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Nanotechnology in vaccine delivery ☆

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Abstract

With very few adjuvants currently being used in marketed human vaccines, a critical need exists for novel immunopotentiators and delivery vehicles capable of eliciting humoral, cellular and mucosal immunity. Such crucial vaccine components could facilitate the development of novel vaccines for viral and parasitic infections, such as hepatitis, HIV, malaria, cancer, *etc.* In this review, we discuss clinical trial results for various vaccine adjuvants and delivery vehicles being developed that are approximately nanoscale (<1000 nm) in size. Humoral immune responses have been observed for most adjuvants and delivery platforms while only viral vectors, ISCOMs and Montanide™ ISA 51 and 720 have shown cytotoxic T cell responses in the clinic. MF59 and MPL® have elicited Th1 responses, and virus-like particles, non-degradable nanoparticles and liposomes have also generated cellular immunity. Such vaccine components have also been evaluated for alternative routes of administration with clinical successes reported for intranasal delivery of viral vectors and proteosomes and oral delivery of a VLP vaccine.
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Keywords: Adjuvant; Immunopotentiator; Humoral immunity; Cellular immunity; Immunization; Nanoparticle; Antigen

Contents

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1. Introduction

The creation of vaccines is one of medicine's most important accomplishments. Their influence on humanity is aptly expressed by vaccinologist Stanley Plotkin when he says:

> "The impact of vaccination on the health of the world's peoples is hard to exaggerate. With the exception of safe water, no other modality, not even antibiotics, has had such a major effect on mortality reduction and population growth." [1]

Diseases such as measles, mumps, rubella, diphtheria, tetanus, pertussis, *Haemophilus influenzae* type b (Hib) disease, polio and yellow fever are now under control because of vaccination [1]. Smallpox has been completely eradicated [1,2] and polio is on the verge of elimination [3], thanks to aggressive vaccination campaigns. Other diseases, including influenza, hepatitis B virus (HBV) and pneumococcal infection are being at least partially controlled by vaccines, but there is still much that needs to be done to eliminate many such diseases, even in the developed world [1].

Throughout history, most vaccines have been developed using live attenuated organisms, killed whole organisms or inactivated toxins (referred to as toxoids). Live vaccines, such as smallpox, polio (oral), measles, mumps, rubella, varicella and adenovirus (and others), have the advantage of producing both humoral and cellular immunity and often require only one boost. Disadvantages of live vaccines include a serious risk of reverting back to their virulent form and intrinsic instability, making them difficult to deliver. Killed or inactivated whole organism vaccines (such as influenza, hepatitis A and others) as well as toxoid vaccines (including diphtheria and tetanus) generate a weaker immune response and typically require multiple doses [1,4].

Recent efforts have focused on utilizing technologies such as recombinant DNA methods to develop DNA and subunit vaccines, as well as conjugate vaccines in which a weak antigen is linked to a stronger immunogen such as a protein or membrane complex. The vaccine against Hib, responsible for nearly eliminating Hib meningitis from infants and young children [1,5] is an example of a conjugate vaccine. DNA vaccines, which contain a gene or genes encoding a particular antigen(s), have so far only been licensed for veterinary use. They have, however, shown promise as vaccines against a wide variety of diseases including tuberculosis [6,7], SARS [8–10] and HIV-1 [11,12]. Subunit vaccines use a portion of the pathogen such as an individual protein as the antigen. These vaccines are attractive because of their increased safety since they cannot revert to a virulent form and their lack of contaminants remaining from the original pathogenic organism. Additionally, the ability to consistently produce large, well defined quantities of antigen from recombinant methods is highly desirable [1,13].

With the development of these new types of vaccines, there exists a critical need for additional delivery vehicles as well as new adjuvants. In many cases, the antigen itself is only very weakly immunogenic; therefore, an adjuvant is needed to intensify the immune response. Adjuvants can also be included in vaccines to guide the type of immune response generated [14,15]. This may be especially important when developing vaccines for cancer [16], HIV [17] or mucosal immunizations [18]. In contrast, a more immunogenic antigen may benefit from a specific delivery vehicle [14]. This component may facilitate targeting and/or controlled release of the antigen to antigen presenting cells. Recent studies utilizing Toll-like receptor ligands have shown that antigens associated with these ligands can produce exceptionally high antibody titers and rapid immune responses [19]. Adjuvants and delivery vehicles have also been shown to protect antigens from degradation, although this generally depends on the nature of adjuvant. For example, while chitosan–alginate nanoparticles were found to stabilize ovalbumin [20], other studies have shown that model protein antigens are actually destabilized by traditional aluminum salt adjuvants [21,22].

Currently, only very few vaccine adjuvants are licensed for use in humans. Although both MF59 and aluminum salts have been approved in Europe, only aluminum salts have been used in licensed human vaccines in the United States [23]. Billions of doses of vaccines containing aluminum salts have been shown to elicit early, high and long lasting antibody titers after a single immunization [24]. Despite their frequent, global use for many decades, the mode of action of aluminum salt adjuvants is not well understood. At least three potential mechanisms are frequently described in the literature [25,26]. One idea is that the formation of a depot at the site of injection allows the antigen to be released gradually, thereby extending the time possible for the antigen to interact with antigen presenting cells and lymphocytes [27,28].

A second proposed mechanism is attributed to the particulate nature of the aluminum salts. Because particles smaller than 10 μm are more easily phagocytosed by macrophages and dendritic cells [29,30], uptake of the antigen adsorbed to a particulate adjuvant should be increased (relative to antigen in solution) thereby improving the efficiency of antigen recognition and presentation. The third suggested mechanism of action is by a direct stimulation of the immune system through enhanced cytokine production. More specifically, aluminum salt adjuvants are known to primarily stimulate a humoral response by increasing production of Th2 cytokines [24]. While enhancement of Th2 cytokine production may be favorable for the treatment of some extracellular infections, a cellular immune response may be preferred for immunization against other disease states (e.g., HIV, malaria, cancer, *etc.*). A fourth mechanism of action was recently proposed after studies revealed a destabilization of proteins adsorbed to aluminum salt adjuvants: the structural perturbations that occur upon adsorption may, in fact, increase the antigen's susceptibility to proteolytic processing by the immune system, leading to enhanced antigen presentation [21].

Besides a bias in the type of immune response elicited by aluminum salt adjuvants, other disadvantages of their use include instability to freezing and drying [23,31,32] and inconsistencies in producing humoral immunity [24]. Additionally, despite maintaining a good safety profile for more than seven decades, there have still been safety concerns regarding the use of aluminum salts. Although the evidence is conflicting,

symptoms such as erythema, allergic responses, hypersensitivity to contact, granulomatous inflammation and subcutaneous nodules as well as macrophagic myofascitis have been reported for patients who received an aluminum salt-containing vaccine [29,33,34]. Due to the infrequent occurrence of these side effects, the verdict remains that aluminum salts exhibit a particularly good safety profile.

While aluminum salts offer an appropriate immune enhancement for some types of vaccines, they are clearly not adequate for all. A critical need exists for alternative immunopotentiators and antigen delivery agents. With the current trend toward "going small," many efforts to develop novel adjuvants have focused on systems at the micro- and nanoscale.

Particulate systems on the order of a micron in size offer several advantages for vaccine delivery [35,36]. For example, microparticles are approximately the same size as many pathogens that the immune system is equipped to attack [37]. Additionally, larger particles generally provide a longer duration of antigen release than equivalent smaller ones, which can have a dramatic influence on immunogenicity [30,38–40]. Moreover, microparticles have been shown to elicit both vigorous cellular [41] and humoral immunity [42].

Smaller particles on the order of 10–1000 nm in size pursued as vaccine delivery systems and immunopotentiators are the focus of this review. An additional characteristic shared by the nanosystems discussed herein is that they have all been at least preliminarily tested in the clinic (with the exception of polymeric nanoparticles).

2. Nanoparticulate vaccine adjuvants and delivery systems

2.1. Viral-vectored vaccines

Viral-vectored vaccines consist of a non-replicating virus that contains some defined genetic material from the pathogen to which immunity is desired. Such vaccines are also commonly referred to as live recombinant vaccines [43]. Since the immune system has evolved to respond to viruses, this would seem to be an ideal way to deliver an antigen. Advantages of virally-vectored vaccines include their ease of production, a good safety profile (at least in some cases), ability to potentiate strong immune responses, potential for nasal or epicutaneous delivery and mucosal immunization [44–46]. In cases where viral-vectored vaccines have been compared to DNA vaccines, the viral vectors have been shown to significantly enhance immunogenicity [47–50].

Adenovirus, which has been administered orally as its own vaccine for decades, has also provided a frequent vector platform for many of these types of vaccines, including delivery systems for Alzheimer's disease [51], influenza [44], tetanus [45] and HIV [52,53] based vaccines. Such systems are also being used for alternative routes of administration (i.e., not the parenteral route, which is typically used for immunization). A recent phase I clinical trial of an adenovirus-vectored flu vaccine administered intranasally and epicutaneously was found to elicit high serum antibody titers with a good safety profile [44]. This study was the first of its kind to show that adenovirus-vectored vaccines are safe for intranasal and epicutaneous administration in humans. Preclinical studies of an adenovirus-vectored tetanus vaccine reported similar results [45].

In addition to adenovirus, a variety of other vectors have shown success in both preclinical and clinical studies. A modified vaccinia virus Ankara (MVA) was well-tolerated and produced a good safety profile in humans infected with HIV-1 undergoing highly active anti-retroviral therapy (HAART) [54]. Additionally, a canarypox vector was used for expression of a cytomegalovirus (CMV) antigen [55]. The resultant clinical results manifested a specific cytotoxic T cell (CTL) response, which is especially important for developing immunity to intracellular viral pathogens [56]. This was the first vaccine to produce this effect for CMV [55]. Preclinical studies have also shown promise for use of CMV as a vector for an immunocontraception vaccine [57] and yellow fever virus as a vector for expression of dengue virus envelope genes [58]. Additional viral-vector technologies that are currently being pursued for vaccine delivery include poxviruses, measles virus, vesicular stomatitis virus [59], HSV and alphavirus [60], among others.

Immune responses generated by virally-vectored vaccines have been found to increase when a prime-boost regimen is employed. Such a procedure involves priming the immune system with one vectored vaccine (often a DNA vector) and boosting with the same pathogen's genetic material in another type of vector or a recombinant protein. While this approach has been successful for HIV vaccines in monkeys, it has not worked well in humans for HIV immunization [61–63] until recently. A clinical study published last year demonstrated that a prime-boost regimen HIV vaccine employing DNA and MVA resulted in "multifunctional HIV-1-specific T cells capable of rapid proliferation in eight out of eight vaccine recipients" [47,64]. A malaria vaccine administered using this dosing regimen also showed promise in the clinic [48,50]. Preclinical trials of viral-vectored vaccines used to immunize against tuberculosis [65], Ebola [66] and SIV [67] using a prime-boost strategy also appear promising.

In virtually all cases, the prime-boost regimen has resulted in strong T cell and/or IFN-γ responses, leading to its current status as an especially promising technology.

2.2. Virus-like particles and virosomes

Virus-like particles (VLPs) and virosomes also use nature's own mechanism and structural principles to trigger the immune system for protective effects. Like viral-vectored vaccines, these macromolecular complexes stimulate an immune response by delivering a material that mimics certain viral properties. VLPs are essentially non-infective viruses consisting of self-assembled viral envelope proteins without the accompanying genetic material. In the case of virosomes, the envelope of one virus is used as a platform to which additional components of the virus or another virus or pathogen are attached or inserted [68,69]. Both types of particles maintain a morphology and cell-penetrating ability similar to infective viral particles [70]. VLPs and virosomes have also been shown to stimulate both cellular and humoral immunity [69,71,72].

A number of virosome-based vaccines have already reached the market. The first of these was Epaxal™, a hepatitis A vaccine registered in 1994 by Berna Biologics Ltd. (Bern, Switzerland) in several European, Asian and South American countries [24,68]. The same company also licensed an influenza vaccine, Inflexal® V in Switzerland in 1997, which is now available in 25 countries [73]. Another flu vaccine utilizing virosomes is Invivac®, which is registered in the Netherlands and Switzerland [70,72]. NasalFlu®, an intranasal flu virosome vaccine that was co-administered with native *E. coli* heat-labile enterotoxin (LT) as a mucosal adjuvant, was marketed in Switzerland by Berna in 2001, but was removed from the market after an increased occurrence of Bell's Palsy was observed in people who had recently received the vaccine [70].

Additionally, several recombinant HBV VLP vaccines have been licensed. The first licensed recombinant HBV vaccines, Recombivax (Merck) and Engerix-B (GSK), were composed of the viral small envelope protein, which upon expression in yeast formed 22 nm VLPs [69,74]. While these were effective, they suffered from a lack of immunogenicity (~5–10% non-responders), which was determined to be due to an absence of Pre-S epitopes on the surface of the VLPs [75]. A more immunogenic VLP vaccine was subsequently described that contained Pre-S1, Pre-S2 and HBV surface antigen [75]. This potential third generation HBV vaccine, BioHepB was found to elicit a strong antibody response and 100% seroconversion and seroprotection rates [76,77], although it has yet to reach the market.

The most recently approved VLP vaccine is Gardasil® for immunization against human papillomavirus (HPV) and subsequent prevention of cervical cancer and genital warts. This vaccine is composed primarily of self-assembled particles of L1 (the major capsid protein) from HPV types 6, 11, 16 and 18 [78–80] and also contains an aluminum salt adjuvant. It has been shown to reduce infection of HPV by 90% and is apparently almost 100% effective against these types [80]. Since two of the four antigens in the HPV vaccine (HPV types 16 and 18) are implicated in 70% of cervical cancers [81], this vaccine is expected to drastically reduce the occurrence of this life threatening disease in women and has subsequently generated significant excitement [80,81].

Several other VLP vaccines have made it into the clinic. A Norwalk virus vaccine has shown humoral, mucosal and cellular immune responses when administered orally suggesting that VLPs may be useful for delivery of vaccines for mucosal immunization [82]. Another study showed that a small peptide of the Der p1 allergen covalently attached to a Qβ bacteriophage VLP was well-tolerated and generated high antibody titers in humans [83].

Additionally, a malaria vaccine composed of a VLP of HBV core antigen containing proteins from the circumsporozoite stage of the *Plasmodium* parasite was shown to produce significant humoral and cellular immune responses when formulated with Alhydrogel® [84]. Further studies revealed that a single dose of this vaccine administered with Montanide ISA 720 (see below) was as immunogenic as results produced by multiple immunizations of the Alhydrogel-adsorbed vaccine [85]. Similarly, a recombinant hybrid p17/p24:Ty VLP used for HIV immunization was found to produce both cellular and humoral immune responses to both components included in the VLP [86].

A variety of other VLP vaccines have been evaluated in preclinical studies and are tabulated in a 2006 *Methods* publication by Grgacic, et al. This table provides a comprehensive summary of the uses of VLPs in vaccines and their status in development [69]. Among those listed in preclinical studies are VLPs for influenza, hepatitis C virus (HCV), Ebola virus, rotavirus and SARS coronavirus [69].

2.3. MF59

While other vaccine delivery vehicles have been included in licensed vaccine formulations, MF59 is the only nano-sized vaccine *adjuvant* approved for human use thus far, although it is not yet licensed in the United States. MF59 is an oil-in-water emulsion composed of <250 nm droplets formed when squalene (4.3% v/v) and two surfactants, polysorbate 80 (0.5% v/v, Tween 80) and sorbitan trioleate (0.5% v/v, Span 85) are emulsified in citrate buffer [87,88]. The strong immunogenicity enhancement of MF59 is clearly seen in preclinical data published by Ott et al. [87]. They reported that guinea pigs showed a 34-fold increase in antibody titers when immunized with glycoprotein D of herpes simplex virus (HSV) type 2 in the presence of MF59 compared to aluminum hydroxide, while goat and baboon showed 9- and 5-fold increases, respectively [87]. The mechanism of adjuvanticity of MF59 is believed to be through direct stimulation of cytokine production [24,87–89].

Similar results have also been observed in the clinic. For vaccines against HIV, HSV and CMV, antibody titers measured in seronegative patients were often greater than those of infected, seropositive patients [87,90–92]. Additionally, strong helper T cell responses were also detected in seronegative patients as a result of the vaccination [87,89,91,93]. An MF59-adjuvanted influenza vaccine, Fluad®, licensed in Europe [24,93–95] as well as experimental vaccines for avian influenza A/H9N2 virus [96] and HBV [97] produced similar behavior in the clinic. Based on these and other studies, the safety, tolerability and adjuvanticity of MF59 in humans seem to be well established.

While these studies evaluated the vaccine delivered parenterally, another clinical study evaluated the immunogenicity of an MF59-adjuvanted flu vaccine administered intranasally (IN). This vaccine also was well-tolerated. The results indicated that a mucosal immune response may be generated upon IN administration, but this route may not be optimal for eliciting a humoral immune response [98]. This study, however, found no enhanced potency of the vaccine in the presence of MF59 compared to unadjuvanted vaccine when administered IN [98].

2.4. Immunostimulating complexes

Another vaccine delivery vehicle with potent adjuvant activity being studied in the clinic is the immunostimulating complex (ISCOM). These are ~40 nm cage-like particles

produced by combining a protein antigen, cholesterol, phospholipid and the saponin adjuvant Quil A, which is derived from the bark of the South American *Quillaia saponaria* Molina tree [99–101]. The matrix that is formed traps the protein antigens (typically hydrophobic membrane proteins) through apolar interactions [23,24,23,99,102,103]. A similar vaccine delivery vehicle and adjuvant has also been developed that uses the same material minus the antigen and is referred to as ISCOMATRIX® [104]. The antigen can be added later to the ISCOMATRIX® during formulation of the vaccine. This material seems to work similarly to ISCOMs, but provides for more general applications by removing the requirement for hydrophobic antigens [104].

A clinical study that compared a classical trivalent flu vaccine with an ISCOM adjuvanted version composed of the same three virus strains revealed a stronger immune response with the ISCOM vaccine eliciting rapid antibody responses as well as T helper and some CTL responses [105]. A separate study of an ISCOM based flu vaccine showed that virus-specific CTL memory was achieved in 50–60% of the patients, compared to only 5% who received the standard flu vaccine [106]. Additional ISCOM/ISCOMATRIX® vaccines have been in the clinic for HIV [102], HSV [102], HPV [100,107], HCV [100,108] and cancer (utilizing NY-ESO-1 as the antigen) [109]. In all cases, the studies have shown a good safety and tolerability profile in humans [106,107,110,111] as well as induction of both humoral and cellular immune responses [100,106,111]. Despite these successes, the actual use of ISCOMs in human vaccines has been deterred by concerns regarding safety since some saponins are toxic at elevated levels [102]. Nevertheless, certain saponins, such as Quil A and QS-21 have not shown major signs of toxicity in humans at the doses administered [102,106,111].

When administered IN in mice, flu ISCOM vaccines were found to elicit strong mucosal (IgG and IgA) responses as well as systemic and CTL responses [103,112]. A similar result was also observed in sheep [113] and baboons [104], but the titers were much lower than those detected in mice [104]. Oral administration of ISCOM vaccines has also been shown to be effective, but this route requires the use of high and frequent dosing [104,114]. A study in sheep also indicated that ISCOM vaccines may be able to elicit strong mucosal immune responses when administered in the pelvic presacral space, which could be useful for immunization against viral infections of the female genital tract [115].

2.5. Monophosphoryl lipid A

Monophosphoryl lipid A (MPL®) is an immunostimulating TLR-4 receptor agonist [116] composed of detoxified lipopolysaccharide (LPS) from *Salmonella minnesota* R595 [24,117]. LPS, a major component of the cell wall of Gram-negative bacteria, is a strong adjuvant, but is highly toxic [118]. Its toxicity has been attributed to the lipid A region of the molecule [24,119]. After detoxification, the resulting MPL® maintains adjuvanticity and is a versatile vaccine adjuvant that may be either included in aqueous formulations or in an oil-in-water emulsion for a more dynamic response [120].

A phase I study of an HIV vaccine in healthy volunteers showed high antibody titers similar to that of infected individuals for individuals administered the MPL®-containing vaccine, but these were not found to be neutralizing. Importantly, a large percentage of patients receiving the emulsion with MPL® in this study reported adverse events, suggesting that this adjuvant may not be well-tolerated under some circumstances [121].

Novel adjuvants/delivery vehicles containing MPL® have also been developed. For example, AS04 and AS02A are adjuvants developed by GlaxoSmithKline that consist of combinations of MPL® and either aluminum salts or QS-21, a purified component of the Quil A described above. These combinations have been in the clinic for a variety of vaccines, including ones for HSV, HBV, *Streptococcus pneumoniae*, malaria and HPV [24,116,117,122]. Furthermore, AS04 is now used in the European-licensed HBV vaccine, Fendrix® [24,122]. AS04 has been shown to enhance the humoral response (characteristic of aluminum salts) but also induces a strong Th1 cell-mediated response (characteristic of MPL®) [122]. Another combination adjuvant known as DETOX™ contains MPL® and *Mycobacterium phlei* cell wall skeletons in a squalene emulsion and has been used in the clinic for melanoma [123], ovarian cancer [124], breast cancer [125] and is included in the Canadian-licensed Melacine® for late-stage melanoma. Such MPL®-containing vaccine formulations have been found to enhance both cellular and humoral immune responses with minimal toxicity (depending on the antigen) relative to non-MPL® formulations [122,123,126].

2.6. Calcium phosphate nanoparticles

Nanoparticles can be generated by combining (while stirring) calcium chloride, sodium phosphate and sodium citrate [127,128]. Since calcium phosphate is naturally occurring in the body, issues surrounding the safety of these materials are reduced [129,130]. Not to be confused with the calcium phosphate gel adjuvant used in the European diphtheria–pertussis–tetanus (DPT) vaccine formulations [130,131], calcium phosphate nanoparticles are less than ~1.2 μm (<1000 nm according to He et al., 2000) in diameter [127,128]. BioSante Pharmaceuticals has been developing this "CaP" technology. A phase I study in healthy volunteers showed that CaP was safe and non-toxic when administered subcutaneously [132]. Preclinical studies indicated that vaccines containing CaP resulted in immune responses similar to or greater than those adjuvanted with aluminum salts, and the duration of the response was longer [128,133]. Additionally, CaP has shown promise as a mucosal adjuvant. Studies in mice utilizing an HSV-2 antigen suggest that CaP administered IN or intravaginally can elicit protective systemic and mucosal immunity [127]. Vaccines utilizing CaP in preclinical studies include anthrax, HBV, flu (H5N1 avian and seasonal) and HSV-2 [127,133].

2.7. Polymeric nanoparticles

A variety of polymers exist from which nanoparticles for drug delivery can be synthesized; however, the most commonly studied

polymers are poly(D,L-lactide-co-glycolide) (PLG) and polylactide (PLA) [134]. These biodegradable, biocompatible polymers have been approved for use in humans (e.g., as sutures, bone implants and screws as well as implants for sustained drug delivery) and have been extensively studied for use in the formulation of vaccine antigens (i.e., proteins, peptides, DNA, *etc.*) [134–136]. In these formulations, antigen can be either entrapped or adsorbed to the surface of the particles. Furthermore, these particles can be tailored to degrade over a range of rates [134]. They can therefore act as a depot from which the encapsulated antigen is gradually released [134]. Additionally, polymeric particles may offer protection to encapsulated antigens delivered orally and facilitate uptake by M-cells in the nasal-associated lymphoid tissue (NALT) when administered nasally, thus serving as a vehicle for mucosal immunization [134,137,138]. Adsorbed antigen, however, may offer improved stability and activity over encapsulated antigen by avoiding exposure to organic solvents used during formulation and acidic pH conditions caused by degradation of the polymer [139].

Preclinical studies have shown that PLG nanoparticles can induce systemic antibody titers comparable to those of aluminum salts [134]. Additionally, a study using tetanus toxoid (TT) found that a synergistic immune response (i.e., four-fold higher mean serum anti-TT IgG response) could be achieved by injecting TT bound to an aluminum salt along with TT-loaded nanoparticles [134,140]. Another study showed that PLG nanoparticles loaded with MPL® and a cancer-associated antigen (MUC1 mucin peptide) were efficiently taken up by dendritic cells [141]. These materials have not entered the clinic for vaccine applications, and therefore, will not be discussed in any greater detail here.

2.8. Non-degradable nanoparticles

In contrast to biodegradable nanoparticles, various non-degradable nanoparticles are being evaluated for their uses as vaccine adjuvants and delivery systems. Among the materials that are being examined are gold, latex, silica and polystyrene [16]. Since these materials may remain in the tissues for extended periods of time, it is thought that the antigen may be presented to the immune system over similar time periods thereby enhancing immunogenicity. Gold particles have been frequently described for vaccine delivery both with and without the aid of electroporation, which has been shown to often dramatically enhance the potency of DNA vaccines by improving delivery into cellular interiors [142]. Combining electroporation with intradermal delivery of DNA and gold particles, an enhanced and accelerated immune response has been observed in mice [143]; however, electroporation may not be applicable in a human clinical setting due to cell mortality resulting from the high-voltage electrical pulses [144]. A study in humans using these particles without electroporation produced a relatively low response rate after vaccination with DNA-gold particle granulocyte-macrophage colony-stimulating factor (GM-CSF) transfected autologous tumor cells [145].

An alternative approach to delivering DNA vaccines employing non-degradable nanoparticles is through particle bombardment, also referred to as particle-mediated epidermal delivery (PMED) or the "gene gun" approach [146,147]. This method involves ballistically firing the DNA-coated gold nanoparticles into the epidermis [147,148]. While the delivery efficiency of this technique is quite low, only small amounts of DNA are required to achieve a significant immune response [147,149]. Clinical trials have shown that this approach can elicit both humoral and cellular immune responses, making it one of the only consistently successful DNA vaccine delivery approaches currently available [146]. Success based on ballistic methods has been observed for vaccines against HBV, influenza and malaria, the latter of which involved a prime-boost regimen [146].

2.9. Liposomes

Liposomes, which are sometimes classified as VLPs, are spherical entities composed of a phospholipid bilayer shell with an aqueous core. For this review, liposomes are considered to be composed of non-viral lipids (i.e., lipids not obtained passively from host cells in viral budding processes). For vaccine delivery, an antigen (or adjuvant) may be either encapsulated in the core of the liposome, buried within the lipid bilayer or adsorbed on the surface for presentation to antigen presenting cells [16,150,151]. These delivery vesicles are considered to be non-toxic when the phospholipids used in their preparation are found in mammalian cells, but the lipids themselves are relatively non-immunogenic. Thus, for vaccine purposes, these particles are considered most useful for *delivering* antigens and adjuvants [16].

In contrast, liposomes can be made immunogenic by modifying the surface of the particle by adding a ligand [16], antigen [150] or another type of lipid. Nakanishi et al. demonstrated that cationic liposomes are much more potent than anionic or neutral liposomes for generating a cell-mediated immune response [152]. An interesting preclinical study in mice conducted by Guan et al. evaluated the effect of the liposome formulation on the type of immune response generated for a MUC1 therapeutic cancer vaccine. This study revealed that liposome-associated (either encapsulated or surface-exposed) MUC1 peptide (BP25) produced a strong specific CTL response; however, an antibody response was only observed for the surface-associated BP25 formulation [153]. Clinical studies have confirmed that L-BLP25 (also known as Stimuvax®, a lyophilized, liposomal formulation of BP25 lipopeptide, MPL® and three lipids [154]) is well-tolerated and elicits a cellular immune response in patients with lung cancer [155]. Stimuvax® is being developed by Merck and Biomira for treatment of non-small cell lung cancer (NSCLC), which accounts for ~80% of all lung cancers [156]. A phase IIB trial showed that the L-BLP25 vaccine increases survival rates for patients with smaller, nonmetastatic NSCLC tumors [154]. A phase III clinical trial is currently underway.

Despite there being a number of liposome-based products on the market in the U.S., there are currently no liposome-based vaccines [157]. Besides the NSCLC vaccine just described, additional liposomal vaccines that have been investigated in human trials include vaccines against malaria, HIV, hepatitis A, influenza [158], prostate cancer [159] and colorectal cancer [157]. These were all found to be safe and highly immunogenic [157–159].

2.10. Proteosomes

The most common forms of proteosomes used for vaccine applications are nanoparticles composed of the outer membrane proteins (OMPs) of *Neisseria meningitidis*. OMPs have been used successfully in a marketed meningococcal vaccine since 1981 and are considered non-toxic and well-tolerated [160,161]. Due to the hydrophobic nature of the OMPs, this immunogenic delivery system is appropriate for delivering apolar or amphiphilic antigens, and generally uses a non-covalent interaction between the proteosome and antigen to form the appropriate complexes [162].

These delivery vehicles have further been qualified as safe and well-tolerated materials through various human clinical trials. In most cases, these trials have involved IN administration of the vaccine. In several cases, a novel adjuvant known as Protollin™ consisting of proteosomes non-covalently complexed with LPS has been used [163]. For example, a vaccine composed of *Shigella flexneri* 2a LPS conjugated to proteosomes was found to elicit an immune response similar to that observed after immunization with the live pathogen [164]. Additionally, monovalent [165] and trivalent [162] influenza A/H1N1-proteosome (no LPS) vaccines administered IN produced high antibody titers in serum as well as in nasal secretions [162,165], suggesting that IN delivery of proteosome-based vaccines may be able to produce both systemic and mucosal immunity. Furthermore, preclinical studies have shown that such a vaccine is capable of protecting mice upon challenge with the infective pathogen [163,166–168].

Another very similar category of vaccines, which is probably more appropriately discussed elsewhere due to its non-particulate character, is the conjugate vaccine. These vaccines consist of a relatively non-immunogenic (especially in infants) antigen linked to a more immunogenic carrier such as a protein or toxoid [161]. The world's best selling vaccine, Prevnar, is an example of such a vaccine [169]. Prevnar is a pneumococcal vaccine manufactured by Wyeth consisting of the saccharides of the capsular antigens of seven serotypes of *S. pneumoniae* conjugated to mutant diphtheria toxoid CRM_{197} [170]. Additionally, the conjugate vaccines for *H. influenzae* type B (Hib) were developed using Hib polysaccharide conjugated to either diphtheria toxoid (PRP-D), OMP of *N. meningitidis* (PRP-OMP), mutant diphtheria toxoid CRM_{197} (HbOC) or tetanus toxoid (PRP-T) to render the Hib antigen immunogenic [171]. The meningococcal vaccine is another example of a conjugate vaccine. The quadrivalent vaccine manufactured by Sanofi Pasteur and marketed as Menactra® contains four meningococcal polysaccharides conjugated to diphtheria toxoid to enhance the immune response [160].

2.11. Montanide™

There are several different types of Montanide™, including ISA 50V, 51, 206 and 720. ISA 50V, 51 and 720 are water-in-oil emulsions while ISA 206 is a water-in-oil-in-water emulsion. ISA 206 and 50V have been used only in veterinary vaccine formulations while the other two are under investigation for use in humans [172]. Emulsions of Montanide™ ISA 51 and 720 are composed of a metabolizable squalene-based oil with a mannide monooleate emulsifier [173–175]. The ISA 720 formulation is slightly different from that of ISA 51 and permits antigens to be released more rapidly [24]. Similar to Incomplete Freund's adjuvant (IFA) in physical character, the biodegradable nature of the Montanide™ eliminates many of the cytotoxic properties of IFA [176]. The immune enhancement produced by the Montanide™ emulsions is believed to be due to the formation of a depot at the site of injection [173].

ISA 51 and 720 have both been shown to induce high antibody titers and CTL responses in a variety of animal species [176–179]. In many cases, the response was greater than that achieved using other types of adjuvants [85,173,173,176,179]. These emulsions have been in phase I and/or II clinical trials for vaccines against malaria, HIV and various cancers [173–175,180–184]. In most cases, the vaccines were found to be safe and fairly well-tolerated. A phase I trial of a trivalent malaria vaccine containing ISA 720 induced both humoral and cellular immune responses [182]. Other trials of a synthetic malaria peptide vaccine, a Wilms' tumor protein vaccine against various malignancies and a melanoma vaccine containing either ISA 51 or 720 all showed strong CTL responses in humans [180,184,185].

2.12. Cholesterol-bearing hydrophobized pullulan nanoparticles

Cholesterol can be conjugated to a variety of carbohydrates, including pullulan, dextran and mannose, rendering the molecules amphiphilic. Such molecules have been shown to self-assemble with and without proteins into 30–40 nm, colloidally stable nanoparticles [186–189] whose size and density can be modified by altering the degree of substitution of cholesterol groups on the polysaccharide [189]. Pullulan is the most popular polysaccharide to which cholesterol has been conjugated, with numerous reports published for studies conducted *in vitro* but only a single one in humans.

Currently, there is only one report of cholesterol-bearing hydrophobized pullulan nanoparticles (CHP) being evaluated in the clinic. A complex of CHP and NY-ESO-1 was shown to enhance the humoral immune response. In this study, the cellular immune response was not evaluated due to seropositive patients possessing activated CD8 T cells [190]. Previously, an *in vitro* study showed that dendritic cells loaded with CHP/NY-ESO-1 complexes induced both CD8 and CD4 T cells [188]. A preclinical study in mice showed that immunization with a complex of the HER2 oncoprotein and CHP induced both humoral and CD8 responses [191]. In all studies, vaccination with CHP seems to be both safe and well-tolerated [190,192].

3. Summary, conclusions and future challenges

Novel vaccine adjuvants and particle-based delivery vehicles are being evaluated in a variety of vaccines, including those against diseases such as cancer, malaria, AIDS, hepatitis, *etc.*, in which a cellular and/or mucosal immune response is desired. In these cases, a humoral response, which may be attainable with the use of aluminum salt adjuvants, may not be sufficient for generating

protective immunity. Clinical studies of various nanoparticulate immunopotentiators and antigen delivery vehicles have shown CTL responses for viral vectors, ISCOMs and Montanide™ ISA 51 and 720. Th1 responses have been elicited for MF59 and MPL®. Additionally, cellular immune responses have also been generated in humans using VLPs, virosomes, non-degradable nanoparticles and liposomes. The breadth of carriers that has shown this desirable response shows promise for the development of new and improved vaccines of a wide variety of types.

Also encouraging are the numbers of vaccine delivery components and adjuvants that are being pursued for alternative routes of administration and the mucosal immune response many have shown. Viral-vectored vaccines as well as proteosomes given IN have shown to be capable of producing systemic antibodies in humans. Moreover, a VLP vaccine administered orally elicited humoral, cellular and mucosal immune responses in the clinic. Preclinical studies have also demonstrated mucosal immunity for IN administration of ISCOM and CaP vaccines as well as for intravaginal delivery of a CaP vaccine.

So, why have only a few of these particle-based vaccine delivery systems made it to the market? The most probable reason is that the clinical trials required for vaccine approval are often very long and difficult. Unlike preclinical studies in which animals can be challenged with the infectious pathogen following immunization to evaluate whether the vaccine is protective, human trials often require waiting for an outbreak before protection can be analyzed. Furthermore, since many vaccines are often administered to healthy individuals, and frequently to infants, it is critical that they are proven safe and well-tolerated in non-human primates before entering human trials.

While the development of novel vaccine delivery systems and adjuvants has been aided by nanotechnology, this field has also resulted in some concerns regarding the toxicity of such small particles. Among perceived potential problems are their high surface area and reactivity, the ability of such small particles to cross biological membranes and the slow biodegradability of some materials [193,194]. Such arguments are supported by data describing the effects of pollutants on human health [138,193–195]. Despite these issues, it seems reasonable to anticipate that in the case of vaccines, the infrequent and low-level exposure to nanoparticles that an individual will encounter during immunization is not enough to cause adverse health problems such as those potentially attributed to nanotoxicity effects. With that being said, the development of any novel vaccine adjuvant or delivery platform, like any other pharmaceutical product, must prove its safety and tolerability before its approval.

Many challenges must be met before new classes of vaccines become available. Ideally, an adjuvant or delivery vehicle will have the ability to stimulate humoral, cellular and mucosal immune responses concurrently or discretely, depending upon the desired treatment strategy. The duration of the response should be long and the vaccine components should be easily metabolized by the body. An additional challenge includes developing alternative, less invasive approaches for the administration of vaccinations. Perhaps most importantly, the cost of producing and distributing new vaccines should remain moderate such that the benefit is available to everyone at risk including persons in less developed parts of the world. As these challenges are met, the prevention and therapy of many previously untreatable diseases should become increasingly possible.

4. Note added in proof

Recent clinical trials of an adenovirus-based HIV vaccine by Merck proved unsuccessful. Furthermore, there was some evidence that vaccinated subjects previously exposed to adenovirus showed a higher incidence of infection by HIV [HIV vaccine failure prompts Merck to halt trial, Nature 449 (2007) 390.] [H. Ledford, HIV vaccine may raise risk, Nature 450 (2007) 325.]

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