Enhancing the Immunogenicity of Subunit Vaccines by Utilisation of Particulate Vaccine Delivery Systems

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Abstract

Control and eradication of a number of infectious diseases are primarily attributed to effective vaccination programs. A concerted effort is still imperative to develop novel vaccines and improve the immunogenicity of existing ones with regards to efficacy, immunogenicity and safety. Rational design of vaccines using subunit vaccines is a potentially safer alternative to conventional vaccines, yet they are poorly immunogenic without additional adjuvant. Using antigen carriers to enhance their immunogenicity in the forms of adsorption or encapsulation with a delivery system has been widely investigated as an alternative to currently available adjuvants. This review aims to elaborate on the existing nanotechnology being used to develop more immunogenic subunit vaccines, with focus on particulate delivery systems for development of prophylactic vaccine candidates.

Keywords: immune system, vaccine, particulate delivery system, liposome, virus-like particles, polymeric nanoparticles

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Introduction

Vaccination, in combination with good hygiene and clean water, has proven to be the most effective strategy for controlling the incidence of infectious diseases. Availability of effective vaccines and successful vaccination campaigns have significantly reduced the incidences of infectious diseases including diphtheria, tetanus, pertussis, mumps, rubella, hepatitis A and B in many countries (André, 2003) and successfully eradicated smallpox, whereas progress is also being made to continuously decrease the incidence of polio and measles (de Quadros, 2002; Shahzad & Kohler, 2009). However, there are other diseases that are of public health importance for which effective vaccines are not available yet, such as tuberculosis, malaria, HIV and vaccine-preventable cancers as each of those diseases presents unique challenges in terms of pathogen complexities and immune correlates of protection (Crompton *et al.*, 2010; Chhatbar *et al.*, 2011; Rappuoli & Aderem, 2011; Kaufmann, 2012; Schlom, 2012; Vaughan & Kappe, 2012). It is therefore imperative that not only novel vaccines are discovered but also immunogenicity of existing vaccines is enhanced.

Traditionally, live-attenuated organisms or whole-killed organisms are used as vaccines (Plotkin, 2005, 2009) and such vaccines are immunogenic as they induce strong, long-lasting humoral and/or cell-mediated immunity. However there are a number of safety reasons associated with such formulations, including but not limited to reversion to more virulent strain and possible compromise of quality with temperature-sensitive vaccines (Diminsky *et al.*, 1999; Bolgiano *et al.*, 2001; Boros *et al.*, 2001). All of the above risks and issues associated with safety and efficacy of 'traditional' vaccines have shifted the focus of vaccine design towards rationally-designed vaccines, which centres on the development of subunit vaccines that include peptide- and protein-based vaccines. These subunit vaccines have the potential to be developed into safer and more immunogenic vaccines whilst also avoiding the risks commonly associated with conventional vaccines (Moyle & Toth, 2013). Proteins as vaccine candidate can be produced using various expression systems (Ledizet *et*

al., 2005; Wang *et al.,* 2006; Coller *et al.,* 2011; Cox, 2012; Lobanova *et al.,* 2012). Peptide epitopes when incorporated into a vaccine formulation can elicit antibody-mediated humoral responses and/or T lymphocyte-mediated cellular responses (Ghosh & Jackson, 1999; Tian *et al.,* 2001; Calvo-Calle *et al.,* 2005; Jackson *et al.,* 2006; Li *et al.,* 2011; Christy *et al.,* 2012; Huang *et al.,* 2013).

In spite of these advantages subunit vaccines have to offer, their immunogenicity is very poor and they require addition of adjuvant to successfully induce the desired immune responses. This is due to the fact that subunit vaccines lack the 'danger signals' to alert the immune system – in particular the antigen presenting cell (APC) – and they are also susceptible to proteases that in turn render their half-life to be relatively short. However, an issue commonly associated with adjuvants is that they are toxic, as such currently only a number of oil-water emulsion and aluminium-based adjuvants have been licensed (Wilson-Welder *et al.,* 2009) and even such aluminium-based adjuvants are lacking in their ability to mediate cellular immune responses despite their effectiveness in eliciting antibody-mediated responses (McElrath, 1995; HogenEsch, 2002; Reed *et al.,* 2009). Although in most cases antibody-mediated humoral responses would be sufficient, yet infections brought about by viruses, intracellular bacteria, or cancer need efficient cellular responses to clear. The aforementioned issues highlight the potential and feasibility of particulate antigen carriers for adjuvanting subunit vaccines as they are inherently immunostimulatory in nature and they provide a depot effect that prolong the exposure time of peptides and proteins to the immune cells (Tao Liang *et al.,* 2006). Particulate delivery systems also offer safeguarding against degradation, facilitation of uptake by APC, feasibility of incorporation of additional immunostimulatory moieties that can further boost immunogenicity by direct targeting of APC. All of these features indeed make them a viable option to enhance the immunogenicity of subunit vaccines so both immunogenicity and safety are achieved. This review article aims to illustrate the recent developments in prophylactic vaccines that utilise particulate delivery systems. The basis of immune recognition on which vaccine design is based on as well as the interaction of these particulate carriers with immune cells will also be discussed.

How the Immune System Works: Antigen Recognition, Processing and Presentation

In order to appropriately select and assess which delivery systems are suitable, it is imperative to understand the basis of immune response induction and the correlates of protection (Oyston & Robinson, 2012). Such knowledge would form the basis for selection of the most fitting delivery system for optimal efficacy and immunogenicity.

When infection occurs, foreign molecules at the site of infection are being recognised by the cells of the innate immune system and this recognition provide signalling to recruit inflammatory cells including macrophages and natural killer (NK) cells. This event is followed by phagocytosis by macrophages and immature dendritic cells (DC) that results in the release of pro-inflammatory cytokines and recruitment of more macrophages. Availability of pathogen-associated molecular patterns (PAMP) as a result of phagocytosis leads to their recognition by pattern recognition receptors (PRR) on the surface of DC –an important APC – and subsequent DC activation. Microorganisms have PAMP structures (Medzhitov & Janeway Jr, 1997; Aderem & Ulevitch, 2000; Takeda *et al.,* 2003; Kaisho & Akira, 2004) that keep the immune system aware of foreign invasion by providing 'danger' signals that will lead to innate immune system activation (Medzhitov & Janeway Jr, 1997). One family member of PRR is the Toll-like receptors (TLR) that are equipped to recognise PAMP as being foreign as they can distinguish between 'self' and 'non-self' (Medzhitov & Janeway Jr., 1997; Medzhitov *et al.,* 1997). Largely expressed in DCs and macrophages (Medzhitov, 2001), there are 10 functional members of human TLR family and 12 TLR members in mice (Kawai & Akira, 2010, 2011). Each TLR has a different function with regards to PAMP recognition (Akira *et al.,* 2006) and they also have different ligands with distinct structure and origin (Medzhitov, 2001; Akira & Hemmi, 2003).

Immature DCs play a role in detecting and capturing pathogens in the peripheral tissues and other sites of entry (Steinman, 1991). They function in the uptake of antigen that can occur by a number of mechanisms: phagocytosis, macropinocytosis and receptor-mediated endocytosis (Banchereau *et al.,* 2000; Banchereau & Steinman, 1998). Following recognition of PAMP, DC undergoes a process called maturation. DC maturation can also be triggered by pro-inflammatory cytokines such as TNF-α or cross-linking of CD40 with CD40 ligand (Reis e Sousa *et al.,* 1999; Rescigno *et al.,* 1999) in addition to the presence of microbial antigens. There are a number of changes that occur during DC maturation including the up-regulation of CCR7 and down-regulation of CCR6 that enables mature DCs to migrate to the draining lymph nodes (Sallusto *et al.,* 1998; Forster *et al.,* 1999). DCs also lose their phagocytic abilityand secrete large amounts of cytokines and chemokines essential for stimulation of T cells (Rescigno *et al.,* 1999). Additionally, co-stimulatory molecules such as CD80, CD86 and CD40 as well as MHC class II molecules are up-regulated on the surface of DCs (Banchereau & Steinman, 1998; Mellman & Steinman, 2001). These changes prepare DCs for presentation of peptide fragments to T cells that lead to activation of naïve T cells. Mature DCs are able to prime naïve $CD4^+$ and $CD8^+$ T cells following recognition of antigen-MHC molecules complex by the T-cell receptors (TCR) with additional signalling from binding of CD40 to its ligand. Activated $CD4^+$ T cells develop into three different subsets of T helper (Th) cells depending on the cytokines secreted by mature DCs, namely Th1-, Th2-, or Th17-type T cells, which differ in the resulting immune responses.Activated $CD8^+$ T cells obtain help from Th1 cells that secrete interferon-γ (IFN-γ) to differentiate into cytotoxic T lymphocytes (CTL) that kill infected cells. Figure 1 summarises the cascade of event involved in adaptive immune responses following activation of dendritic cells.

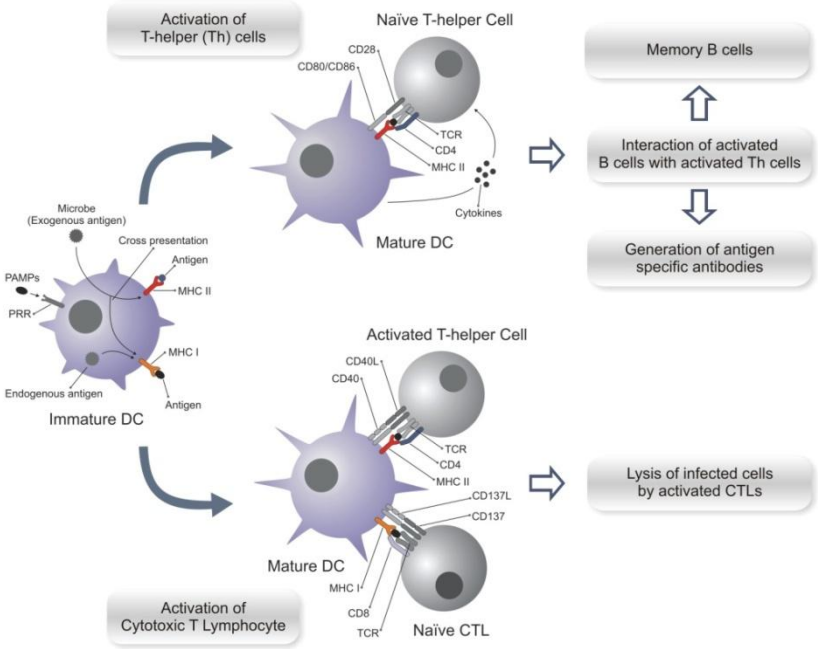

Figure 1. Activation of dendritic cells (DCs), T-helper (CD4) cells (Th cells) and $CD8^+$ cytotoxic T lymphocytes (CTL). Activation (maturation) of DC through PAMP signaling (licensing); uptake of exogenous antigen, degradation and presentation of antigen by MHC. Naïve Th cells are activated by mature DC through TCR (T cell receptor) recognition of antigen presented in the context of MHC II, and co-stimulation (interaction of CD28 with CD80/CD86 of mature DC). Presentation of antigen in the context of MHC II by activated B cells (not shown) to activated Th cells leads to the generation of antibodies and memory B cells. Activation of naïve CTL by mature DC occurs through TCR recognition of antigen presented in the context of MHC I, which normally requires stimulation by activated Th cells and co-stimulation by interaction of CD137 with CD137L (adopted from Moyle & Toth, 2013).

Following capture and internalisation by DC, the antigen will then be processed into peptide fragments that will associate with MHC molecules before being transported to the cell surface for presentation to T cells. MHC molecules are highly polymorphic and as such enables the presentation of an array of different peptides. Depending on their origin, different antigens are presented in the context of MHC class I or II molecules. Endogenously-derived antigens such as those of tumour or viral origin are typically presented by MHC class I molecules to $CD8^+$ T cells (Rock & Goldberg, 1999; Grommé & Neefjes, 2002; Rock *et al.*, 2004), while extracellular antigens derived from endocytosis or phagocytosis are presented by MHC class II molecules to $CD4^+$ T cells (Villadangos, 2001; Watts, 2004). The MHC class I and II molecules have different preferred peptide lengths for processing. The class I molecules prefer peptides of 8-9 residues in length (York *et al.*, 2002; Rock *et al.*, 2004). Peptides that result from degradation by the multi-catalytic and ubiquitous protease complex, the proteasome, range from 2-3 residues up to more than 20 amino acid in length (Kisselev *et al.*, 1999; Rock & Goldberg, 1999). Therefore, peptides that are longer than the preferred size undergo further trimming in the ER (Rock *et al.*, 2004). On the other hand, the optimal peptide length for binding to class II molecules is approximately 18-20 amino acids long (O'Brien *et al.*, 2008).

Another mechanism of antigen presentation is known as 'cross presentation' where extracellular antigens are presented to $CD8^+$ T cells in the context of MHC class I molecules.This mechanism is crucial for generation of cell-mediated immunity as well as for immune surveillance (Heath *et al.*, 2004; Groothuis & Neefjes, 2005; Rock & Shen, 2005). DC is the main immune cell responsible for cross presentation (Ackerman & Cresswell, 2004; Heath *et al.*, 2004) and antigens that can be cross presented include those derived from whole proteins, peptides, DNA, and RNA (Rock & Shen, 2005) as well as intracellular bacteria, parasites, immune complexes and soluble proteins (Heath *et al.*, 2004). The exact mechanism of cross presentation is yet to be elucidated although a number of mechanisms have been proposed, including the endosome to ER pathway where antigens of endosome origin are degraded in the ER cytosol and the peptides are subsequently transported by TAP to MHC class I molecules (Shen & Rock, 2006) and the phagosome-cytosol and phagosome-lysosome pathways that are usually associated with particulate antigens phagocytosed by DC and macrophages (Khor & Makar, 2008).

Particulate Vaccine Delivery Systems

Adjuvants are defined as molecules, compounds or macromolecular complexes that are able to augment antigen-specific immune responses with regards to their magnitude and duration (Wack & Rappuoli, 2005). Adjuvants exert their effects by a number of mechanisms, either by providing a depot effect to ensure prolonged exposure to APC (for aluminium- or emulsion-based adjuvants), promoting antigen uptake by APC (for particulate adjuvants) or targeting APC by means of compounds that bind to receptors on APC surface (for example TLR ligands) with a common aim of a more efficient antigen processing and presentation (Marciani, 2003). Particulate antigen carriers are especially attractive as they boast all of the above characteristics of adjuvant. To date there are numerous particulate delivery systems that are being investigated in vaccine formulations, some of which have been approved for human use and more are in pre-clinical and clinical trials (Kushnir *et al.*, 2012; Correia-Pinto *et al.*, 2013).

At the central of immune response induction is DC as the only APC capable of efficiently activating naïve T cells to subsequently activate the adaptive immune responses. It is therefore a very important cell to target in vaccination (Belz *et al.*, 2004) and the rationale behind formulation of subunit vaccines with particulate delivery system. The hallmark of particulate delivery system is indeed their particulate nature that mimics pathogens with regards to their size, shape and molecular structure (Bachmann & Jennings, 2010).The similar sizes and structure with pathogens facilitate better uptake by APC of antigens associated with particulate carriers compared to soluble antigens. Soluble antigens are internalised mostly via endocytosis, whereas uptake of particulate antigens are usually mediated by phagocytosis (Burgdorf & Kurts, 2008). Phagocytosis by DC delivers

antigenic cargo into early phagosomes, which are important organelles that can facilitate cross presentation of antigen for presentation in the context of MHC class I molecules (Ackerman *et al.*, 2003). This highlights the potential of particulate carriers to direct the immune response towards cell-mediated response, opposite to soluble antigens which are usually associated with induction of humoral responses.

Other attractive features of particulate antigen carriers are that they are amenable to insertion or fusion of different protein antigenic sequences within its structure as well as to chemically-conjugated antigens on its surface. These features highlight their versatility in terms of the feasibility of not only carrying a variety of antigen cargos but also tailoring specific types of immune responses. As such there are different kinds of delivery systems being investigated, each with their own characteristics and advantages.

Liposome

Liposomes are spherical structures made of phospholipid bilayers encapsulating an aqueous centre. Liposomes are safe and well-tolerated, biodegradable as they are composed of lipid bilayers that are naturally found in cell membranes, exhibit low reactogenicityand very versatile in terms of the types of lipid components and methods of vesicle preparation to cater for specific physicochemical properties as desired (Watson *et al.*, 2012). Liposomes have been reported as potent enhancer of immune responses when used in delivery of protein (Leserman, 2004; Hansen *et al.*, 2008; Thueng-in *et al.*, 2010), DNA vaccine (Nakanishi *et al.*, 1999; Wang *et al.*, 2010b), peptide epitopes (Ludewig *et al.*, 2000; Chikh & Schutze-Redelmeir, 2002) and cancer vaccine (Zhang *et al.*, 2013). Schematic representation of subunit vaccine delivery by liposome is shown in Figure 2.

There are a number of key parameters in the design of liposomal vaccines, some of which are method of antigen attachment, vesicle size and charge (Watson *et al.*, 2012), as well as lipid composition, lamellarity, pegylation and the type of targeting moiety (Giddam *et al.*, 2012). Liposomes can be made from lipids that are positively or negatively charged to produce cationic or anionic liposomes, respectively (Ulrich, 2002). There are various methods by which antigen can be incorporated into liposomes (Giddam *et al.*, 2012). Soluble antigens can be encapsulated in the aqueous core, embedded within the lipid bilayer or absorbed on the lipid membrane surface; peptide antigens can be coupled to the membrane; and charged antigens can be associated electrostatically with oppositely-charged membrane.

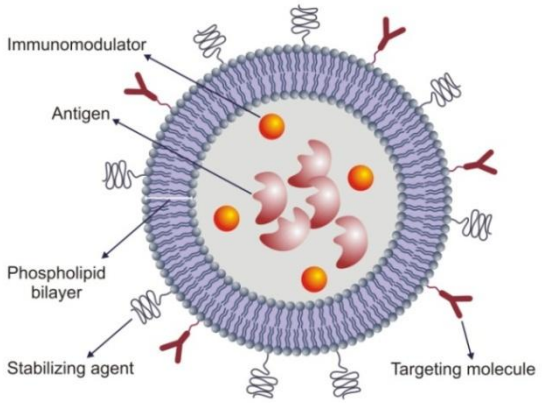

Figure 2. Schematic representation of subunit vaccine antigen delivered by liposome. Liposome particles can be armed by targeting molecule e.g. antibody fragments for targeting to APC, and stabilizing agents such as polyethyleneglycol (PEG).

A number of studies showed that the resulting immune response against liposomal-associated antigens is influenced by the positioning of antigens. In general, conjugation of antigen on liposome surface promotes better antibody-mediated immune response compared to encapsulated antigen, whereas no significant differences were observed between these two methods of antigen association with regards to cell-mediated immunity (Shahum & Therien, 1994, 1995; Chen & Huang, 2008). This is perhaps due to ease of recognition of antigen by B cells when it is located on the surface of liposomes (White *et al.*, 1995). Additionally, vesicle size also affects the resulting immune response where vesicles sized between 250-700nm elicit better Th1-skewed response (Mann *et al.*, 2009; Henriksen-Lacey *et al.*, 2011). Moreover, one factor that significantly determines the immunogenicity of liposomal antigens is the surface charge of lipid membranes, where charged liposomes are better than neutral ones in eliciting antibody and cell-mediated responses with positively-charged liposomes being the most potent inducer of immune

responses (Nakanishi *et al.*, 1999; Badiee *et al.*, 2009).

Virus-like Particles (VLP)

Virus-like particles (VLP) are non-infectious particles formed by self-assembly of envelope and/or capsid proteins of viruses and thus they mimic the overall structure of virus without the infectious genetic material (Ludwig & Wagner, 2007; Noad & Roy, 2003). Preparation of VLPs from a number of viruses such as human papillomavirus (HPV), hepatitis B and C virus, human immunodeficiency virus (HIV) as well as influenza viruses have been documented (Liu *et al.*, 1998; Yao *et al.*, 2003; Mihailova *et al.*, 2006; Szécsi *et al.*, 2006; Karpenko *et al.*, 2007; Kang *et al.*, 2009a). VLPs can be produced using various expression systems, such as bacterial, yeast, insect and mammalian cells (Watanabe *et al.*, 2001; Zheng *et al.*, 2004; Mena *et al.*, 2006; Santi *et al.*, 2008; Baek *et al.*, 2012). An example of VLP assembly from HPV is depicted in Figure 3.

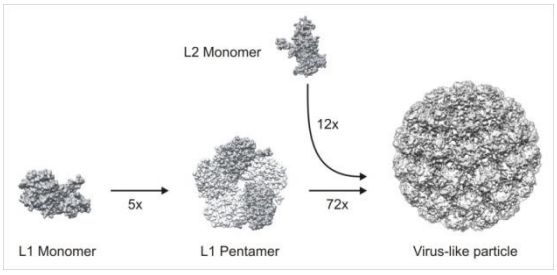

Figure 3. Assembly of human papillomavirus (HPV)-like particle. Interaction of five L1 proteins, the major viral capsid protein, yields a L1-pentamer (capsomer). 72 of these pentamers and L2 proteins, the minor capsid protein, assemble to a virus-like particle (VLP) mimicking the outer shell of an authentic virion (adopted from Schiller & Lowy, 2012).

Immunogenicity of VLP is mainly attributed to their particulate structure that is preferred for uptake by APC and their high density display of epitopes (Grgacic & Anderson, 2006; Roy & Noad, 2008). As a vaccine VLP can induce both humoral and cellular responses (Sailaja *et al.*, 2007; Quan *et al.*, 2008; Song *et al.*, 2010b) as well as providing protective immunity following their administration (Kang *et al.*, 2009b; Song *et al.*, 2010a; Hossain *et al.*, 2011) via stimulation of APC that subsequently prime naïve T cells (Moron *et al.*, 2002; Lenz *et al.*, 2003; Buonaguro *et al.*, 2006). Their superior immunogenicity as vaccines, their non-infectious and particulate nature have prompted the utilisation of VLPs as antigen delivery systems as well (Garcea & Gissmann, 2004; Teunissen *et al.*, 2013). Additionally, their wide range of target pathogens and versatility in production systems have led to a number of candidates undergoing clinical trial with a handful of VLP-based vaccines already entering the market (Kushnir *et al.*, 2012). Their versatility as antigen carrier has been shown in a variety of cargos such as peptide epitopes (Gilbert *et al*., 1997; Liu *et al.*, 2000; Woo *et al.*, 2006; Cheong *et al.*, 2009; Krammer *et al.*, 2010) and DNA vaccines (Karpenko *et al.*, 2007; Jariyapong *et al.*, 2013).

VLP can be used to display different peptides on their surface without interfering with their assembly (Peabody *et al.*, 2008; Takahashi *et al.*, 2008; Caldeira *et al.*, 2010). The peptide epitope can also be incorporated within the VLP to substitute certain epitopes creating a chimeric VLP, such as in the construction of hepatitis B virus surface antigen VLP (Cheong *et al.*, 2009) and simian virus 40 VLP (Kawano *et al.*, 2014) containing embedded influenza virus CTL epitopes. A number of studies have demonstrated that VLPs are potent DC stimulator (Lenz *et al.*, 2003; Sailaja *et al.*, 2007) and also showed how administration of antigen-VLP complexes resulted in induction of high titers of antigen-specific antibodies (Slupetzky *et al.*, 2007; Caldeira *et al.*, 2010; Krammer *et al.*, 2010), CTL responses and protective immunity (Liu *et al*., 2000; Cheong *et al.*, 2009; Kawano *et al.*, 2014). Other studies also showed that VLP as antigen carrier can elicit mucosal and systemic immune responses against the antigen (Richert *et al.*, 2012; Rivera-Hernandez *et al.*, 2013) which correlate to their potential as inducer of mucosal immunity and facilitator of intranasal vaccine delivery.

Polymeric Nanoparticles

Vast numbers of synthetic polymers have been used as biomaterials for production of nanoparticles, such as poly(ethylene glycol) (PEG) (Vila *et al.*, 2004a), poly(D,L-lactic-coglycolic acid) (PLGA) (Elamanchili *et al.*, 2004; Sapin *et al.*, 2006; Hamdy *et al.*, 2011; Prasad *et al.*, 2011; De Temmerman *et al.*,

2012; Colonna *et al.*, 2013), poly(D,L-lactide-co-glycolide) (PLG) (Vila *et al.*, 2004a; Rajkannan *et al.*, 2006; Singh *et al.*, 2006), poly(g-glutamic acid) (g-PGA) (Akagi *et al.*, 2005; Matsuo *et al.*, 2007; Shima *et al.*, 2013; Toita *et al.*, 2013), and polystyrene (Minigo *et al.*, 2007). Out of these synthetic polymers, PLGA and PLG have received considerable attention and are the most investigated materials for particulate delivery systems as their biocompatibility and biodegradability are outstanding (Panyam & Labhasetwar, 2003; Peek *et al.*, 2008; Danhier *et al.*, 2012).

Natural polysaccharide-based polymers have also been utilised in preparation of delivery systems, which include chitosan (Nagamoto *et al.*, 2004; Vila *et al.*, 2004b; Amidi *et al.*, 2010; Chua *et al.*, 2011; Gordon *et al.*, 2012), alginate (Mata *et al.*, 2011; Li *et al.*, 2013), inulin (Layton *et al.*, 2011), pullulan (Dionísio *et al.*, 2013). Chitosan in particular has been intensively investigated as they are non-toxic, highly biocompatible and biodegradable, and amenable to various modification of shapes and sizes (Amidi *et al.*, 2010; Chua *et al.*, 2011). Numerous studies have demonstrated increased immunogenicity of antigen delivered in chitosan nanoparticles (Prego *et al.*, 2010; Zhao *et al.*, 2011; Tafaghodi *et al.*, 2012) and that these nanoparticles are indeed favoured for uptake and presentation by APC (Koppolu & Zaharoff, 2013).

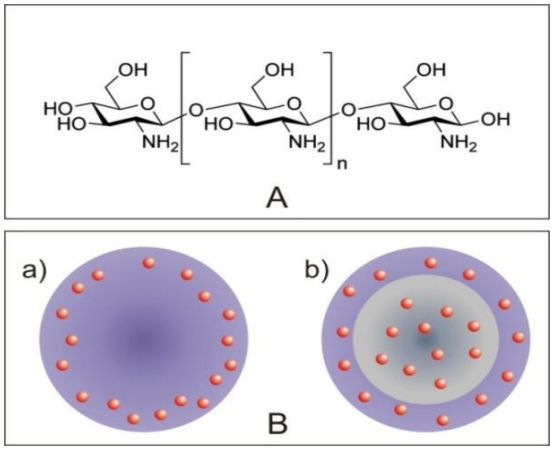

Figure 4. **A**. Chemical structure of chitosan. **B**. Schematic representation of a protein (e.g. BSA, in red) loading patterns in chitosan-based microspheres prepared **a**) by one-step crosslinking with glutaraldehyde or **b**) by two-step crosslinking with p-phtaldehyde and glutaraldehyde (adopted from Wei *et al.*, 2008).

Chitosan-based delivery systems have been shown to be a viable platform for development of mucosal vaccines with strong and long-lasting immune responses (van der Lubben *et al.*, 2001; Nagamoto *et al.*, 2004; Vila *et al.*, 2004b) due to the chitosan's ability to facilitate transport of peptides and proteins across mucosal barriers (Amidi *et al.*, 2006; Illum *et al.*, 2001). Pulmonary administration of chitosan nanoparticles containing DNA vaccine encoding T cell epitopes from *Mycobacterium tuberculosis* resulted in higher IFN-γ production compared to soluble form of plasmid DNA and when intramuscular administration was used (Bivas-Benita *et al.*, 2004). N-trimethyl chitosan (TMC), a more soluble chitosan derivative, has also been developed and investigated as a delivery system (Amidi *et al.*, 2006; Sayın *et al.*, 2008). TMC is also found to be viable for intranasal administration of vaccines and enhances immunogenicity of its encapsulated antigen (Amidi *et al.*, 2007; Verheul *et al.*, 2008).

Targeting Particulate Delivery Systems to Dendritic Cells

The highly immunogenic nature of particulate delivery systems is evident in the resulting antigen-specific immune responses following their administration. However, with regards to developing better vaccines, utilising additional ligands or molecules for direct targeting to DC has proven to be an efficient means to further augment the resulting immune responses. DC-targeting ligands allow for direct DC activation, ensuring the cascade of events leading to activation of adaptive immunity. Improved immunogenicity may translate to lower doses required, which would be beneficial in the event where vaccine demands increase significantly such as in a pandemic setting.

In the case of VLP as vaccines, incorporation of DC-targeting ligands evidently is an efficient approach to further enhance their immunogenicity. For example, incorporation of CD40 ligand into chimeric simian immunodeficiency virus-like particles (SIVLP) enhanced their immunogenicity (Skountzou *et al.*, 2007) and a more recent study using CD40 ligand into SIVLP demonstrated a significant increase in DC maturation markers (CD83, CD40, and CD86)

and cytokines indicating the activation of DCs, as well as overall increase in the magnitude of humoral and cellular responses against SIV (Zhang *et al.*, 2010). DC-stimulating cytokines such as Flt3 ligand and granulocyte macrophage colony-stimulating factor (GM-CSF) when inserted into budding VLP also successfully increased VLP immunogenicity by virtue of triggering DC activation (Sailaja *et al.*, 2007). Other studies showed that incorporation of TLR ligands into VLP resulted in strong antibody responses (Wang *et al.*, 2008), as well as cellular responses and protective immunity (Wang *et al.*, 2010a; Schneider-Ohrum *et al.*, 2011).

This approach has been shown to be feasible as well in improving the immunogenicity of liposome-associated antigens. For example, cationic liposomes complexed with either a TLR3 or TLR9 agonist have successfully delivered their cargo to be cross presented resulting in cellular response (Zaks *et al.*, 2006). Cell-mediated immunity against *M. tuberculosis* was also obtained following administration of liposome that incorporated mycobacterial heat shock protein 65 and IL-12 (Yoshida *et al.*, 2006). A more recent study using a TLR3 agonist-incorporated cationic liposomes demonstrated that such liposomes generated a much better tumour-specific cell-mediated immune response and IFN-γ production compared to the same liposome without the TLR3 agonist (Wang *et al.*, 2012).

Concluding Remarks

The potential of particulate delivery systems for adjuvanting subunit vaccines is evident in numerous studies. With a variety of delivery system to choose from, we can select the most appropriate antigen carrier with regards to the type of resulting immune responses we desire. This is especially important for diseases that require cell-mediated immunity as only a few adjuvants are capable of eliciting cellular responses. Improvement in formulation and addition of immunostimulatory molecules provides an alternative to formulate an even more immunogenic delivery system.

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