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Nanovaccines: nanocarriers for antigen delivery

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Abstract – Vaccination has become one of the most important health interventions of our times, revolutionizing health care, and improving the quality of life and life expectancy of millions all over the world. In spite of this, vaccine research remains a vast field for innovation and improvement. Indeed, the shift towards the use of sub-unit antigens, much safer but less immunogenic, and the recognized need to facilitate the access to vaccines in the global framework is currently stimulating the search for safe and efficient adjuvants and delivery technologies. Within this context, nanocarriers have gained particular attention over the last years and appear as one of the most promising strategies for antigen delivery. A number of biomaterials and technologies can be used to design nanovaccines that fulfill the requirements of new vaccination approaches, such as single-dose and transmucosal immunization, critical for achieving a widespread coverage while reducing the overall costs in relation to traditional forms of vaccination. Here we present an overview of the current state of nanocarriers for antigen delivery, developed with the perspective of contributing to the global vaccination goal.

Key words: Vaccination / sub-unit antigens / single-dose vaccination / mucosal immunization / nanoparticles

Résumé – Nanovaccins : transporteurs pour l'administration des antigènes.

La vaccination est devenue l'une des plus importantes interventions sanitaires de notre époque. Elle a révolutionné l'approche de la santé et amélioré la qualité et l'espérance de vie de millions de personnes dans le monde entier. Malgré tout, la recherche vaccinale reste un vaste domaine ouvert à l'innovation et l'amélioration. En effet, l'évolution vers l'utilisation de sous-unités antigéniques, beaucoup plus sûres mais moins immunogènes, et la nécessité reconnue de faciliter l'accès aux vaccins au niveau mondial est aujourd'hui une source de stimulation pour la recherche d'adjuvants sûrs et efficaces et de technologies pour leur administration. Dans ce contexte, les nanovecteurs, qui ont suscité une attention particulière au cours des dernières années, apparaissent comme une des stratégies les plus prometteuses pour l'administration d'antigènes. Plusieurs biomatériaux et technologies peuvent être utilisés pour faire des nanovaccins sur mesure, qui répondent aux exigences des nouvelles approches de vaccination, telles que la vaccination à dose unique et l'immunisation transmuqueuse, critiques pour obtenir une couverture généralisée et réduire le coût global par comparaison avec les formes traditionnelles de vaccination. Nous présentons ici un aperçu de l'état actuel des nanovecteurs utilisés pour l'administration d'antigènes, développés dans la perspective de contribuer à l'objectif de la vaccination globale.

Mots clés : Vaccination / sous-unités antigéniques / vaccination à dose unique / immunisation muqueuse / nanoparticules

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Abbreviations

APCs,	antigen presenting cells
CFA,	complete Freund's adjuvant
CS,	chitosan
CS-MCC,	negatively charged mono-N-carboxymethyl CS derivative
CS-TMC,	positively charged N-trimethyl CS derivative
DCs,	dendritic cells
GALT,	gut-associated lymphoid tissue
HBsAg,	Hepatitis B surface antigen
IFA,	incomplete Freund's adjuvant
ISCOMs,	immunostimulating complexes
JE,	Japanese encephalitis
MALT,	mucosal associated lymphoid tissue
M-cells,	microfold-cells
NALT,	nasal-associated lymphoid tissue
NP,	nanoparticle
OVA,	ovalbumin
PCL–PEG,	poly(ε-caprolactone-co-ethylene glycol)
PEG-CS,	PEGylated CS
PLA,	polyesters poly(D,L-lactide)
PLGA,	poly(D,L-lactic-co-glycolic acid)
TLRs,	toll-like receptors
TT,	tetanus toxoid

Framework and technological needs

The first scientific attempt to control an infectious disease through the deliberate use of vaccination was brought forth by Edward Jenner more than 200 years ago. Jenner's work turned the scientific community attention towards the development of vaccines and their potential for prevention and eradication of life-threatening diseases, such as smallpox, polio, diphtheria or tetanus (Riedel, 2005). The implementation of vaccination has increased over time, being nowadays recognized as a greatest milestone in health protection. Furthermore, vaccination remains an interesting and vast field for innovation (WHO, 2005; Peek *et al.*, 2008; Plotkin & Plotkin, 2011).

A major goal in vaccination is to achieve worldwide coverage against highly infectious diseases, such as tuberculosis, polio, diphtheria, tetanus, cholera, pertussis and to focus research on emerging or re-emerging diseases such as HIV or malaria. Currently, the population with access to the existing vaccines is extremely reduced and some infectious diseases exhibit complex pathogenesis mechanisms, responsible for great difficulties in the development of efficacious vaccines. The benefits of achieving an effective immunization program go beyond the life-saving objective, particularly in developing countries where vaccination allows to reduce the sickness burden, thus contributing to global development. This crucial observation has led several public and private institutions, such as the World Health Organization (through the Global Alliance for Vaccines and Immunization) and the Bill and Melinda Gates Foundation, to commit themselves to the challenge of improving current vaccination programs (WHO *et al.*, 2009; Gates, 2011).

Traditionally developed vaccines consist of either live attenuated or inactivated pathogenic agents. Live attenuated vaccines stand out for their unique capacity to enhance strong innate and long-lasting immune responses without needing additional adjuvant components. However, their instability and potential to revert to the virulent form represents a serious risk for the patient health. To overcome this problem, by the end of the 19th century, researchers developed inactivated whole organism vaccines as well as toxoid vaccines. These vaccines were shown to be safer than the previous ones, despite the limitation of yielding weaker immunity levels, thus requiring the use of an adjuvant, *i.e.* alum, and multiple-dose vaccination programs. More recently, vaccine research has been oriented towards the development of purer, safer and easier to produce antigens, namely 1) sub-unit vaccines in which a unique fraction of the pathogenic agent is used as antigen; 2) DNA vaccines; and 3) conjugate vaccines, which consist of the presentation of the antigen covalently linked to a protein or membrane complex with stronger immunogenicity (Peek *et al.*, 2008; Plotkin & Plotkin, 2011). Regardless of the promising features of these forms of vaccination, their use in a global perspective is still limited, essentially because of their low immunogenicity, thus making the search for new adjuvants, a critical need for improving vaccination coverage (Perrie *et al.*, 2008; Skwarczynski & Toth, 2011).

Besides the development of new adjuvants, the current technological challenges in vaccination are aimed at improving the stability of the commercially available vaccines and decreasing the number of doses needed for an efficient immunization, altogether reducing the costs of this type of health intervention. Indeed, it is known that the assumed commitment to a cold chain protocol for the worldwide distribution of vaccines is frequently infringed with the subsequent risk of irreversible antigen damage (Matthias *et al.*, 2007; Vicente *et al.*, 2010b). On the other hand, current schedules typically require the parenteral administration of several vaccine doses in order to reach adequate levels of protection, a fact that represents a real challenge in developing countries (Giudice & Campbell, 2006; Vicente *et al.*, 2010b).

The goals are therefore set for progress and innovation in the vaccination field with the final aim of reaching universal accessibility to vaccines that are simultaneously effective, affordable and safe. The most remarkable advances in vaccination reported to date,

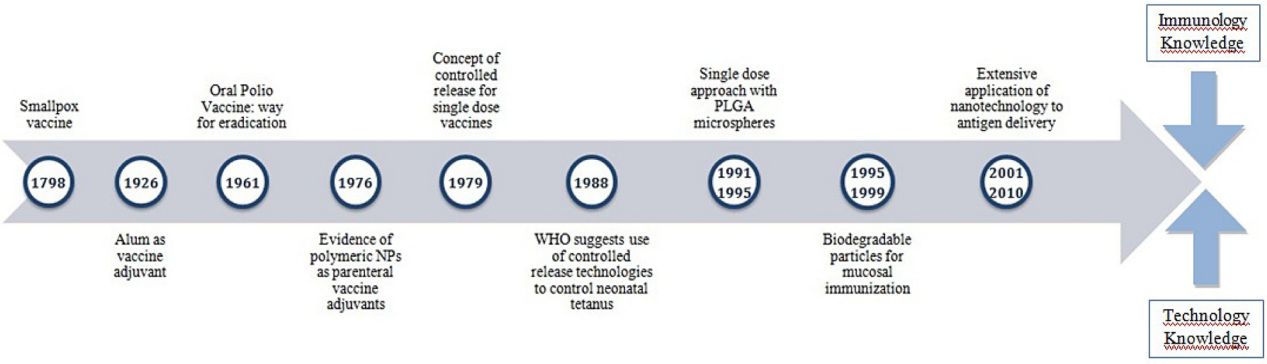

Fig. 1. Milestones in vaccination and evolution of vaccines towards the development of single-dose and needle-free approaches. NPs: nanoparticles; WHO: World Health Organization; PLGA: poly(D,L-lactic-co-glycolic acid).

and the role of nanotechnology are reviewed in the following sections and summarized in Figure 1.

The essential role of adjuvants in vaccination

The term adjuvant encloses a heterogeneous group of compounds with different activities and functions, intended to enhance the quality, length and extent of a specific immune response (Reed *et al.*, 2009). The discovery and rational design of new compounds with adjuvant properties is becoming a key point in vaccination research that benefits from the knowledge gathered in the field of immunology. Indeed, advances in the study of activation mechanisms of antigen presenting cells (APCs) and the discovery of pattern recognition receptors for highly conserved structures of pathogens, such as the toll-like receptors (TLRs), are having a major impact in the development of new adjuvants (Steinhagen *et al.*, 2011).

The first significant adjuvants developed for human vaccines are the aluminum compounds depicted for the first time in 1926 and generally referred to as *alum*. Despite of its favorable safety profile, alum is not adequate for the recently developed sub-unit vaccines, due to the probable loss of antigenicity of these antigens when adsorbed to this adjuvant (Lindblad, 2004). Another concern of alum is its sensitivity to damage upon freezing (Matthias *et al.*, 2007).

Emulsion technologies represent a second approach to the development of new adjuvants. The first attempt involved the use of water-in-oil emulsions, known as Complete and Incomplete Freund's Adjuvant (CFA and IFA). Since then, toxicity concerns have led to the development of new oil-in-water emulsions made from highly purified emulsifiers, which have successfully led to some marketed vaccines for influenza (Fluad®, Focetria® and Prepandrix®) and human papilloma virus (HPV) (Cervarix®) (O'Hagan & De Gregorio, 2009; Correia-Pinto *et al.*, 2012).

In parallel to the development of emulsions, vaccine research has focused on exploring alternative lipid-based antigen delivery technologies. Liposomes are the main class of delivery systems explored for drug delivery applications, and consequently, they have been widely investigated as antigen delivery systems in vaccination. In this field, it is worth highlighting the therapeutic tuberculosis vaccine RUTI® which is now undergoing clinical development (Gregoriadis *et al.*, 1999; Peek *et al.*, 2008; O'Hagan & De Gregorio, 2009; Correia-Pinto *et al.*, 2012).

The integration of functional viral envelope glycoproteins into liposomes has led to an interesting type of antigen adjuvants named virosomes. Importantly, they retain the cell binding and membrane fusion properties of the native virus, therefore manifesting an improved capture by the APCs and antigen processing. Vaccines against influenza (Inflexal V®) and Hepatitis A (Epaxal®) have already reached the market, and several are undergoing clinical trials (Huckriede *et al.*, 2005).

Colloidal structures constituted by a combination of phospholipids, cholesterol and saponines, named immunostimulating complexes (ISCOMs), have been explored for their ability to associate hydrophobic and hydrophilic antigens, their good stability, and ability to induce both humoral and cellular immune responses. However, their potential use in human vaccines is hampered by toxicity concerns attributed to certain saponines as Quil A and QS-21 (Malliaros *et al.*, 2004).

Other advanced lipidic formulations are the synthetic biomimetic supra-molecular Biovector™ (SMBV) particles, consisting in a polysaccharide core of positive or negative particles, surrounded by a phospholipid layer, which allows the incorporation of distinct active compounds. These structures are particularly studied in the area of mucosal vaccine

delivery and have reached the clinical development status (von Hoegen, 2001).

Regardless of the interesting features of lipid-based adjuvants, there is a trend to explore the potential of biocompatible polymeric nanostructures for antigen delivery. These delivery systems present several advantages and are promising adjuvants in vaccination, as highlighted in the next sections.

The potential of polymeric nanostructures as adjuvants in vaccination: nanovaccines

In 1976, Kreuter and Speiser reported for the first time the potential use of polyacrilic nanoparticles as an adjuvant for an influenza vaccine (Kreuter & Speiser, 1976). Following this pioneering work there has been a significant number of reports on the development of micro- and nano-sized particulate delivery systems (O'Hagan *et al.*, 2006; Jones, 2008; Peek *et al.*, 2008).

Throughout the last years, different studies have been carried out to understand the importance of particle size in vaccination. Recent works have made clear that small and large nanoparticles reach the lymph nodes by different mechanisms and interact differently with APCs. While nanoparticles with a size inferior to 100 nm have shown an improved ability to drain to the interstitial flow and be transported to the lymph nodes for antigen presentation to resident dendritic cells (DCs), larger particles typically reach the lymph nodes in a cell-associated manner (Gregoriadis *et al.*, 1999; Li *et al.*, 2011). Similarly, the internalization mechanisms by APCs are dependent on the size of the particle engulfed: small nanoparticles are usually taken up by DCs through receptor-mediated endocytosis into clathrin-coated pits or through caveolae, while larger particles are generally phagocytosed specially by macrophages (Mottram *et al.*, 2006; Xiang *et al.*, 2006). In this regard, the route of internalization is gaining interest for the understanding of immune responses, and receptor-mediated endocytosis has been related to cross-presentation processes and induction of combined cellular and humoral responses (Shen *et al.*, 2006; Okamoto *et al.*, 2008; Hirosue *et al.*, 2010).

For the engineering of nanovaccines, the component materials must be biocompatible and biodegradable and possess a good safety record. Biodegradable polymeric delivery systems exhibit a number of advantages as vaccine adjuvants: (i) they reproduce the natural particulate form of pathogenic agents, passively targeted to APCs; (ii) they can be engineered to specifically interact with certain cell populations as microfold-cells (M-cells) and DCs (Azizi *et al.*, 2010); (iii) they can accommodate immunopotentiators as TLR agonists for an increased response; (iv) they can control the release of the antigen and prolong the exposure and duration of the immune response; (v) they can be administered by alternative non-invasive transmucosal routes; (vi) in the case of dry powder formulations generated upon freeze-drying, these adjuvants are known stabilizers of the associated antigens (Csaba *et al.*, 2009a; Vicente *et al.*, 2010b; De Koker *et al.*, 2011; De Temmerman *et al.*, 2011).

Among the materials studied, the polyesters poly(D,L-lactide) (PLA) and poly(D,L-lactic-co-glycolic acid) (PLGA), as well as their PEGylated derivatives, have been widely explored, first for the preparation of microparticles, and a few years later for the development of smaller nanosized particles (Alonso *et al.*, 1994; Blanco & Alonso, 1997; Tobío *et al.*, 1998). Other biomaterials, such as oils, polyethylenoxides, and cationic polymers, have subsequently been incorporated to PLA/PLGA formulations in the search for vaccines with improved properties (Tobío *et al.*, 1999; Csaba *et al.*, 2006; Martínez Gómez *et al.*, 2008; Paolicelli *et al.*, 2010). Further optimization of the adjuvant properties can be achieved by modification of their surface properties with immunostimulatory components such as the TLRs agonists CpG oligonucleotides (Fischer *et al.*, 2009).

Among the natural polymers that have been disclosed for the preparation of nanovaccines, special mention should be given to the biodegradable polysaccharide chitosan (CS), which has either been used as a polymeric coating or as the core forming material of nanovaccines (Vila *et al.*, 2004b; Prego *et al.*, 2006; Vicente *et al.*, 2009; Prego *et al.*, 2010). Nanovaccines based on CS are promising systems for the development of vaccines, in particular for transmucosal vaccination due to its mucoadhesive character, as discussed in the section entitled "The promise of nanovaccines for transmucosal vaccination". Other CS derivatives as PEGylated and quaternized CSs have similarly been explored for this application (Prego *et al.*, 2006; Hagenaars *et al.*, 2010).

As stated above, nanoparticulate delivery systems can be prepared from a variety of biomaterials with defined properties and are promising technologies in the search for efficient adjuvants for labile and low-immunogenic sub-unit antigens. Interesting results concerning the application of polymeric nanoparticles for single-dose and needle-free vaccination that have been reported to date are disclosed in the next sections and summarized in Table 1.

Controlled release technologies for the development of single-dose vaccines

In the late 70s, Preis and Langer showed that the release of active macromolecules could be extended

Table 1. Relevant examples of nanosystems for single dose and needle-free vaccination: immunization results in animal models (results obtained with model antigens are omitted).

Type of Nanostructure	Immunization route	Antigen	Dose	Immunization scheme	Key observations	Ref.
PLA/PLGA nanoparticles	Intramuscular	TT	30 μg	Single dose	Antibody titers generated by the NP lasted for over 5 months, an improved immune response compared to that of a saline solution of the antigen.	(Raghuvanshi *et al.*, 2002)
CS nanocapsules		HBsAg	10 μg	Single dose Weeks 0, 4	A single-dose approach: protective IgG levels comparable to those obtained for the alum-adsorbed antigen in a two-dose administration schedule.	(Vicente *et al.*, 2010a)
PGA nanoparticles	Intraperitoneal	JE vaccine BIKEN	1 μg	Single dose	Effective protection against JE virus in levels similar to those obtained with the conventional vaccine administered with alum.	(Okamoto *et al.*, 2008)
PLA-PEG / PLA nanoparticles	Nasal	TT	40 μg	Single dose	PLA-PEG NP facilitated antigen transport through nasal route in comparison with PLA NP.	(Tobío *et al.*, 1998)
CS nanoparticles		TT	10 μg 30 μg	Days 1, 8, 15	IgA titers generated by the NP in saliva and broncho-alveolar/intestinal lavages, were much higher than those obtained with the antigen in solution.	(Vila *et al.*, 2004b)
		HBsAg	10 μg 20 μg	Days 1, 28	Increasing anti-HBsAg IgG levels (seroprotective) over time.	(Prego *et al.*, 2010)
CS-coated nanocapsules		HBsAg	10 μg	Days 0, 28	Increasing IgG levels over time for up to 112 days. Significantly increased response when the immunostimulant imiquimod was incorporated into the oily core.	(Vicente *et al.*, 2009)
PEG-CS / CS nanoparticles		DT	10 μg	Days 0, 7, 14	Both formulations achieved mucosal and systemic immune responses, although the PEG coating allowed higher antibody titers.	(Rezaei-Mokarram *et al.*, 2005)
TMC / MCC / CS nanoparticles		TT	5 Lf	Days 0, 22	Positively charged TMC and CS NP showed better results than the negatively charged MCC NP.	(Sayın *et al.*, 2008)
PLA-PEG / PLA nanoparticles	Oral	TT	498 μg	Single dose	The PEG coating was found to be crucial for the stabilization of the nanoparticles in the gastrointestinal environment, explaining the improved absorption of the antigen after oral administration to rats.	(Tobío *et al.*, 2000)
Lectin-decorated PLGA nanoparticles		HBsAg	10 μg	Week 0, 2	Lectinized NP provided higher anti-HBsAg titers than the plain PLGA NP, probably due to selective targeting to the M-cells. IgG titers elicited were comparable between orally administered lectinized NP and intramuscularly administered alum-HBsAg.	(Mishra *et al.*, 2010)

Abbreviations: NP: nanoparticle; PLA: poly(lactid acid); PLGA: poly(lactic-co-glycolic acid); CS: chitosan; PGA: poly(γ-glutamic acid); PEG: polyethylene glycol; PEO: polyethylene oxide; TMC: *N*-trimethyl chitosan; MCC: mono-*N*-carboxymethyl chitosan; PCL: poly(ε-caprolactone); Lf: limit of flocculation; TT: tetanus toxoid; HBsAg: recombinant Hepatitis B surface Antigen; DT: diphtheria toxoid; OVA: ovalbumin; JE: Japanese encephalitis.

for periods that exceeded 100 days, upon association to ethylene-vinyl acetate beads. The most remarkable finding was that, as a consequence of this sustained release, the stimulated immune response was comparable to the secondary response induced by the same total dose of antigen emulsified in CFA (Preis & Langer, 1979). This discovery introduced the idea of single-dose vaccination achievable through controlled delivery of the antigen.

In 1988, the WHO proposed the global use of controlled release technologies in the development of single-dose vaccines, a grand challenge in global health as it intends to increase the compliance with the immunization schedule and the effectiveness of vaccination in developing countries. Following this indication, in the next decade, a number of prototypes based on PLGA or PLA microspheres were designed and studied, as can be read next (also see previous section).

The tetanus toxoid (TT) was the first antigen to be considered for a single-dose approach because of the high incidence of neonatal tetanus in developing countries. Alonso *et al.* (1994) provided the first insight on the potential of PLGA microspheres to enhance and prolong the immune response to TT. Despite these promising results, PLGA microspheres presented as main limitation the degradation of the encapsulated antigen and the consequent loss of antigenic activity. This fact was attributed to the harsh technologies applied for their preparation (organic solvents and strong shear forces) and to the acidification of the microenvironment due to the erosion of the polymeric matrix (Schwendeman *et al.*, 1996; Jiang *et al.*, 2005). To answer this problem, revolutionary solutions were presented. An interesting approach consisted in the isolation of the antigenic protein in oil-based cores surrounded by outer PLGA shells (Sanchez *et al.*, 1996). Another proposal was to develop microparticles from an intimate blend of PLGA and polyethylene oxide (poloxamer 188), a non-ionic polymeric surfactant, able to block the interaction of PLGA with the antigen and prevent its degradation (Tobío *et al.*, 1999). In view of these results, the same strategy was later applied for the development of improved PLGA nanostructures (Csaba *et al.*, 2006; Santander-Ortega *et al.*, 2010).

PLGA microparticles have successfully been used for encapsulation of a variety of antigens, including influenza (Hilbert *et al.*, 1999), diphtheria toxoid (Johansen *et al.*, 1999) and Hepatitis B (Feng *et al.*, 2006) among others, showing in all cases the possibility to enhance and prolong the release of the encapsulated antigens. PLGA microparticles have also been used for the encapsulation of plasmid DNA (pDNA) designed to express the Hepatitis B surface Antigen (HBsAg) (DNA vaccination) (He *et al.*, 2005).

In the 90s, PLGA nanoparticles were also developed by our group, on the face of the promising results achieved with microparticles, and considering the potential advantages of smaller size (Blanco & Alonso, 1997). PLA and PLGA nanoparticles showed that the intramuscular immunization of rats with TT-loaded nanoparticles provided anti-TT antibody titers that persisted for more than 5 months, which was significantly better than the immune response elicited by saline solutions of TT (Raghuvanshi *et al.*, 2002). The immune response peaks achieved were higher for nanoparticles than for the corresponding microparticles, leading to the concept that particle size has a major influence in the immune response produced. The same study also concluded that the hydrophobicity of the vehicles is a very important factor, since the PLA nanoparticles provided better immunization results than the PLGA ones, in agreement with previous studies performed with PLA/PLGA microparticles (Alonso *et al.*, 1994). To study the possibility of improving the results of the immunization with these systems, this group also tested the possibility of administering simultaneously TT-loaded PLGA nanoparticles and alum. The results suggested a synergistic effect between the two adjuvants (Raghuvanshi *et al.*, 2002).

Improved PLGA/poloxamer nanosystems previously developed to deliver DNA vaccines (Csaba *et al.*, 2006) were conveniently adapted for the association of more complex antigens, *i.e.* virus-like particles such as HBsAg, and additionally coated with CS to further improve the presentation of the nanocarrier to immunocompetent cells (Paolicelli *et al.*, 2010). CS-coated PLGA/poloxamer nanoparticles delivered HBsAg in a controlled manner for up to 14 days, fully preserving the integrity and antigenicity of the released antigen. The long-lasting delivery properties of these nanostructures evidence their potential for single-dose vaccination, but this possibility has yet to be evaluated in animal models.

Another promising delivery carrier with a potential for single-dose immunization are the so-called CS nanocapsules. In this case, HBsAg was adsorbed onto the nanocapsule surface, through the electrostatic interactions between the negatively charged antigen and the positively charged CS. These systems were evaluated *in vivo* through intramuscular administration to mice of a 10 μg dose of HBsAg both in a single-dose and two-doses schedule, and compared with the antigen adsorbed in alum. The protective antibody levels induced by the single-dose administration of this system were comparable to the ones elicited by the alum-adsorbed antigen in a two-doses schedule, proving that these CS nanocapsules are a valid prototype for a single-dose vaccination approach (Vicente *et al.*, 2010a).

Polyaminoacids have also been studied for their use in drug delivery and vaccine nanocarriers (González-Aramundiz *et al.*, 2012). More specifically, hydrophobically modified polyglutamic acid (PGA) nanoparticles were developed for association of the Japanese encephalitis (JE) vaccine BIKEN (a formalin-inactivated mouse brain-derived vaccine) and treatment of JE. The results showed that, after a single intraperitoneal dose to mice, nanoparticles provided effective protection from lethal JE virus. The level of protection resulted to be comparable to the JE vaccine BIKEN administered with alum (Okamoto *et al.*, 2008).

Overall, the above-disclosed information has made evident the potential of micro- and nanoparticles as single-dose vaccine formulation approaches. It is however worth noting that the most investigated delivery carriers, PLGA micro- and nanoparticles, have not reached the clinical development status yet, with only a small trial of clinical evaluation (Correia-Pinto *et al.*, 2012). Irrespective of the commercial justifications for the implementation of these technologies, it seems reasonable to conclude that it is necessary to make further progress in the development of safe, inexpensive and efficacious polymer particles, which may one day represent single-dose formulation vaccines.

The promise of nanovaccines for transmucosal vaccination

Regardless of the intended achievements in parenteral immunization, needle-free vaccination has been recognized as a great challenge in global health. Needle-free vaccination facilitates compliance with immunization schedules, decreases pain and suffering, requires less healthcare training for vaccination, enables faster vaccine delivery, avoids the risk of incorrect or repeated use of injection devices and allows an eventual cost reduction (Giudice & Campbell, 2006). This section is focused on mucosal immunization, among the different needle-free delivery approaches, as this is a suitable and already established vaccination protocol for some specific vaccines, *i.e.* polio, rotavirus, *Salmonella typhi* and adenovirus type 4 and 7 vaccines for oral administration, and influenza vaccine for nasal administration.

The interest in mucosal vaccines relies not only in the induction of systemic immune responses but also of mucosal responses, thus providing additional protection against pathogens even at their site of entry, since most infections start at or affect specifically mucosal surfaces. The use of antigen delivery systems in mucosal vaccination aims at the improvement of the antigen stability and the facilitation of its penetration across the mucosal surface, so that the intact and active antigen could be taken up by APCs and transported from the mucosal associated lymphoid tissues (MALT) to the lymph nodes or other secondary lymphoid organs (Holmgren & Czerkinsky, 2005). As an example of this process, Figure 2 represents schematically the pathways involved in intranasal vaccine delivery.

Depending on the organ or site to which it is associated, the MALT may have some differences in structure. For example, in the digestive tract this tissue is often referred to as gut-associated lymphoid tissue (GALT) and is mainly composed of Peyer's patches and M-cells, which are capable of carrying antigens in a particulate formulation from the absorption site until they reach APCs, which migrate to the regional lymph nodes and trigger the adequate immune response. In the human nasal cavity this tissue is known as "diffuse" nasal-associated lymphoid tissue (NALT) and is composed of a collection of isolated subepithelial lymphoid follicles and the lingual, palatine and nasopharyngeal tonsils (adenoids) (Holmgren & Czerkinsky, 2005; Csaba *et al.*, 2009a).

In all cases, the first barrier to overcome in mucosal vaccination is a dense and dynamic mucus layer that covers and protects the underlying epithelium. For a successful mucosal vaccination, the vehicles should overcome the mucus barrier, adhere to or penetrate the epithelium and deliver the immunologically active antigen in a controlled-release fashion. The following sections are intended to analyze information on the most relevant technologies developed for needle-free nasal and oral vaccination as well as the most remarkable immunization results obtained from these vaccination approaches.

Nasal vaccine delivery systems

Some relevant characteristics of the nasal cavity physiology that make it especially attractive for vaccination are its relatively reduced enzymatic activity, a moderately permeable epithelium, and its high amount of available immune-reactive sites. For the success of needle-free nasal immunization, nanoparticles appear to be an interesting approach, as they are known to increase the residence time upon intranasal deposition and improve the interaction with the mucosal epithelium.

As highlighted in this review, PLGA and PLA have been the most studied polymers for the design of antigen delivery systems, being the first to be evaluated for needle-free nasal vaccination in the early 90s. Almeida *et al.* (1993) demonstrated that PLA microspheres, administrated through the nasal route to guinea pigs, could effectively enhance the immune response of the adsorbed TT, when compared to the soluble antigen.

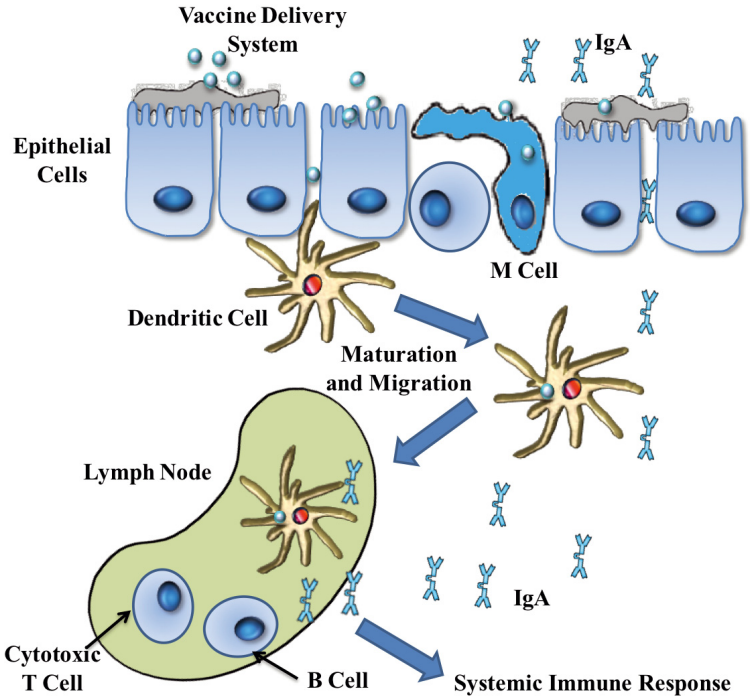

Fig. 2. Schematic illustration of the general pathways involved in mucosal immune response following intranasal vaccination.

The first nanoparticulate compositions to be evaluated for nasal delivery of antigens were nanoparticles composed by PLA or the PEGylated derivative PLA-PEG. Experiments in rats performed with nasally administered radiolabelled tetanus toxoid ^{125}I-TT have shown that PLA-PEG nanoparticles successfully prolonged the residence time, antigen release and transport to a greater extent than conventional PLA nanoparticles (Tobío *et al.*, 1998). The hydrophilic coating was proved to be essential for increasing the stability of the nanoparticles upon contact with the mucosal fluids, thereby enhancing their interaction with the epithelium (Tobío *et al.*, 2000). These results were corroborated in subsequent studies showing that through the careful control of the nanoparticle size and density of PEGylation, it is possible to modulate the interaction of these nanocarriers with the epithelium and hence its mucosal transport. PLA-PEG nanoparticles with an elevated PEG coating density (20–35%) evinced a significantly increased transport versus the PLA control nanoparticles (Vila *et al.*, 2004a).

Optimized nanoparticles of PLGA blended with polyoxyethylene derivatives have also been studied for DNA vaccination through the nasal route (Csaba *et al.*, 2006). These improved nanoparticles could effectively overcome the mucus layer upon intranasal deposition and enter epithelial cells. Most importantly, immunization studies proved the ability of pDNA-loaded nanoparticles to elicit a fast and strong immune response, with IgG antibody titers against the encoded protein significantly higher than those corresponding to the naked pDNA (Csaba *et al.*, 2006). Further studies demonstrated the suitability of these nanostructures to associate other types of antigens as proteins and even virus-like particles (Paolicelli *et al.*, 2010; Santander-Ortega *et al.*, 2010).

Besides the promise of PLA/PLGA nanoparticles for nasal delivery of antigens, CS-based nanocarriers hold a great potential for this application, mainly due to the mucoadhesive properties of this polysaccharide. Vila *et al.* (2002, 2004b) reported the first studies performed with CS nanoparticles for nasal immunization and showed the positive role of CS in improving the transport of antigens across the nasal mucosa. With the purpose of exploring how the polysaccharide properties could influence the immunization process, nanoparticles loaded with TT were prepared from CS polymers of different molecular weights (ranging from 23 to 70 kDa). Upon intranasal administration to mice, results showed comparable levels of IgA antibody in saliva, broncho-alveolar and intestinal lavages, irrespective of the CS molecular weight, as highlighted in Fig. 3. In all cases, the titers were superior to those obtained in animals immunized with the antigen solution (Vila *et al.*, 2004b).

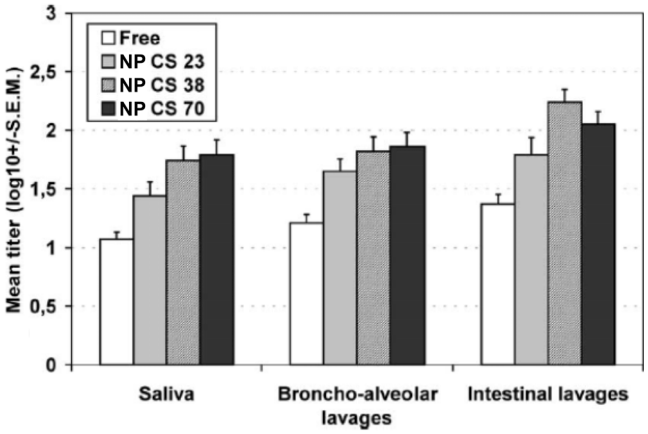

Fig. 3. IgA responses obtained after intranasal immunization of mice with 10 μg of tetanus toxoid encapsulated into nanoparticles prepared with chitosan polymers that differ in their molecular weight: 23 KDa (NP CS 23), 38 KDa (NP CS 38) and 70 kDa (NP CS 70). As control, the antigen was administered in saline. Titers are presented as the geometric mean titer (GMT) per group. Adapted from (Vila *et al.*, 2004b) with permission.

Chitosan nanoparticles also resulted to be suitable for the encapsulation of more complex antigens such as HBsAg, without altered antigenicity (Prego *et al.*, 2010). Intranasal immunization to mice was performed at two different priming HBsAg doses (10 and 20 μg) and a boost dose after 28 days. The anti-HBsAg IgG levels showed a low but increasing immune response over time, regardless of the dose administered. In both cases, the antibody concentrations were considered to be seroprotective against Hepatitis B although the rather low overall response indicates the need for further optimization of the delivery system for this specific antigen.

Stealth PEGylated CS (PEG-CS) nanoparticles have also been studied for intranasal delivery of different antigens and the possible role of PEG in improving the observed immune response was explored (Rezaei-Mokarram *et al.*, 2005; Csaba *et al.*, 2009b). For example, PEG-CS and CS nanoparticles were able to induce systemic and mucosal immune responses against diphteria toxoid (DT) upon intranasal administration to mice. Besides, PEG-CS achieved significantly higher systemic antibody titers in comparison to the ones obtained for CS nanoparticles (Rezaei-Mokarram *et al.*, 2005).

Other CS derivatives have similarly been explored for antigen delivery purposes. The most recurrent modifications involve the methylation of CS amine groups (Amidi *et al.*, 2010). Nanoparticles made of oppositely charged methylated CS derivatives (positively charged N-trimethyl CS-TMC and negatively charged mono-N-carboxymethyl CS-MCC) were loaded with TT and intranasally administered to mice. The results have shown that positive CS and TMC nanoparticles, with a size ranging from 300–400 nm, induced higher serum IgG titers than negative MCC nanoparticles of 90 nm. These results emphasized the assumption that the nature of the nanoparticles surface, as well as the particle size, have a crucial role in obtaining an enhanced immune response (Sayın *et al.*, 2008; Hagenaars *et al.*, 2009).

Mangal *et al.* (2011) have recently reported the superiority of TMC nanoparticles *vs.* classical CS nanoparticles with regard to their ability to elicit anti-HBsAg antibody titers following intranasal administration to mice. This was attributed to the improved mucoadhesion of TMC nanoparticles, followed by an enhanced antigen uptake.

Interestingly, CS-coated nanocapsules, formed by an oily nanocore stabilized with phospholipids and surrounded by a CS shell, as already described in section on "Controlled release technologies", have also shown a great capacity of association of HBsAg. The most remarkable advantage of this type of improved nanostructure is its versatility, as it is feasible to efficiently incorporate simultaneously lipophilic immunostimulants in the oily core, *i.e.* imiquimod, and the antigen in the polymer shell (Vicente *et al.*, 2009). The results of the *in vivo* experiments performed with this novel formulation have shown a progressive increase of the specific IgG levels over time, achieving seroprotection against the HBsAg for up to 112 days. This response was significantly higher than the one obtained with the nanocapsule formulation in the absence of imiquimod.

Nanoemulsions have similarly been explored for the stabilization and nasal delivery of different molecules, in particular ovalbumin (OVA), porcine intestinal alkaline phosphatase (AlkP), and HBsAg (Makidon *et al.*, 2010). *In vivo* results show that upon intranasal administration to mice of HBsAg associated to the nanoemulsion, the reported serum anti-HBsAg IgG antibody titers were comparable to the ones obtained for the antigen adsorbed on aluminum hydroxide and given intramuscularly. These results were similarly reported in other animal species (rats and guinea pigs), in a single- or two-doses scheme (Makidon *et al.*, 2008).

Despite the promising examples reported here, there is no doubt about the need to further improve the design and, thus, the efficiency of intranasal delivery vehicles. Nevertheless, the accumulated knowledge and the cross-disciplinary approaches currently underway will hopefully define the way to proceed in the optimization of the design of nanostructures for intranasal antigen delivery.

Oral vaccine delivery systems

While live-attenuated vectors have shown promising results for oral immunization, problems related to their safety made it necessary to find new solutions for oral vaccination. This route is particularly challenging due to the harsh conditions of the gastrointestinal environment and the need to confront the intestinal mucosa. Nano-sized delivery systems are expected to have several functions in oral vaccine administration: (i) to improve the antigen stability in the gastric environment and increase its bioavailability; (ii) to overcome the mucus layer and interact with the underlying epithelium; (iii) to increase the uptake of these systems by the epithelial and M-cells (Brayden, 2001).

The physicochemical properties of particulate vehicles are known to affect the uptake of the antigen along the intestine and by APCs. The smaller size of nanoparticles is considered to be a key parameter influencing the uptake and immunogenicity of these delivery systems, as nanoparticles are better taken up by intestinal cells than microparticles (O'Hagan, 1996; Florence, 2005; des Rieux *et al.*, 2006). With respect to the surface properties, both the hydrophobicity and the surface charge affect colloidal stability, mucoadhesion properties and absorption of the carriers (des Rieux *et al.*, 2006). In addition, targeting specific receptors on the apical surface of M-cells may enhance the entry of antigens, triggering the immune response and leading to effective protection against mucosal pathogens (Azizi *et al.*, 2010). Nanoparticles can be conveniently targeted to M-cells by surface modification with selective ligands such as lectins or certain bacterial surface proteins (O'Hagan, 1996; Florence, 2005).

The importance of the surface properties on the ability of particles to efficiently deliver antigen through the oral route was evidenced by Tobío *et al.* (2000). Similarly to what was observed when the nanoparticles were administered to the nasal mucosa, the coating of PLA nanoparticles with a hydrophilic and stabilizing PEG coating was found to be crucial in order to avoid nanoparticle aggregation in gastro-intestinal fluids and upon contact with the mucosa. This increased stability has been the explanation for the improved absorption of ^{125}I-labelled TT after oral administration to rats in PLA-PEG nanoparticles (Tobío *et al.*, 2000).

Poly(anhydrides) were also used for the preparation of a specific type of polymer named Gantrez® AN (Arbós *et al.*, 2002). Nanoparticles prepared with this polymer present interesting bioadhesion characteristics and were therefore studied for oral immunization with OVA. Nanoparticles provided better immune response than that obtained for the OVA in solution. The survival rate of 100% achieved by the OVA-loaded nanoparticles versus the 40% obtained with the OVA-solution after challenge with an intraperitoneal injection of this allergen also proves that this can be an interesting alternative for oral immunization (Gómez *et al.*, 2007).

Other recent studies have aimed at exploring the possibility to target the M-cells, through the development of carriers that mimic the entry of pathogens. An example of this approach was the study of PLGA nanoparticles decorated with a specific type of lectin (*Lotus tetragonolobus – LTA*). These nanoparticles interacted with Peyer's patches M-cells, as confirmed by confocal microscopy, and provided anti-HBsAg titers, which were higher than those corresponding to the control PLGA nanoparticles. This positive behavior was attributed to the lectin-mediated selective targeting (Mishra *et al.*, 2010).

The RGD peptide (a small sequence composed of L-arginine, glycine, and L-aspartic acid which is involved in cell recognition processes) was also grafted to the PEG residues of the poly(ε-caprolactone-co-ethylene glycol) (PCL–PEG) copolymer used to prepare nanoparticles. Unfortunately, despite the targeting ability evidenced *in vivo*, the improvement of the immune response achieved by any of the RGD-grafted formulations was minimal (Garinot *et al.*, 2007). Thus, these results put in question the interest in targeting the M-cells for achieving adequate immune responses.

Overall, considering the studies described, the evidence of the potential of nanostructures for oral vaccination is still scarce. Besides, the positive role of a specific targeting to M-cells needs to be confirmed. Despite this, the reported results have paved the way towards the optimized design of nanoparticles intended for oral vaccination. Considering the whole perspective on transmucosal vaccine delivery, it becomes clear that this approach is a valid and interesting pathway for future developments. Research about new delivery vehicles for this type of administration is therefore expected to play a very important role in global health improvement and may represent a new milestone in vaccine research.

Conclusions

Over the past few decades, the search for new adjuvants capable of enhancing the immunogenic properties of current and developing antigens has driven the attention of the scientific community towards the design of particulate antigen delivery systems. These new adjuvants are not only able to enhance the immunogenicity of safer but poorly effective antigens but may also allow the targeting of these antigens to the adequate immune-competent cells. Beyond this adjuvant capacity, polymer-based micro/nanostructures

can be now presented as promising single-dose and transmucosal vaccination approaches. Giving room to new routes of administration, nanovehicles are influencing decisively the pathways of vaccinology and gaining an essential place in this field. Hopefully, these progresses in immunization strategies will help to achieve the universal goal of satisfactory immunization coverage to life-threatening diseases worldwide.

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