

Working together: interactions between vaccine antigens and adjuvants

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Abstract: The development of vaccines containing adjuvants has the potential to enhance antibody and cellular immune responses, broaden protective immunity against heterogeneous pathogen strains, enable antigen dose sparing, and facilitate efficacy in immunocompromised populations. Nevertheless, the structural interplay between antigen and adjuvant components is often not taken into account in the published literature. Interactions between antigen and adjuvant formulations should be well characterized to enable optimum vaccine stability and efficacy. This review focuses on the importance of characterizing antigen–adjuvant interactions by summarizing findings involving widely used adjuvant formulation platforms, such as aluminum salts, emulsions, lipid vesicles, and polymer-based particles. Emphasis is placed on the physicochemical basis of antigen–adjuvant associations and the appropriate analytical tools for their characterization, as well as discussing the effects of these interactions on vaccine potency.

Keywords: vaccine adjuvant, vaccine antigen, physicochemical characterization

Introduction

Modern vaccines based on recombinant antigens generally require adjuvant help to generate adequate immune responses. Even live attenuated or inactivated vaccines contain intrinsic adjuvant structures [McKee *et al.* 2007]. Thus, vaccines can be considered to consist of two principal components: antigen and adjuvant. In general, the mechanism of action of each of these two components is heavily investigated before a vaccine reaches licensing stage. For example, vaccine antigens are carefully screened based on bioinformatic and experimental approaches for their ability to elicit protective immunity [Flower *et al.* 2010]. Likewise, specific receptors and immune signaling cascades are well known for immunostimulators such as Toll-like receptor (TLR) agonists or C-type lectin receptor (CLR) agonists [Duthie *et al.* 2011]. While there may be less consensus regarding mechanisms of action of particulate-based adjuvants such as aluminum salts and oil-in-water emulsions, even these adjuvants have been investigated at length to generate data on a range of potential biological mechanisms [Kool *et al.* 2012; O'Hagan *et al.* 2012]. However, a thorough analysis of the physicochemical interactions between antigen and adjuvant, and the

resulting optimization of those interactions, is too often lacking in the literature. The purpose of this review is to highlight the work that has been reported regarding antigen–adjuvant interactions and generate interest in the need for more investigation in this area in order to optimize vaccine formulations for stability and bioactivity.

Adjuvants are often simplistically classified as immunostimulatory molecules (TLR ligands, CLR ligands, NOD-like receptor (NLR) ligands, saponins, etc.) or delivery systems (aluminum salts, emulsions, lipid vesicles, etc.). In reality, most adjuvants are a combination of these two classes. For instance, aluminum salts and emulsions are not just delivery vehicles since they clearly generate adjuvant activity besides their potential antigen delivery functions. Likewise, immunostimulatory molecules are rarely employed in isolation; in general, they are formulated in some particle-based platform. The best illustration of this concept is perhaps the adjuvant AS04 in the Cervarix® vaccine, approved by the US Food and Drug Administration (FDA) in 2009, which is composed of aluminum oxyhydroxide and a TLR4 ligand (MPL®). Thus, the aluminum salt may serve as an adjuvant itself as well as a delivery vehicle for MPL® and/or the vaccine antigen.

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In this review, emphasis is placed on adjuvant formulations rather than unformulated immunostimulatory molecules. While a few vaccines in development contain soluble unformulated immunostimulatory molecules, little information is available regarding interactions of these adjuvants with the antigen. For instance, the most advanced vaccine candidate containing a soluble immunostimulatory molecule is Dynavax's HEPLISAV, which has completed phase III clinical testing although an FDA committee decided in November 2012 that there was insufficient data to support the safety of the vaccine [FDA, 2012]. While HEPLISAV's hepatitis B surface antigen forms small particles, the adjuvant itself (a CpG-based TLR9 ligand known as 1018 ISS) is apparently not formulated in any particle-based platform, which may explain why a relatively high dose of 3 mg is necessary, and no information is available regarding interactions between the antigen and adjuvant in HEPLISAV [Heyward, 2012; Sablan *et al.* 2012].

Most vaccines that contain immunostimulatory molecules employ some type of particle-based formulation for the adjuvant molecule for stabilization, delivery, or dose-sparing purposes. For example, using tetanus toxoid antigen, Diwan and colleagues demonstrated that 10-fold dose sparing of CpG adjuvant is feasible when the adjuvant is formulated in polymeric nanoparticles compared with soluble CpG [Diwan *et al.* 2004]. Thus, the complete formulation (comprising the immunostimulatory molecule and the particulate platform) becomes the entity of interest when investigating interactions with the antigen. In the following sections, we focus on the interactions of adjuvant formulations with vaccine antigens, beginning with the adjuvant most widely used in vaccines today and for the last century: aluminum salts. Owing to their ubiquity, there are multiple studies delineating the effect of antigen adsorption to aluminum salts, including the affinity of the adsorption interaction and corresponding effects on antigen structure and bioactivity. Emulsions and lipid vesicles will then be addressed, the latter forming one of the most versatile formulation platforms since the antigen can be encapsulated in the vesicles or surface-conjugated. Finally, other formulations falling outside of the above traditional platforms will be discussed.

Aluminum salts

Aluminum salt adjuvants are the most commonly used class of adjuvants and were the first class of

adjuvants approved for use in human vaccines [Vogel and Powell, 1995]. The safety and efficacy of these adjuvants are well established; however, their adjuvanticity is not entirely understood. Potential mechanisms of action include serving as a depot for slow antigen release, enhancing the recruitment of and subsequent uptake by immune cells, or direct stimulation of the immune system [Gupta, 1998; Gupta *et al.* 1995; Hem and Hogenesch, 2007; Noe *et al.* 2010]. Several detailed reviews on aluminum adjuvants have been written [De Souza Reboucas *et al.* 2012; Kamerzell *et al.* 2011; Wilson-Welder *et al.* 2009]; here we will focus on the physical properties of antigen–adjuvant interactions.

Several types of aluminum salt adjuvants are approved for use, including aluminum oxyhydroxide and aluminum phosphate, of which Alhydrogel® and Adju-Phos® are respective commercial examples [White and Hem, 2000]. At neutral pH, the difference in the physical properties of these two adjuvants is quite significant. Aluminum oxyhydroxide has a point of zero charge (PZC) at pH 11 and aluminum phosphate has a PZC at pH 4–5.5 [al-Shakhshir *et al.* 1995; Seeber *et al.* 1991]. Adsorption of antigen by adjuvant is thought to be important for immune response, so when selecting which aluminum salt to use, antigen and adjuvant charge is a critical consideration. Electrostatics are often a dominant force in antigen adsorption by adjuvant [al-Shakhshir *et al.* 1995]; however, hydrogen bonding, van der Waals forces, hydrophobic interactions, and ligand exchange have also been shown to play a role [al-Shakhshir *et al.* 1995; Iyer *et al.* 2004; Peek *et al.* 2007]. A recent report describes the generation of functionalized aluminum oxide for covalent conjugation of small molecule haptens [Maquieira *et al.* 2012]. Furthermore, pH, ionic strength, and buffer and excipient selection can have a significant effect on antigen adsorption and adjuvant degradation [al-Shakhshir *et al.* 1995; Peek *et al.* 2007; Salnikova *et al.* 2008].

Antigen adsorption isotherms are easily constructed, since aluminum salts are denser than aqueous solutions and can be removed by centrifugation [Iyer *et al.* 2004]. The strength of adsorption of antigen by aluminum salts is antigen specific and can have a significant effect on immunopotentiality; modulation of the strength of adsorption by aluminum oxyhydroxide can be achieved by pretreatment with phosphate salts [Hansen *et al.* 2011]. Antigen adsorption may not

always be important for immunogenicity, as has been demonstrated by Romero Mendez and colleagues [Romero Mendez *et al.* 2007]. In this study, three model vaccines in which protein was not adsorbed by aluminum phosphate produced antibody titers that were similar to those produced by protein adsorbed by aluminum phosphate. Confocal microscopy results suggested that the antigens used in this study were trapped in the void spaces between adjuvant aggregates and that this facilitated uptake of the antigen by dendritic cells. In the case of AS04 with human papilloma virus antigens, the TLR4 agonist (and half the dose of aluminum) could be administered 1 hour, but not 24 hours, before administration of the antigens (with the other half dose of aluminum) and still elicit similar antibody response as contemporaneous administration [Didierlaurent *et al.* 2009].

Proteins adsorbed by adjuvant are frequently less stable than in solution [Peek *et al.* 2007] and may be more susceptible to physical and chemical degradation [Estey *et al.* 2009; Vessely *et al.* 2009]. Differences in pH at the adjuvant surface can have a significant effect on the rates of antigen degradation [Ljutic *et al.* 2012], and the pH of the microenvironment around aluminum oxyhydroxide has been shown to be 2 pH units higher than the bulk [Wittayanukulluk *et al.* 2004]. One must perform a detailed study of the protein on adjuvant and not rely solely on antigen solution studies. When selecting excipients, pH, buffers, and other storage conditions for antigen adsorbed by adjuvant, it is important to be able to evaluate the stability and integrity of the antigen. Peek and colleagues studied the effects of various excipients on the stability of protein in solution and adsorbed by adjuvant [Peek *et al.* 2007]. They found that many of the same excipients that stabilized a protein in solution also stabilized the protein on adjuvant; however, the adsorbed protein remained less stable than in solution.

Many of the spectroscopic techniques traditionally used to assess antigens are not suitable or must be modified due to the turbidity of solutions containing aluminum salt. Intrinsic tryptophan fluorescence can be used to assess the tertiary structure of the antigen using a traditional fluorometer and a cuvette designed for front-face fluorescence. Attenuated total reflection Fourier transform infrared (ATR-FTIR) and Raman spectroscopies can be used to examine properties of the secondary structure of the antigen. Differential

scanning calorimetry (DSC) can be used to determine the thermal stability of the antigen–adjuvant system, and isothermal titration calorimetry (ITC) can be used to more thoroughly characterize antigen adsorption; however, cleaning the calorimeter after the experiment may be difficult. Ausar and colleagues describes a high-throughput screening (HTS) approach that uses a real-time polymerase chain reaction (RT-PCR) instrument and extrinsic fluorescence dyes such as SYPRO Orange to rapidly screen for stabilizers of protein adsorbed by adjuvant [Ausar *et al.* 2011]. To assess the chemical stability of the antigen, traditional approaches (high-performance liquid chromatography [HPLC], mass spectrometry, capillary isoelectric focusing) may be used if antigen can be desorbed and isolated from the aluminum salts. Several methods have been used to desorb protein from aluminum including incubation with succinate, urea, phosphate, or various surfactants [Estey *et al.* 2009; Katz, 1987; Rinella *et al.* 1998; Vessely *et al.* 2009]. Aluminum salt adjuvants have been imaged by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) [Burrell *et al.* 2000; Harris *et al.* 2012; Lee *et al.* 2009b]. A recent study describes an ultrasonication treatment of Alhydrogel® to generate finely dispersed crystals that can be negatively stained for visualization of protein on the surface (Figure 1) [Harris *et al.* 2012].

Emulsions

Emulsion-based adjuvant systems have also been widely employed in vaccine development and formulation. Several different classes of emulsions exist, such as oil-in-water (o/w) emulsions, water-in-oil (w/o) emulsions, water-in-oil-in-water (w/o/w) emulsions and protein-stabilized emulsions. O/w emulsions are formulated as oil nanoparticles suspended via surfactant in an oil phase, and have been observed to be more stable in protein-containing formulations than w/o/w emulsions [Barnett *et al.* 1996]. The w/o emulsions are essentially the inverse of o/w emulsions and are gentle enough on proteins to maintain enzymatic activity of chymotrypsin in the aqueous droplet [Lee and Brody, 2005]. The w/o/w emulsions contain water droplets within larger oil droplets, which are themselves suspended within a bulk aqueous solution. Whereas o/w emulsions are generally preferred for human applications, both w/o and w/o/w emulsions are widely used in veterinary vaccines containing attenuated or inactivated antigens [Aucouturier *et al.* 2001]. Protein-stabilized

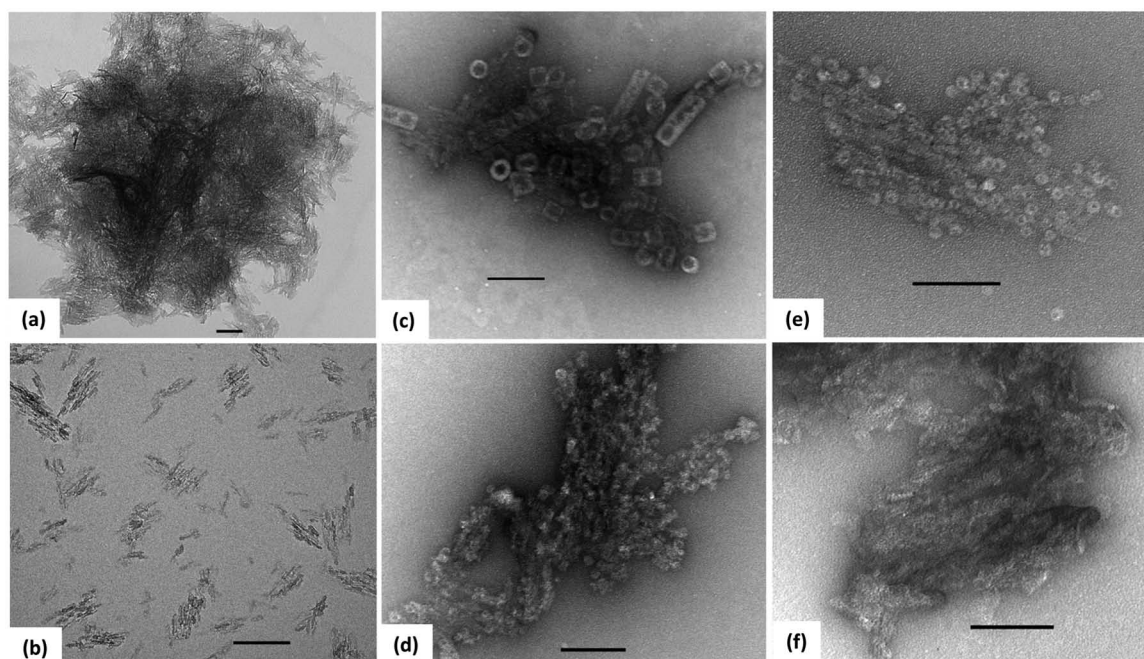


Figure 1. TEM images of Alhydrogel untreated (a), or ultrasonicated with a probe sonicator for 10 min (b)–(f). Panels (c)–(f) contain protein adsorbed to Alhydrogel and negatively stained with uranyl acetate. Keyhole limpet hemocyanin (c), respiratory syncytial virus nucleocapsid protein RSV n-RNA (d), anthrax protective antigen PA63 (e), and *Escherichia coli* outer membrane protein OmpF are shown. Scale bars indicate 100 nm. [Reproduced with permission from Harris *et al.* [2012]. Copyright © 2012 Elsevier.]

emulsions have been extensively characterized and are a class of o/w emulsions, however with proteins as the primary surfactant species. While protein-stabilized emulsions are generally studied for food science applications, the knowledge gained is often relevant for other systems such as vaccines, and for this reason we cite several food science studies below. In each of these systems, hydrophobic and electrostatic forces govern antigen–adjuvant interactions that result in important interprotein interactions and adsorbed protein conformational shifts (Figure 2).

Within the context of protein-stabilized emulsions, hydrophobicity has been demonstrated to be the primary interaction mechanism [Junghans *et al.* 2010], in which emulsifying proteins can be easily denatured [Jutila *et al.* 2000] or displaced by non-ionic surfactants such as polysorbates [Courthaudon *et al.* 1991; Dickinson and Gelin, 1992; Rampon *et al.* 2003a, 2003b; Stevenson *et al.* 1997] and sorbitan esters [Cornec *et al.* 1996], among others [Courthaudon *et al.* 1991]. This desorption tends to proceed as a gradual replacement of protein with surfactant [Rampon *et al.* 2003a] at the oil–water interface resulting from a change in protein binding exchange kinetics [Dickinson and Gelin, 1992] in a

manner that appears to be a function of protein structure [Cornec *et al.* 1996; Stevenson *et al.* 1997] and the timing of surfactant introduction [Courthaudon *et al.* 1991].

Electrostatic interactions pertain most significantly to protein mixed with membranes generated with ionic surfactants [Chang *et al.* 2008; Junghans *et al.* 2010] or ionic proteins [Chen and Dickinson, 1995a, 1995b, 1995c]. These interactions are driven by charge differentials between components, and as such are highly impacted by formulation pH and ionic strength [Chang *et al.* 2008; Chesko *et al.* 2005; Tokle and McClements, 2011]. Emulsion particle size is another important factor in protein–emulsion adsorption likely because the net surface area of an emulsion (with constant oil-phase and surfactant concentrations) increases when the particle size decreases, making more surface area available for protein adsorption. Depending on protein concentrations, protein monolayers or multilamellar protein shells can form around emulsions [Chang *et al.* 2008].

Another significant consideration regarding proteins adsorbed to oils and emulsions is interprotein interactions. The strongest of these interactions

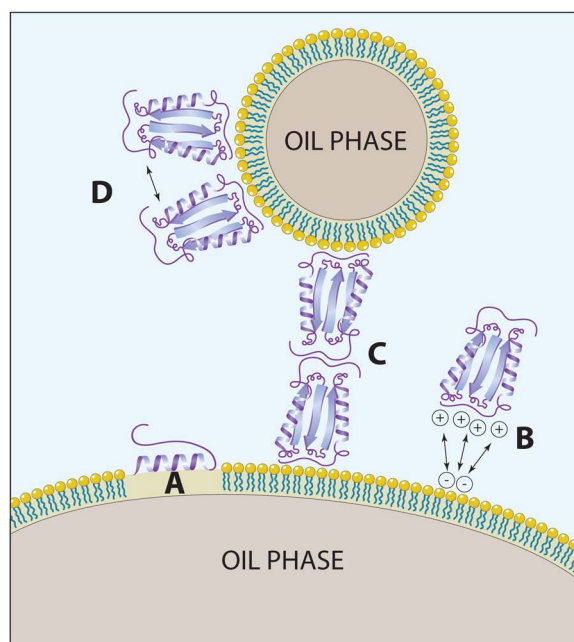


Figure 2. Common protein interactions with emulsion systems. (A) Hydrophobic interactions with the oil phase resulting in α -helical transitions. (B) Electrostatic interactions binding charged surfaces of antigen and emulsion. (C) Protein—protein interactions resulting in flocculation between particles. (D) Protein—protein interactions on the surface of the emulsion, resulting in increased surface viscosity. Image courtesy of Alessandro Baliani © 2013. Adapted from Lucien Barnes' original artwork.

is covalent aggregation via disulfide bridges, as has been observed in some o/w emulsions following formulation, perhaps due to oil-phase oxidation products or other low concentration reactive species [Miles *et al.* 2005]. Strong surface protein interactions between emulsions can result in flocculation events [Rampon *et al.* 2003a; Zhai *et al.* 2011]; however, these can commonly be abated by increasing electrostatic repulsion [Zhai *et al.* 2011], or by adding cosolvents such as glycerol and sorbitol that increase aqueous phase viscosity (slowing particle velocity) and repulsive colloidal interactions between particles, in addition to altering the conformation of adsorbed proteins to avoid exposure of hydrophobic or cysteine residues [Chanasattru *et al.* 2007]. Further, these interprotein interactions on a single surface can produce dense viscoelastic protein films [Zhai *et al.* 2011], potentially explaining why high protein concentrations can reduce overall formulation stability [Zhu *et al.* 2011].

Substantial shifts in protein secondary and tertiary conformation have been observed following adsorption to emulsions and oils. Although some studies have claimed only minor changes in structure using atomic-level techniques such as electron paramagnetic resonance [Berzofsky *et al.*

1976], advanced spectroscopic methodologies that are sensitive to protein secondary and tertiary structural rearrangements are required to fully understand these interaction systems. FTIR studies have captured changes in secondary structure for proteins adsorbed to emulsions [Jorgensen *et al.* 2004]. Further, circular dichroism (CD) spectroscopy studies have detected a general decrease in β -sheet structure concurrent with an increase in α -helical content upon adsorption to oil and emulsions, which is lost during desorption [Lee *et al.* 2009a; Zhai *et al.* 2011, 2012]. Tertiary structural changes have also been observed via fluorescence spectroscopy such as blue shifts and increases in quantum yield that occur when proteins are adsorbed to oils and emulsions, suggesting that changes in tertiary structure result in shielding of tryptophan residues from solution [Castelain and Genot, 1994; Fox *et al.* 2012; Husband *et al.* 2001; Jorgensen *et al.* 2004]. These structural rearrangements appear to be more resilient to thermal denaturation than nonadsorbed proteins [Zhai *et al.* 2011, 2012], potentially due to α -helical conformation support imparted by the oil microenvironment. Moreover, the oil phase may have a considerable impact on adsorbed protein structure in comparison to native protein conformation, as oil polarity and

chain disorder can vary folding of adsorbed proteins [Herrero *et al.* 2011; Stevenson *et al.* 1997; Venien *et al.* 2000; Zhai *et al.* 2011, 2012].

Given the multitude of potential interactions and conformational changes between protein antigens and emulsion formulations, it is perhaps surprising that in some cases these interactions, though detectable, do not appear to affect bioactivity [Fox *et al.* 2012; Ott *et al.* 1995]. Likewise, significant association between antigen and emulsion does not appear to be critical for adjuvant effects. For example, Ott and colleagues showed that the o/w adjuvant emulsion MF59® does not detectably bind the herpes simplex virus (HSV) antigen gD2 but still generates high-titer antibodies to the antigen [Ott *et al.* 1995]. Similarly, the HSV antigen gB2 could be made to bind to MF59 to some extent, but this did not improve the elicited immune response at the normal MF59 dose; interestingly, at a lower MF59 dose, the effect of bound gB2 on elicited antibody titers was significant, indicating that a certain density of antigen/emulsion droplet may provide boosted immune responses. A recent report of recombinant HIV protein, gp140, interactions with MF59 found little or no association of the protein with the oil phase as indicated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and only minor changes in protein conformation after extraction from the adjuvant as determined by monoclonal antibody enzyme-linked immunosorbent assays (ELISAs), whereas polymer-based formulations had more significant effects on protein conformation [Lai *et al.* 2012]. Finally, MF59 could be administered 24 hours before the antigen or 1 hour after the antigen and elicit the same antibody responses as jointly administered adjuvant and antigen. In a similar study, the o/w adjuvant emulsion AS03 could be injected 1 hour before the antigen and induce the same antibody response to an influenza antigen as contemporary administration with the antigen [Morel *et al.* 2011]. Further, even though a low level of association was detected between antigen and AS03, administration of premixed vaccine compared to administration with two syringes at the same time (one with adjuvant and one with antigen) did not alter the immune response.

Lipid vesicles

Lipid vesicles comprise a class of formulations that generally consist of some sort of lipid bilayer encapsulating an aqueous core. Liposomes,

composed of phospholipids and cholesterol, are the most prominent example, with several liposome-based drug delivery products in use as well as GlaxoSmithKline's liposome-based adjuvant formulation, AS01, in malaria vaccine phase III clinical trials. However, virosomes (liposomes with embedded fusogenic viral proteins) are the more accomplished formulation in the vaccine adjuvant field, with two virosome-based vaccine products licensed in Europe (Inflexal® and Epaxal®). Niosomes are formed with nonionic surfactants instead of phospholipids and have also shown promise as vaccine adjuvant formulations.

The main advantage of lipid-vesicle-based systems is their versatility. A superb review by Watson and colleagues of liposome-based vaccine development, with emphasis on formulation parameters, was recently published [Watson *et al.* 2012]. Here, we focus only on the antigen association aspect of lipid vesicle adjuvants with emphasis on the most recent reports from the literature. As a general rule, it is thought that some form of association of the antigen with the liposome is desirable, although this may substantially increase the complexity of manufacturing and maintaining stability [Haensler, 2010; Watson *et al.* 2012]. For example, GlaxoSmithKline's liposomal system AS01 is apparently a simple mixture with the RTS,S malaria antigen, with no available information regarding extent or mechanism of antigen–adjuvant association. Indeed, Yanasarn and colleagues discovered that simple mixtures of anionic antigens with negatively charged anionic liposomes produced equivalent immune responses to cationic liposomes (even though the cationic liposomes demonstrated a higher level of association with the antigen), although this adjuvant activity depended on the anionic lipids employed [Yanasarn *et al.* 2011]. In contrast, developers of the CAF01 adjuvant formulation, comprising cationic lipid with the immunostimulatory molecule trehalose dibehenate, have demonstrated that electrostatic association of antigen with adjuvant is necessary for optimal adjuvant activity [Henriksen-Lacey *et al.* 2010].

Besides electrostatic association, antigens may be covalently bound or chelated to modified lipids or intercalated into the bilayer. For example, virosome adjuvant activity is best when the antigen of interest is associated with the virosome, either through encapsulation (for optimal T-cell responses) or anchored to the bilayer via hydrophobic protein domains or protein lipidation (for

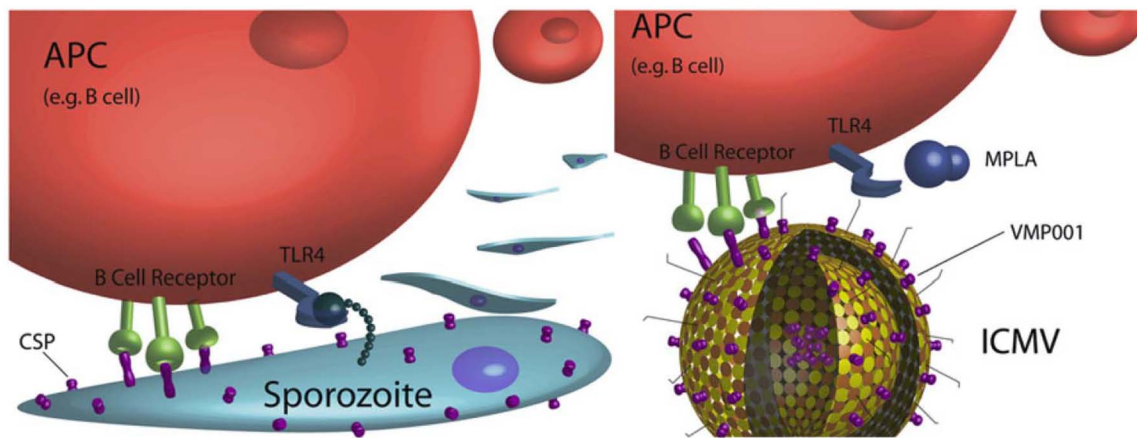


Figure 3. Interbilayer crosslinked multilamellar vesicles (ICMVs) enhance immune responses by presenting surface-linked malaria antigen along with TLR4 agonist to antigen-presenting cells (right), mimicking the malaria sporozoite structure (left). (Reproduced with permission from Little [2012]).

optimal antibody responses) [Moser, 2011]. Antigen conjugation to a lipid vesicle surface can be accomplished by covalent or noncovalent attachment, with the conjugation method having a potential effect on the adjuvant activity. For instance, covalent attachment of antigen compared with noncovalent attachment via metal chelation demonstrated improved adjuvant activity; furthermore, antigen attachment via trivalent metal chelation was not better than monovalent linkage despite the higher association affinity of the former [Watson *et al.* 2011]. New click chemistry methods have shown potential for more straightforward and/or controlled linking of antigen to adjuvant nanoparticles [Mahon *et al.* 2012], but more studies are needed to evaluate their practical product potential for vaccine applications.

Another important but often overlooked consideration is the antigen density per particle [Vorup-Jensen, 2012; Watson *et al.* 2012]. Appropriate antigen density on the surface of lipid vesicles and other nanoparticles may result in B-cell receptor cross-linking and enhanced immune responses [Little, 2012]. Moon and colleagues developed a novel multilamellar lipid vesicle delivery system (ICMV) with recombinant malaria antigen chemically linked to the vesicle surface or encapsulated in the vesicle interior (Figure 3), resulting in significantly enhanced antibody titers compared with encapsulated antigen; this lipid vesicle delivery system, in combination with TLR4 agonist, induced a balanced Th1/Th2 type response, broadened antibody specificity while increasing

avidity, and caused expansion of helper T cells [Moon *et al.* 2012]. A well-controlled click chemistry technology was used by Elias and colleagues to show that an intermediate density of targeting proteins on a nanoparticle surface provided better cell binding compared with lower or higher densities [Elias *et al.* 2012]. Antigen density in virosomes has also been shown to be an important factor for immunogenicity and protection [Homhuan *et al.* 2004].

Other adjuvant formulations

IC31 is a binary polyelectrolyte complex consisting of the TLR9-agonist oligodeoxynucleotide mixed with an excess of the antimicrobial peptide KLKL(5)KLK. For negatively charged antigens, adhesion is likely due to coulombic forces. At pH 7.9, any unbound antigen Ag85B-ESAT-6 (pI 4.88) was not detectable by SDS-PAGE and Western blot in the supernatant after ultracentrifugation of the antigen–adjuvant complex for 1 hour at 100,000g, and the Western blot of preformulated antigen to antigen that had been adsorbed and then desorbed from IC31 showed that the antigen was not significantly degraded by adsorption to the adjuvant [Agger *et al.* 2006]. However, to the best of the authors' knowledge no characterization of bound antigen structure has been evaluated. Adsorption efficiencies and kinetics depend on the pH of the vaccine formulation, antigen pI, and antigen hydrophobicity. The extent of antigen adsorption and its adsorbed conformation are likely important physicochemical properties of the vaccine formulation. Indeed, association of the

antigen with the adjuvant may be necessary for uptake by dendritic cells [Aichinger *et al.* 2011].

Chitosan nanoparticles, another polyelectrolyte formulation, can be formulated to have either a positive or negative surface charge. The surface charge, buffer type, pH, and antigen, all determine the type and strength of interaction, as well as reactogenicity *in vitro* and *in vivo*. A complex of chitosan and dextran sulfate can be formulated to be cationic (excess chitosan) or anionic (excess dextran sulfate). The capsid protein from HIV-1, p24, bound to both positively and negatively charged particles regardless of pH [Drogoz *et al.* 2008]. Complexes with excess chitosan bound p24 more efficiently than complexes with excess dextran sulfate. Furthermore, acetic acid and ammonium phosphate buffer disrupted adhesion, suggesting that hydrogen bonding is involved in the immobilization process [Drogoz *et al.* 2008; Schatz *et al.* 2003]. Adsorption and desorption kinetics depend strongly on the excess electrolyte in the complex. Particles with excess dextran sulfate in a pH 6.2 solution had very rapid sorption kinetics, reaching equilibrium after only 15 hours. Below the isoelectric point of p24, adsorption efficiency was low and reached equilibrium at 35% binding after 24 hours on the same chitosan particles [Drogoz *et al.* 2008]. Drogoz and colleagues found sorption capacity to be above that predicted by a monolayer model as a function of solid content suggesting absorption into the particle under certain conditions. Thus, the immobilization process is not only directed by the charge of the protein and polyelectrolyte particle, but also by protein/particle ratio and the protein partition coefficient between the solution and the colloid. Another report investigating an analogous protein-polymer interaction concluded that proteins may overcome overall charge repulsion through more specific interactions of polymer with oppositely charged protein patches, which releases counter ions and increases system entropy [Rosenfeldt *et al.* 2004].

There are a variety of other polymer particles in development as putative vaccine antigen delivery systems. Suitable polymers include poly(ϵ -caprolactone), poly(ethylenimine), poly(γ -glutamic acid), poly(D,L-lactic acid), poly(methyl methacrylate), and poly(uridylic acid), in addition to the chitosan polymer described above [Ferreira *et al.* 2012]. Polymer particles can be formulated with a variety of immunostimulatory molecules

and have been explored in the context of various diseases and both parenteral and mucosal delivery routes [Davis, 2006; Kirby *et al.* 2008; Murillo *et al.* 2002; Roman *et al.* 2008]. As with chitosan and IC31 particles, the charge differential between the antigen and polymer particle is one of the primary drivers of adsorption; as such adsorption is highly impacted by formulation pH and ionic strength [Chesko *et al.* 2008]. The optimal condition for electrostatic binding is the pH at which surface charge differentials are greatest, although non-Coulombic forces can overcome even charge repulsion [Chesko *et al.* 2005, 2008]. Thus, adsorption depends on the surface pI and hydrophobicity of the protein, the formulation PZC and surface chemistry, and the pH and ionic strength of the solution [Chesko *et al.* 2008]. Investigations into polymeric particle development with protein have yielded a variety of methods for modulating important properties such as protein adsorption, size, release rate, and encapsulation efficiency [Blanco and Alonso, 1997; Ho *et al.* 2008; Jiang and Schwendeman, 2001; Yang *et al.* 2000]. A recent report by Dey and colleagues employed dynamic light scattering and surface plasmon resonance (SPR) to show that a recombinant HIV glycoprotein, gp140, bound to polyanionic polymer at low pH and slowly disassociated when the pH was raised to 7.4; SDS-PAGE, monoclonal antibody ELISA, and SPR assays confirmed that the protein was stable for several hours at low pH in the presence of the polymer, although some conformational changes were apparent due to reduced antibody binding [Dey *et al.* 2012]. Interestingly, a complex emulsion-polymer combination adjuvant enhanced antibody responses to gp140 more than either formulation alone [Dey *et al.* 2012; Lai *et al.* 2012].

Immune stimulating complexes (ISCOMs) are a class of vaccine adjuvant formulations developed in the mid-1980s [Morein *et al.* 1984]. These adjuvants consist of mixtures of saponin, cholesterol, and phospholipids in specific stoichiometric ratios. Antigen is either formulated into the ISCOM without chemical modification [Homhuan *et al.* 2004], or by covalent modification via conjugation to phospholipid or fatty acid, then formulated into the ISCOM [Pedersen *et al.* 2012; Reid, 1992]. Because ISCOM particles have a negative surface charge [Kersten *et al.* 1991; Pedersen *et al.* 2012] as do many soluble proteins, strong adhesion of most soluble proteins is not observed. Attempts to improve adhesion of soluble proteins by treating with extreme pH conditions (pH 2.5) and high lipid

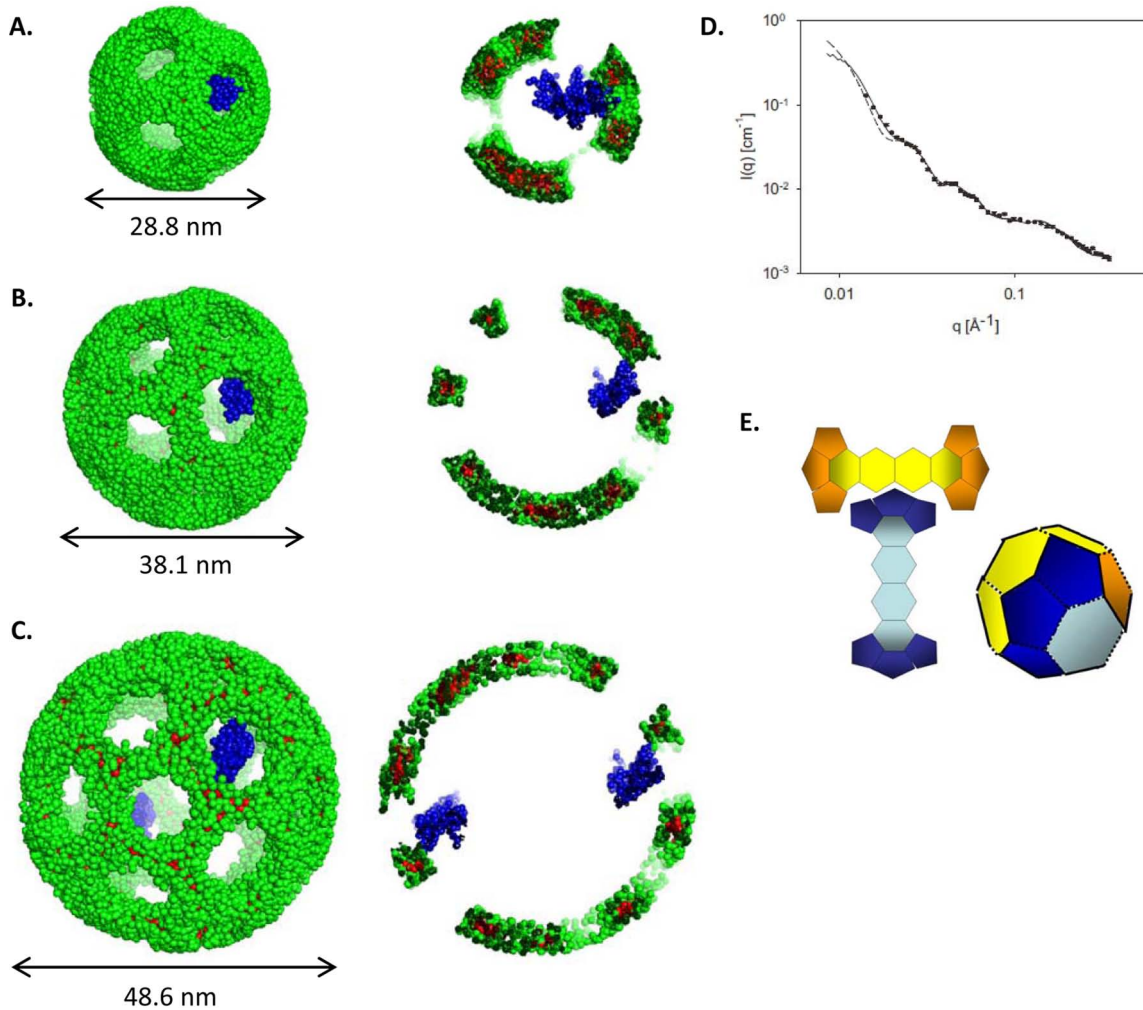


Figure 4. Immune stimulating complex (ISCOM) model structures with antigen positions (blue) from small angle x-ray scattering data (SAXS). (A) Icosahedral structure with a single associated antigen molecule. (B) 'Tennis ball' structure with a single associated antigen molecule. (C) 'Football' structure with two associated antigen molecules. (D) Experimental SAXS data of ISCOM with loaded antigen. The dashed line is the nonsmeared fit of the data. The solid line is the actual fit of the data with instrumental smearing; modeling by Monte Carlo integration. (E) Schematic of the 'tennis ball' model structure showing the orientation of the 12 pentagonal and 8 hexagonal openings. The other structures have only hexagonal openings. (Reproduced with permission from Pedersen *et al.* [2012]. Copyright © 2012 Elsevier.)

concentration increased recovery to 14% [Morein *et al.* 1990]. Based on the more efficient incorporation of amphipathic antigens such as envelope proteins [Morein *et al.* 1984], and the need for lipids for incorporation of antigen [Lovgren and Morein, 1988], the major mechanism of association between the ISCOM and antigen is likely hydrophobic in nature. Small-angle X-ray scattering spectroscopy (SAXS) and modeling by Monte Carlo integration suggest that lipid-modified antigen is incorporated into the ISCOM inside of the cage-like structure rather than on the external surface (Figure 4) [Pedersen *et al.* 2012]. Furthermore, this study

showed that ISCOMs likely consist of three particles with different geometry: an icosahedral structure with a mean diameter of 28.8 nm accounting for 10% of the mixture, a tennis ball structure (12 pentagonal and eight hexagonal openings) with a mean diameter of 38.1 nm accounting for 76% of the mixture, and a football (soccer ball) structure with a mean diameter of 48.6 nm accounting for 11% of the mixture. Based on modeling of the SAXS data, the smaller structures contain only one antigen molecule per particle; the larger structure contains two antigen molecules per particle. Positioning of the antigen is likely an important

factor in the nature, strength, and duration of the immune response to the vaccine.

Conclusion

There is much to be learned regarding how antigens and adjuvant formulations interact. In many cases, it appears that antigen–adjuvant association behavior could be further characterized and subsequently optimized for maximum vaccine stability and biological activity. On the other hand, in some cases, direct antigen–adjuvant association may not be necessary or even desirable. For example, adjuvant stockpiling in preparation for an influenza pandemic favors separate vialing of adjuvant and antigen since the dose and strain of the latter is not knowable until the pandemic arrives. However, even in this case, a thorough understanding of antigen–adjuvant interactions is essential to ensure stability after mixing and to prevent unforeseen interaction effects from decreasing vaccine efficacy. Just as regulatory bodies only approve adjuvants in the context of a vaccine product and not by themselves, vaccine developers must also consider vaccines containing adjuvants as a unit with integral interactions and associations, and perform the requisite characterization studies to understand each antigen–adjuvant combination.

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Conflict of interest statement

The authors report no financial conflicts of interest.

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