; JE, Japanese encephalitis; TLR, Toll-like receptor; IgE, immunoglobulin E.

delivering an additional immunostimulating molecule with the antigen in a single nanostructure, and open room for the potential of modulating immunity towards the cellular pathway using a needle-free vaccination approach.

A chitosan derivative with higher water solubility and pH-independent cationic nature, N-trimethyl chitosan (TMC), has also been evaluated for the preparation of nanovaccines. The intraduodenal immunization of OVA-loaded TMC nanoparticles to mice, provided a similar immune response than conventional chitosan nanoparticles, in terms of IgG levels, and showed an improved ability to induce DC maturation (Slütter et al., 2009). The behaviour of OVA-loaded TMC nanoparticles was also compared with that of OVA-loaded PLGA and OVA-loaded TMC-coated PLGA nanoparticles, upon intranasal or intramuscular administration to healthy mice. While all formulations could enhance the immune response following intramuscular administration, only TMC nanoparticles were able to elicit an immune response after intranasal administration, a fact that was attributed to their increased interaction with the nasal mucosa (Slütter et al., 2010a). The efficacy of these nanoparticles was also compared with that of TMC–OVA conjugates, previously developed for intramuscular administration, which had shown adjuvant activity similar to that of TMC nanoparticles (Slütter et al., 2010c). Upon intranasal administration of both TMC–

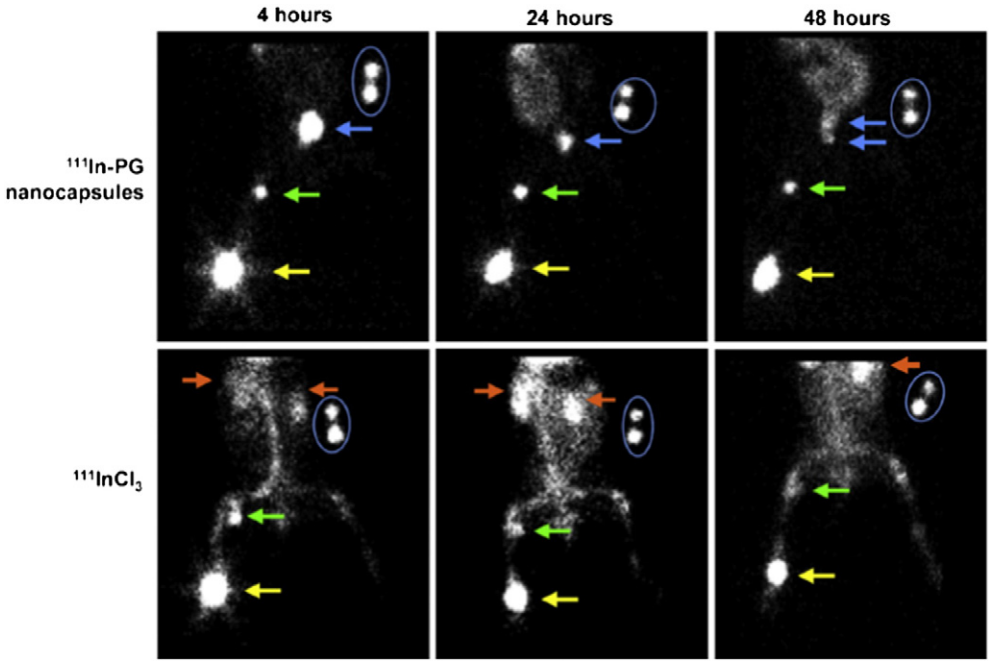

Fig. 2. Images of the lower body of rabbits injected with ^{111}In-radiolabelled chitosan nanocapsules (upper row) or with a control solution of $^{111}InCl_3$ (lower row), acquired 4, 24, and 48 h post injection. A depot formation in the injection site, as well as a slow drainage and further accumulation in the popliteal lymph node, can be observed in the case of the nanocapsules. Yellow arrow: injection site (rear foot); green arrow: popliteal lymph node; blue arrow: iliac lymph nodes; orange arrows: kidneys; circle: external standard.
Adapted with permission from Vicente et al. (2014).

OVA conjugates (30 nm) and OVA-loaded TMC nanoparticles (300 nm), results have shown the conjugates' superiority in terms of the IgG and IgA levels elicited, a fact attributed to the higher uptake of these structures by the nasal epithelium (Slütter et al., 2010b).

In another study, TT-loaded TMC nanoparticles were compared to TT-loaded chitosan nanoparticles, upon either intranasal or subcutaneous administration to mice, with the corresponding polymer solution as a control. The results evidenced the similar behaviour of both types of nanoparticles in terms of IgG response (Sayın et al., 2008). Finally, TMC has also been used in combination with hyaluronan (HA) (Verheul et al., 2011). Despite the observation of an immune response upon intranasal administration to mice, the benefits of incorporating HA to the formulation remain unclear, since comparative studies with plain TMC nanoparticles were not included in this work.

The last example corresponds to OVA-loaded TMC nanoparticles that co-encapsulate additional immunostimulant molecules, namely lipopolysaccharide (LPS, TLR-4 agonist), CpG (TLR-9 agonist), Pam_3CSK_4 (TLR-2 agonist), muramyl dipeptide (MDP, NOD-like receptor 2 ligand) and cholera toxin B subunit (CTB, GM1 ganglioside receptor ligand) (Bal et al., 2012). Upon intranasal administration to healthy mice, a Th2-biased response was reported in most cases (except for CpG), being the highest response achieved with nanoparticles co-associating MDP.

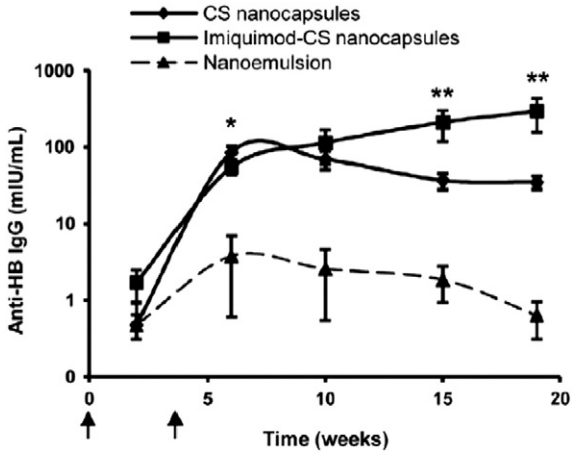

Fig. 3. Serum IgG levels achieved after intranasal immunization (two doses at 0 and 4 weeks, indicated by the arrows) of healthy mice with rHBsAg-imiquimod-loaded chitosan nanocapsules, rHBsAg-loaded chitosan nanocapsules (without imiquimod) and rHBsAg-loaded nanoemulsion (control group without chitosan). *$p < 0.05$ between rHBsAg-loaded chitosan nanocapsules (with and without imiquimod) and the rHBsAg-loaded nanoemulsion; **$p < 0.05$ between rHBsAg-imiquimod-loaded chitosan nanocapsules and the other two formulations.
Reproduced with permission from Vicente et al. (2013b).

3.1.2. Nucleic acid-based nanovaccines

3.1.2.1. Parenteral vaccination with chitosan/nucleic acid-based nanovaccines. Chitosan nanoparticles have also been developed for the delivery of plasmid DNA for immunization purposes (Gomes et al., 2014). In a couple of examples, chitosan nanoparticles have been loaded with plasmid DNA encoding for the recombinant major outer membrane protein of *Chlamydia trachomatis* (Cambridge et al., 2013) or for three T-cell epitopes of Esat-6, a *Mycobacterium tuberculosis* antigen critical for virulence (Feng et al., 2013). In the first case, results showed high expression levels of the *C. trachomatis* protein after intramuscular administration to mice, both at the site of injection and also systemically (i.e. spleen) (Cambridge et al., 2013). In the second example, the results obtained after intramuscular administration to mice indicated a protection against a *M. tuberculosis* challenge associated to strong Th1 and cytotoxic T lymphocyte (CTL) responses (Feng et al., 2013).

3.1.2.2. Mucosal vaccination with chitosan/nucleic acid-based nanovaccines. Chitosan nanoparticles have been tested for intranasal immunization against several pathogens. One example refers to chitosan nanoparticles that associate a plasmid encoding a multi-epitope protein against *M. tuberculosis* (pHSP65pep). This formulation provided the administered mice with an adequate protection against bacterial challenge, protection that was associated to significant T-cell responses

(Ai et al., 2013). In another study, also for *M. tuberculosis* immunization, chitosan nanoparticles were loaded with a plasmid encoding for antigen 85B together with a second plasmid encoding an autophagy-inducing factor (myc-mTOR) (Meerak et al., 2013). After immunization of mice with a subcutaneous prime and two intranasal boosts (2-week intervals), results proved a synergistic effect, due to the co-administration of both plasmids, in terms of immune response. Chitosan nanoparticles have also been evaluated against hepatitis B, upon association of a plasmid encoding the hepatitis B surface antigen (pRc/CMV-HBs) (Khatri et al., 2008b). Results showed a Th1-biased response after intranasal administration to mice, which was accompanied of increased IgA levels in nasal, salivary and vaginal secretions.

In an attempt to further improve their efficacy as DNA carriers for intranasal immunization, chitosan nanoparticles loaded with a plasmid encoding the nucleocapsid protein of severe acute respiratory syndrome coronavirus (pVAXN) were decorated with a bifunctional fusion protein (bfFP) targeted at the DC surface receptor DEC-205, and also with an antibody binding the DC receptor CD40, to stimulate these cells. Mice were immunized either intranasally or intramuscularly with (i) naked pVAXN, (ii) pVAXN-loaded nanoparticles, (iii) bfFP decorated pVAXN-loaded nanoparticles, and (iv) bfFP and aCD40 decorated pVAXN-loaded nanoparticles. The results indicated that the nanoparticles decorated with both molecules (bfFP/aCD40 approach), were more efficient in terms of IgG and IgA responses irrespective of the administration route (Raghuwanshi et al., 2012). The incorporation of immunostimulatory molecules in chitosan-based nanocarriers was also evaluated. In detail, Heuking et al. (Heuking and Borchard, 2012; Heuking et al., 2009) grafted TLR agonists to TMC and prepared nanoparticles with the model plasmid pGFP. *In vitro* results showed that the incorporation of Pam_3Cys (TLR-2 agonist) and 9-benzyl-8-hydroxyadenine (TLR-7 agonist) enhanced IL-8 release, a cytokine responsible for the attraction of leukocytes to the local of infection.

Chitosan nanovaccines have also been tested for oral immunization (Li et al., 2009; Oliveira et al., 2012). An interesting work refers to the association of plasmid DNA encoding Der p2, an allergen from house dust mites, to chitosan nanoparticles, which were orally administered to healthy mice. The results evidenced high IgG2 and low IgE levels, together with high IFN-γ and low IL-4 secretion, in accordance with a reduction of the allergic response (Li et al., 2009). In another work, a chitosan derivative containing imidazole moieties (CSimi) was used in the preparation of nanoparticles for the delivery of a plasmid encoding a *Schistosoma mansoni* antigenic protein (Rho1-GTPase) (Oliveira et al., 2012). After oral administration of three doses of pDNA-loaded CSimi nanoparticles to healthy mice (weeks 1, 3 and 5), the animals were challenged with the pathogen and results evidenced a reduction of the main pathogenic effect, liver granulomatosis, in comparison with the control animals that received PBS. Interestingly, one of the control groups that received blank chitosan nanoparticles (without antigen) also promoted a strong worm burden reduction in comparison with the control group, a fact that was attributed to an immune reaction against chitosan, since other similar carbohydrates are present in the parasite structure.

Chitosan-coated liposomes have also been described for mucosal immunization (Chen et al., 2013; Khatri et al., 2008a). In detail, glycol chitosan-coated liposomes, which associated a plasmid encoding the surface hepatitis B antigen (pRc/CMV-HBs), were administered intranasally to mice (Khatri et al., 2008a). The results, in terms of IgG responses, were similar to those observed in the control mice receiving the conventional alum-adsorbed encoded antigen (HBsAg) intramuscularly. However, the coated liposomes promoted a superior cell response as evidenced by the IL-2 and IFN-γ levels. Moreover, IgA levels in nasal, vaginal, and salivary secretions were only detected in the animals immunized with the chitosan-coated liposomes. In another example, chitosan-coated liposomes were loaded with a plasmid encoding a surface antigen of *Streptoccocus mutans*, for dental caries prevention, and their efficacy was compared to that of plasmid-loaded chitosan nanoparticles and plasmid in solution (Chen et al., 2013). After intranasal administration to healthy mice, chitosan-coated liposomes elicited an improved immune response, which was related to the controlled antigen released and to the improved interaction of the plasmid-loaded nanoparticles with the nasal mucosa.

Globally, these results evidence the increasing interest in chitosan-based nanovaccines particularly for either parenteral or mucosal immunization strategies using both protein antigens and nucleic acid-based antigens. This potential is associated to the ability of chitosan-based nanosystems to interact with mucosal tissues and to their specific distribution into the lymphatic system. This accumulated information is firmly consolidating the potential of this polysaccharide as a biomaterial for the engineering of new vaccines.

3.2. Dextran as a biomaterial for nanoengineering antigens

Dextran is one of the most studied α-glucans in drug and antigen delivery. This polymer of α-(1,6)-glucan with α-(1,3) branches is obtained from bacteria, particularly from *Lactobacillus*, *Leuconostoc* and *Streptococcus* species (Raemdonck et al., 2013). Native bacterial dextran has high molecular weight and is water soluble, which is a useful characteristic for pharmaceutical formulation purposes. Also, its structure, particularly its hydroxyl groups, allow for easy functionalization and chemical modifications (Baldwin and Kiick, 2010). Finally, the biocompatibility and biodegradability of this biomaterial, together with its high availability and reduced cost of production, make it very attractive for the nanoengineering of antigens (Baldwin and Kiick, 2010; Raemdonck et al., 2013).

It is important to mention that dextran has GRAS status given by the FDA and has been used in humans for a very long time, mainly as plasma volume expander and anti-thrombotic agent (de Belder, 1996). Its sulfated derivative, dextran sulfate (DS), has also been approved by the FDA as a component of apheresis columns, used to remove low density lipoprotein (LDL) from the blood (www.fda.gov). Since the 70s, the immunomodulating properties of dextran sulfate and its potential use as adjuvant have also been studied. Overall the results reported so far have shown its ability to increase the antibody- and cell-mediated immune responses in animal models (Kerlin and Watson, 1987; McCarthy et al., 1977). This derivative, when administered "ad libitum" with drinking water at a 3% (w/v) concentration, has also been associated with strong pro-inflammatory effects, being used as an inducer of inflammatory diseases such as colitis in mice for the development of animal models of this disease (Laroui et al., 2012). Diethylaminoethyl (DEAE)-dextran is another derivative with adjuvant properties, studied mostly for veterinary vaccines (Finnerty et al., 1994). Nevertheless, the mechanism of action of this derivative is still not well understood and requires further research (Cox and Coulter, 1997; Petrovsky and Cooper, 2011).

3.2.1. Protein nanovaccines

As in the case of chitosan, the polysaccharide dextran has been explored as a biomaterial for nanoengineering of protein antigens. However, in this case, and despite its reported immunostimulant properties, the activity has been limited to a few studies, as described below.

3.2.1.1. Parenteral vaccination with dextran-based nanovaccines. Dextran nanoparticles containing OVA in combination with LPS were developed and administered intravenously to mice (Shen et al., 2013). The results showed that these carriers were efficiently internalized by DCs, and induced OVA-specific T-cell proliferation ($CD4^+$ and $CD8^+$ T cells), as well as a stronger Th2-biased immune response, in comparison with the controls (empty dextran nanoparticles, LPS-loaded nanoparticles and OVA-loaded nanoparticles). Dextran nanoparticles have also been evaluated for the encapsulation of immunostimulant molecules such as CpG or poly(I:C) (Peine et al., 2013). *In vitro* studies show as, upon incubation with macrophages, these nanosystems elicited higher production of

cytokines than the respective free molecules, demonstrating an improvement in the adjuvant potential of poly(I:C) and CpG.

The incorporation of dextran into chitosan-based nanosystems has recently been reported for the delivery of the capsid protein of HIV-1 (p24) and pertussis toxoid (PTX) (Drogoz et al., 2008; Sharma et al., 2012). Anionic and cationic chitosan/dextran nanoparticles were prepared by modulation of the mass ratio of both polysaccharides, and subsequently loaded with p24. After subcutaneous administration to healthy mice, both cationic and anionic nanoparticles rendered IgG titres that were comparable to those elicited by the Complete Freund's Adjuvant (CFA) (Drogoz et al., 2008). In the case of chitosan/dextran nanoparticles loaded with PTX (Sharma et al., 2012), the IgG response observed after subcutaneous administration to mice was significantly higher than the one elicited by the alum-adsorbed antigen.

Dextran nanoparticles were also prepared in combination with polyvinylalcohol (PVA), for the encapsulation of a recombinant *Bacillus anthracis* antigen (rPA), or resiquimod, a TLR 7/8 agonist (Schully et al., 2013). These nanoparticles were subcutaneously administered to mice, either separately or in combination with the alum-adsorbed antigen. The first observation was a balanced Th1/Th2 immune response in animals receiving resiquimod. Indeed, the highest levels of IgG and cytokine production were observed with the co-administration of resiquimod-loaded nanoparticles in combination with rPA-loaded nanoparticles, or alum-adsorbed rPA; both combinations were responsible for 100% animal survival upon three challenges with the pathogen.

3.2.1.2. Mucosal vaccination with dextran-based nanovaccines. Dextran-based nanoparticles have been barely explored for intranasal vaccination. In a study (Sharma et al., 2013), IgA was associated to dextran nanoparticles as a possible targeting moiety towards the M cells present in the nasal mucosa. After a single intranasal administration of fluorescent-labelled nanoparticles to healthy mice, the fluorescence levels for IgA-decorated dextran nanoparticles, analysed by confocal microscopy, were particularly high in the nasal-associated lymphoid tissue (NALT) cells in comparison with other nasal tissue areas. Therefore, these preliminary data suggest the potential of these nanoparticles for intranasal immunization.

3.2.2. Nucleic acid-based nanovaccines

Despite the number of studies reported on the use of chitosan nanoparticles for DNA vaccines, no studies have been identified incorporating dextran into the nanostructures or any other application of dextran-based nanocarriers in the development of genetic vaccines.

Overall, regardless of the limited number of studies reported so far, the results achieved with dextran-based systems open room for a deeper research in this field, particularly in terms of the combination of this polymer with other immunomodulatory molecules.

3.3. Mannans as biomaterials for nanoengineering antigens

The term “mannan” refers to storage polysaccharides very abundant in nature, consisting in D-mannose monomers connected by β-(1,4) bonds. The interest of these polysaccharides relies on their ability to interact with C-type lectins, a group of receptors expressed in APCs (DCs and macrophages). These receptors are responsible for the recognition of different carbohydrates present in the cell wall of certain pathogens and include the mannose receptor (MR), the mannose-binding lectin (MBL), and others more specific to DCs such as DC-SIGN or DEC-205 (Apostolopoulos et al., 2013).

The recognition process described above leads to the endocytosis or phagocytosis of pathogens, with major importance in the innate immunity process, eliciting complement activation and triggering inflammation (Petersen et al., 2001; Petrovsky and Cooper, 2011). Moreover, interactions with specific DC C-type lectins have been correlated with DC trafficking and induction of both humoral and cellular responses (Apostolopoulos et al., 2013).

The targeting of nanovaccines to APCs using this approach has been applied to different antigens, as discussed below. Nevertheless, it is important to be aware of a limitation of this strategy, the ubiquitous presence of mannan receptors in a variety of cells, including immune cells, epithelial cells (in the retina), mesangial cells (in kidney) and muscular (in trachea) cells (Joshi et al., 2012).

3.3.1. Protein nanovaccines

The functionalization of nanovaccines with mannose residues for specific targeting of protein and peptide antigens to DCs, macrophages and B cells, has been evaluated in murine and human *in vitro* models (Al-Barwani et al., 2014; Ghotbi et al., 2011; Thomann-Harwood et al., 2013).

Polymeric nanoparticles based on a copolymer of poly-ε-caprolactone (PCL) and polyethyleneglycol (PEG), functionalized with mannan, were optimised for the encapsulation of the human basic fibroblast growth factor (bFGF), for cancer immunotherapy, and administered to healthy mice (Gou et al., 2008). The fundamental role of mannan was evidenced in view of the significantly higher IgG levels achieved, in comparison with the control formulations, i.e. non-targeted nanoparticles and the alum-adsorbed antigen. Moreover, the immune response was biased towards the Th1 pathway, as shown by an increased level of IgG2a, which is particularly important to confront tumour cells. In another study, OVA-loaded mannan-decorated PLGA nanoparticles were also found to elicit higher T-cell responses when compared with OVA-loaded non-decorated PLGA nanoparticles (Hamdy et al., 2011), after subcutaneous administration to mice. Finally, *in vitro* macrophage uptake studies performed using poly(2-hydroxiethyl methacrylate-co-methacrylic acid) (P(HEMA-co-MAA)) nanogels, loaded with OVA, revealed a high internalization that was accompanied of a high production of co-stimulatory molecules, such as CD86, CD40 and CD80 (Durán-Lobato et al., 2014).

Glucomannan, a polymer of β-1,4 linked D-mannose and D-glucose, has also been studied for the preparation of nanoparticles (Alonso-Sande et al., 2006, 2009; Harde et al., 2014; Jain et al., 2014a,b). Bovine serum albumin (BSA)-loaded chitosan nanoparticles were functionalized with glucomannan with the aim of achieving an improved interaction with macrophages (Harde et al., 2014). Upon oral administration of these decorated nanoparticles to mice, the immune response, in terms of IgG and IgA titres and cytokine production, was significantly higher with respect to non-functionalized nanoparticles. A novel approach recently explored comprises the modification of bilosomes (non-ionic surfactant-based nanovesicles containing bile salts) with glucomannan for oral antigen delivery (Jain et al., 2014a,b). BSA and TT were used as model antigens and *in vitro* studies showed that glucomannan-modified bilosomes had an increased uptake by macrophages in comparison with the unmodified ones. This translated into stronger systemic and mucosal immune responses following oral administration to mice.

3.3.2. Nucleic acid-based nanovaccines

Nanoengineering of nucleic acids with mannan has been mainly achieved through the synthesis of cationic derivatives of mannan (Lu et al., 2007; Ruan et al., 2014; Tang et al., 2008, 2009). For example, a cationic mannan-spermine copolymer was engineered for complexation of a model pDNA (pGL3), and the transfection efficiency was tested in a panel of immortalised cell lines, including macrophage and dendritic cells. The results showed high transfection levels in macrophages, which were associated to the receptor-mediated internalization of the mannan complexes (Ruan et al., 2014).

Polymer conjugates of mannan (in the oxidized or reduced form) and poly-L-lysine (PLL) have also been used for the complexation of pDNA encoding MUC1, a tumour-associated antigen, for anticancer immunotherapy. Upon intradermal immunization, these nanovaccines lead to increased T cell activity and antibody production, which protected animals against a tumour challenge with MUC-1 expressing

B16 melanoma cells (Tang et al., 2008). Additional data also showed the ability of these nanovaccines to induce DC maturation, through a TLR-2 dependent pathway, and to mediate the cross-presentation of antigens *in vivo* (Tang et al., 2009).

Mannan-modified cholesterol has also been synthesized and used for the preparation of functionalized cationic liposomes that associate a plasmid encoding a melanoma-associated antigen (pUb-M) (Lu et al., 2007). Mice were immunized through intraperitoneal route with this prototype, using liposomes prepared with unmodified cholesterol and naked plasmid DNA as controls. Results evidenced increased gene delivery in splenic macrophages and DCs, achieved via mannose receptor, and a higher specificity of CTL activity with the functionalized liposomes. More importantly, after challenging the animals through the injection of tumour cells, the group treated with the mannan-targeted nanosystems showed rejection of the injected cells, suppression of their growth and increasing survival.

In general, this work highlights the potential interest of mannan as a targeting agent that may improve the ability of nanosystems to interact with the immunocompetent cells. Further studies are necessary in order to assess the specific benefit of this strategy.

3.4. Beta glucans as biomaterials for nanoengineering antigens

β-Glucans are a group of very different polymers of glucose, varying in chain length as well as in number and position of branches. These polysaccharides can be found in the cell walls of many organisms, from bacteria to yeast and even some species of seaweed (Goodridge et al., 2009). Some of them, such as β-1,3(D)-glucan (known as baker's yeast, from *S. cerevisae*) and β-glucans from *Ganoderma lucidum* mycelium and from *Aureobasidium pullulans*, have GRAS status given by the FDA and are used as food ingredients (www.fda.gov). In terms of immunomodulating properties, most of the research has been done with non-cellulosic β-(1,3) or β-(1,6)-glucans such as curdlan (from *Alcaligenes faecalis*), laminarin (from *Laminaria digitata*), lentinan (from *Lentinus edodes*), pleuran (from *Pleurotus ostreatus*) and zymosan (from *Saccharomyces* spp.) (Goodridge et al., 2009; Petrovsky and Cooper, 2011).

The role of beta glucans in the immune response has already been explored for several decades. Their presence in the structure of different bacteria and fungi makes them easily recognizable by the PRRs, acting as natural PAMPs (Soltanian et al., 2009). However, the adjuvant properties of one specific beta glucan cannot be generalized to others, due to differences in parameters such as size, branching and molecular structure (Adams et al., 2008; Barsanti et al., 2011; Sletmoen and Stokke, 2008; Soltanian et al., 2009).

The intervention of beta glucans in immunity is mainly due to their interaction with specific cell receptors, as pictured in Fig. 4. Though both types of recognition lead to similar biological effects, it is worth describing in further detail each one of them. On one hand, CR3 (complement receptor 3), widely present in myeloid cells such as macrophages, DCs and natural killer (NK) cells, was the first receptor described for the recognition of beta glucans. Though the biological effects of this specific interaction are not yet fully understood, it has already been shown that it leads to complement activation and subsequent opsonisation, followed by internalization of the structures (Bose et al., 2013; Goodridge et al., 2009; Soltanian et al., 2009). On the other hand, Dectin-1, a type II transmembrane protein present in myeloid cells, is the most studied β-glucan receptor. The interaction of beta glucan-containing microorganisms or synthetic particles with this receptor mediates several processes such as reactive oxygen species (ROS) and cytokine production, being also a trigger for the internalization of pathogens via phagocytosis (Goodridge et al., 2009; Lipinski et al., 2013; Mochizuki and Sakurai, 2011).

This interaction has led to the use of beta glucans also as targeting moieties for the enhancement of specific immune responses. For example, in the work of Dube et al. (2014), chitosan-coated PLGA nanoparticles were functionalized with 1,3-β-glucan from *Euglena gracilis* for Dectin-1 targeting. As a result, an increased intracellular delivery of

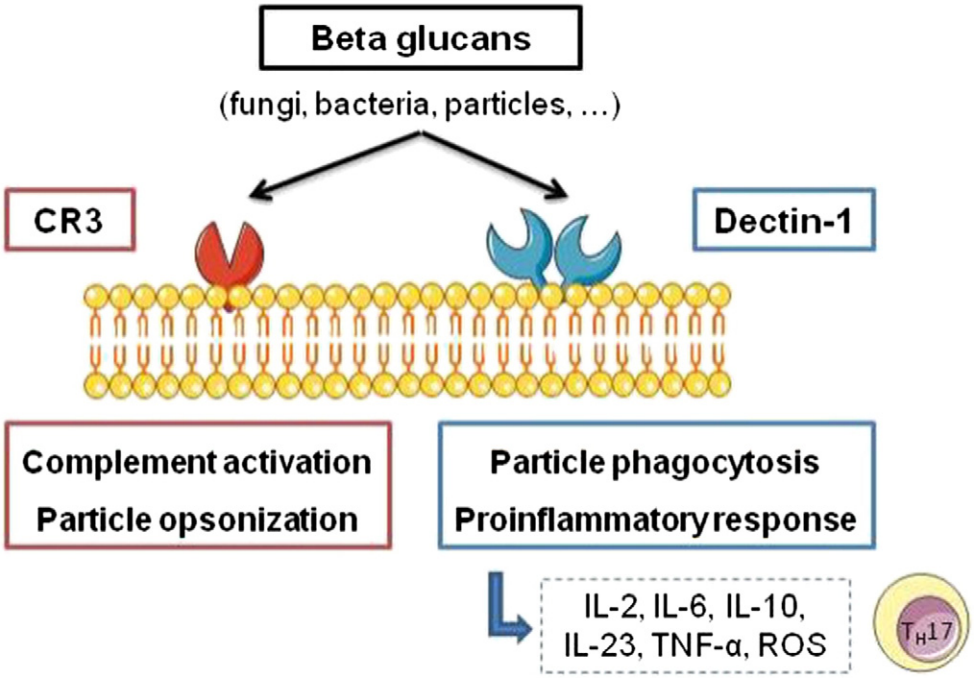

Fig. 4. Schematic representation of beta glucan recognition by immune cell receptors. The two main beta glucan receptors in APCs such as macrophages and dendritic cells are CR3 (complement receptor 3) and Dectin-1. The interaction of beta glucan-containing fungi and bacteria species, as well as synthetic glucan nanoparticles, with these cells may lead, on one hand, to the complement activation and particle opsonisation for phagocytosis (CR3 recognition), or, on the other hand, to the secretion of proinflammatory cytokines and ROS, together with the enhancement of particle phagocytosis by other pathways (Dectin-1 recognition). Moreover, in this last case, the ability to trigger the action T helper (Th17) cells in the adaptive immune response process may also be a relevant feature of beta glucan-containing structures once recognized by Dectin-1.

the encapsulated anti-tuberculosis drug, as well as an enhanced pro-inflammatory reaction with relevant production of reactive oxygen and nitrogen species was reported.

3.4.1. Protein nanovaccines

The above indicated properties of the beta glucans have attracted the attention of a few researchers who have explored their use as antigen carriers. A conjugate of β-mannan, tetanus toxoid and laminarin (from *Laminaria digitata*) was evaluated for the development of a vaccine against *Candida albicans* (Lipinski et al., 2013). This new conjugated antigen was able to promote receptor-based DC uptake mediated by the interaction of laminarin with Dectin-1, altogether resulting in an enhanced cytokine production (IL-4, IL-6 and TGF-β) and strong IgG antibody responses after intraperitoneal or subcutaneous administration to mice, with IFA as adjuvant (Lipinski et al., 2013).

Given the potential exhibited by beta glucans for immunomodulation, it is expected an increasing interest in their use for the development of nanovaccines. However, to the best of our knowledge, only one example relates to the use of the polysaccharide PS4 (isolated from *G. lucidum* mushrooms), with strong immunomodulating properties, based on the regulation of TNF-α, nitric oxide (NO) and cytokine production through interaction with TLR 2, for the preparation of nanosized complexes with poly(I:C) (Tincer et al., 2011). IgG responses observed after intraperitoneal administration of OVA-loaded PS4 nanoparticles to mice were higher than those elicited by the controls (i.e. physical mixtures of PS4 and OVA, or poly(I:C) and OVA). This positive *in vivo* response was correlated to an increased cytokine production after incubation of these nanoparticles with macrophages.

3.4.2. Nucleic acid-based nanovaccines

Some studies have also highlighted the potential of beta glucans for the delivery of nucleic acid antigens. Synthetic cationic glucans obtained from *G. lucidum* have been reported to efficiently associate a model pDNA (pGL-3 encoding luciferase), and to achieve an adequate transfection in human embryonic kidney transformed cells (HEK293T) (Wang et al., 2012). Another example is the one making use of schizophyllan (SPG) complexed with TNF-α oligonucleotides (ODN) for delivery to macrophages, which are their therapeutic target in induced hepatic damage. Upon intraperitoneal administration of either the SPG-ODN complex or both components individually to mice bearing LPS-induced hepatic damage, the ODN were efficiently delivered to APCs through Dectin-1 targeting and exhibited an encouraging therapeutic effect (Mochizuki and Sakurai, 2011).

Overall, beta glucans present intrinsic properties that increase the interest in their use for vaccine development, as demonstrated in this review. Nevertheless, the results achieved with these polymers are still preliminary, particularly in the case of the development of antigen nanocarriers, and the real potential of beta glucans in this field is yet to be demonstrated.

4. Concluding remarks

Throughout the past decades, several innovative biological techniques have been applied to the design and development of safer and more effective vaccines. However, the need for potent adjuvants that can enhance the immune response elicited by modern antigens has also grown and is nowadays an important research field. Within this frame, nanotechnology has been playing an increasingly important role, given the appropriate features offered by nanocarriers to the administration of antigens. These include their particulate nature, controllable particle size, ability to protect antigens from degradation and to deliver them in a controlled manner, possibility to overcome mucosal routes, and ability to include co-stimulatory molecules that may enhance the global adjuvant effect of the nanocarrier.

In this context, the use of polysaccharides in the development of novel nanovaccines has represented a crucial step. Due to the interesting intrinsic properties of chitosan, such as its mucoadhesiveness and effect in the activation of macrophages, both particularly relevant for vaccine delivery, this polysaccharide has been the most studied one in the development of nanovaccines. Nevertheless, the use of other polysaccharides in this field is slowly growing and even though many of them are barely known as nanovaccine components, their recognized immunomodulating properties open room for further research and progress in this particular area.

Globally, recent work in nanovaccine development has demonstrated the potential of this strategy to confront current barriers in vaccination. Nevertheless, it is important to take into consideration that the combination of the biomaterials used in a specific formulation, the chosen antigen and the route of administration of the nanovaccines will be critical factors influencing the final outcome of the vaccination strategy.

5. Future perspectives

The use of biomaterials, especially natural polymers, for the preparation of antigen delivery systems, is one of the main tendencies on the field of vaccination. However, the natural origin of these materials is often responsible for high variability in the characteristics of each specific polymer and, therefore, may lead to variable and even controversial results in terms of their adjuvant activity. This is also a challenge in terms of the progress of these innovative vaccine formulations towards clinical development and commercialization. Therefore, it is expected that the next few years bring deeper knowledge in terms of the mechanism of action of these polymers, which might drive the path for a more rational design of polysaccharide-based nanoparticle adjuvants.

Other particular issues, such as the achievement of thermally stable formulations that can avoid the cold chain of vaccine storage, might be one of the challenges nanovaccines are likely to face in the next steps of the way. Finally, the optimization of the preparation methods used in nanovaccine development in order to adequately manufacture them at the industrial level is a major topic that should be addressed in the near future of this field. The combination of this accumulated knowledge could be the necessary driving force to take polysaccharide-based and other nanovaccines towards the next level and ultimately to achieve clinical use.

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