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Nano-microparticles as immune adjuvants: correlating particle sizes and the resultant immune responses

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Abstract

The development of novel immune adjuvants is emerging as a significant area of vaccine delivery based on the continued necessity to amplify immune responses to a wide array of new antigens that are poorly immunogenic. This article specifically focuses on the application of nanoparticles and microparticles as vaccine adjuvants. Many investigators are in agreement that the size of the particles is crucial to their adjuvant activities. However, reports on correlating the size of particle-based adjuvants and the resultant immune responses have been conflicting, with investigators on both sides of the fence with impressive data in support of the effectiveness of particles with small sizes (submicron) over those with larger sizes (micron) and vice versa, while other investigators reported data that showed submicron- and micron-sized particles are effective to the same degree as immune adjuvants. We have generated a list of biological, immunological and, more importantly, vaccine formulation parameters that may have contributed to the inconsistency from different studies and made recommendations on future studies attempting to correlate the size of particulate adjuvants and the immune responses induced. The information gathered could lead to strategies to optimize the performance of nano-microparticles as immune adjuvants.

Keywords

adjuvants; immune responses; microparticles; nanoparticles; particle size; vaccine formulation

The increasing attention on vaccine development is greatly justified based on the continuous emergence of deadly pathogens that are difficult to manage [1]. Over the years, it has become clear that vaccination is an effective and affordable measure to treat and prevent diseases/infections, which is achieved by the activation of innate, nonspecific defenses and the subsequent development of adaptive immune responses to fight intruding pathogens [2–4]. In order to ensure the quality and quantity of immune responses, it is fundamentally important that the immune systems are presented with antigens (from the pathogens in questions) at the right location and amount [5,6]. Ideally, the goal of vaccination is to ensure the production of strong and lasting immune responses after a single dose of antigen without the need for booster

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doses [7,8]. Success from recombinant DNA technology has afforded the production of antigens that are well preferred over traditional antigens based on safety reasons [4,9]. Traditional antigens obtained from whole or part of live, attenuated or killed pathogens are highly immunogenic, but can potentially pose health hazards when applied in immune-compromised individuals or if the antigens revert to virulent form [6,10]. A limiting factor with antigens made from recombinant DNA technology is that they are often weakly immunogenic on their own and require the inclusion of an immune adjuvant to enhance the resultant immune responses [11–13]. An immunologic adjuvant is defined as any substance that acts to accelerate, prolong or enhance antigen-specific immune responses, but is not immunogenic itself [14, 15]. Clinically, the list of approved adjuvants is very limited. For decades, aluminum hydroxide or phosphates (alum) remained as the only approved adjuvants in the USA [16]. In late 2009, the US FDA approved the Cervarix® vaccine (GlaxoSmithKline Plc; Middlesex, UK), which contained both aluminum hydroxide and AS04 (3-*O*-desacyl-4′-monophosphoryl lipid A) as adjuvants. Although alum has a long track record of safety, it only improves the induction of humoral immune responses, and does not help cell-mediated immune responses. As such, there have been tremendous efforts to develop alternative adjuvants [8,9,17–20]. Materials that have been investigated as immune adjuvants may be divided into immune potentiators (e.g., mineral salts, immunostimulatory compounds, microorganism-derived compounds and poly-saccharides) and vaccine-delivery systems (e.g., particulates and liposomes) [14,21,22]. In this article, we have focused on particles with diameters in the nanometer or micrometer ranges that have been investigated as potential immune adjuvants [5,12,23].

In general, the performance of particulate carriers as vaccine adjuvants in the literature has been attributed to a number of functions, which include the following:

- Particulate carriers can serve as an effective antigen delivery system and, thus, enhance and/or facilitate the uptake of antigens by antigen-presenting cells (APCs) such as dendritic cells (DCs) or macrophages [24,25];
- Particle-based antigen carriers may serve as a depot for controlled release of antigen, thereby increasing the availability of antigens to the immune cells. It has been reported that extended antigen release may enhance not only the level, but also the quality of immune responses [26,27];
- Particle-based adjuvants may possess the ability to modulate the type of immune responses induced when used alone or in combination with other immunostimulatory compounds [22];
- Particulates have the ability to protect the integrity of antigens against degradation until delivered to the immune cells [28]. This is particularly important in oral vaccine formulations where antigens must be protected from the harsh acidic conditions of the stomach and enzymatic degradation in the GI tract [29]. However, it is important to caution that the right balance must be maintained between antigen protection and antigen release. Entrapment of the antigen of interest within the particle matrix may achieve satisfactory antigen protection, but the entrapped antigen may not be released at the right time, concentrations or location, which could lead to a weak immune response [15,30];
- Particulate vaccines can potentially cross-present antigen, and antigen cross-presentation is especially important to generate $CD8^+$ T-cell responses against viral infections [31,32].

Considering the potential effectiveness of particulate-based immune adjuvants, a close review of literature in the field has shown areas of improvement or optimization if particulate-based adjuvants are to be used in vaccines. It is well reported that formulation and process parameters, such as particle size, methods of antigen loading and surface properties (e.g., surface charge),

play important roles in influencing the activity of particle-based adjuvants [11,24,33]. However, there have been discrepancies from various studies on the nature of the influence of formulation and process parameters on the resultant immune response [26,34,35]. Taking the particle size as an example, reports on the correlation of sizes of particle-based adjuvants and the resultant immune responses have been conflicting, with investigators on both sides of the fence reporting excellent data either supporting the effectiveness of small particles over larger particles and vice versa [36,37]. Other investigators presented equally convincing data showing that submicron-sized and micron-sized particles were effective to the same degree as immune adjuvants [35,38,39]. The lack of consistency will bring into question the practicality and feasibility of potential clinical applications of particle-based immune adjuvants. Since the size of the particulate adjuvants is a central parameter, we have attempted to focus on the extent and nature of the effect of the size of particulate adjuvants on the resultant immune responses and offered a few possible reasons that may help in explaining the rather conflicting data in the literature. It is hoped that the information gathered will assist in the preparation of optimized vaccine formulations using reproducible processes so as to achieve and sustain the production of strong immune responses as desired. Application of standardized (optimized) vaccine formulations will hold great promise in achieving the translation of newly developed vaccine formulations from bench to clinic.

Desired qualities of an ideal particle-based vaccine

Many investigators in the field are beginning to share the opinion that the success of vaccination is not only dependent on the nature of vaccine immunogens but also on the delivery system [24,40,41]. In order to design better vaccines and realize the full potential of particulate adjuvants, some of the ideal qualities of a good vaccine formulation are listed:

- The vaccine formulation must be safe and easy to administer;
- The vaccine formulation should be capable of eliciting the desired immune responses, humoral, cellular or both, after a single dose without the need for a booster dose(s);
- The vaccine preparation process should be simple, affordable, reproducible and easy to scale up. In this respect, it is important that all the components are commercially available, safe, affordable and nontoxic;
- The vaccine formulation should be stable with respect to size, surface morphology and size distribution throughout the process of preparation, storage and administration;
- The antigen should be chemically and physically stable throughout the process of antigen loading. There also should not be premature release/leakage of antigen;
- The vaccine preparation process should be amenable to secondary processes, which may include sterilization, drying (such as lyophilization, spray drying or vacuum drying), packaging and reconstitution of the dried powder. These processes should not distort the original particle size and size distribution of particulate vaccine formulations.

Nano-microparticles as immune adjuvants

Examples of materials that have been used to prepare nano-microparticles as vaccine-delivery systems include polymers [42], copolymers [43] and lipids [44–46]. The choice of material in particle preparation is guided by many factors, such as biocompatibility, degradation rate, hydrophilicity or lipophilicity, and polarity. The effects of these factors can be grouped into two sections pertaining to the properties of resultant particles and the induced immune responses. The effects relating to properties of particles themselves will encompass the following properties: the size, stability, antigen loading and antigen-release kinetics [42], while

the effects on the induced immune responses will include factors such as antigen stability, antigen release, particle interaction with APCs, antigen presentation and processing by APCs [47]. Polymers that have been used in the preparation of particles include, but are not limited to, poly(lactic acid) (PLA), poly(ortho esters) and the copolymer poly(lactic-co-glycolic acid) (PLGA), bioeliminable polyethylene glycol [48], and polyphosphazene [49]. In addition, a good number of natural polymers have been used in vaccine candidate formulations, such as albumin, gelatin [50], collagen, chitosan and alginate [51]. The attractiveness of some of these polymers in making particulate immune adjuvants is that they are biodegradable or biocompatible polymers with the US FDA's approval for human use in suture material or in drug-delivery systems [52]. Solid lipid nanoparticles prepared with materials such as emulsifying wax [44,45] or lecithin-glyceryl monostearate have also been explored [46]. A number of techniques have been employed to prepare particles for application as immune adjuvants, including emulsification/solvent evaporation, spray drying, coacervation and (micro)-emulsification [53,54]. Although the best possible protection to antigens is offered when antigens are entrapped within particles [30], a limiting factor is that antigens added during the process of particle formation may be potentially unavailable upon administration (i.e., poorly released), and they are also subjected to physical or chemical degradation in the entrapment process [37]. In this respect, other investigators have applied antigen adsorption or conjugated antigen to particle surfaces [11,46,55]. Irrespective of the process employed to make particles, it is important to pay close attention to important factors relating to formulation and that will influence the performance as immune adjuvants.

Comparison of microparticles & nanoparticles as immune adjuvants

Correlating size of particulate adjuvants with the resultant immune responses

Many investigators have used the terms 'nanoparticles' and 'microparticles' interchangeably in the literature to describe various particles that have been used as vaccine adjuvants [37, 56]. Theoretically, nanoparticles are solid particles ranging in size from 1 to 1000 nm (1 μm) while microparticles are particles that have sizes that range from 1 to 1000 μm [57]. For all practical purposes pertaining to targeted-delivery systems, small-sized particles are considered more effective than large-size ones [58]. This is because, compared with large-sized particles, small-sized particles are more efficient in permeating biological barriers, passing through capillaries after injection and achieving stability in blood circulation [59]. Thus, in targeted drug delivery, nanoparticles with a diameter of 100 nm or less are preferred [58,60,61]. However, for vaccine delivery, reports from studies are conflicting as to the optimum size ranges that will generate stronger and lasting immune responses [62]. A few examples are summarized in Table 1 and briefly discussed.

For instance, using bovine serum albumin (BSA) as a model antigen entrapped into particles of different sizes (200, 500 and 1000 nm) prepared with PLGA, Gutierro *et al.* reported that the 1000-nm particles elicited a stronger serum IgG response than the 500- or 200-nm nanoparticles [36], and the immune response induced by the 500-nm particles was similar to that induced by the 200-nm particles by subcutaneous and oral routes [36]. Similarly, Kanchan and Panda reported that the hepatitis B surface antigen (HBsAg) entrapped in PLA particles of 2000–8000 nm induced a stronger anti-HBsAg antibody response than HBsAg entrapped in PLA particles of 200–600 nm [63]. On the contrary, data from other studies showed that the adjuvant activity of small nanoparticles was more potent than that of the large particles. For example, Jung *et al.* studied the effect of particle size on the immune responses induced by tetanus toxoid (TT) adsorbed onto particulates prepared from sulfobutylated poly(vinyl alcohol)-graft-PLGA and showed that small particles of 100 and 500 nm induced significantly higher antibody titers then larger ones (>1000 nm) after oral (p.o.) or intranasal (in.) administration [37]. Yet, Wendorf *et al.* reported that comparable levels of immune responses were induced in mice by protein antigens (Env from HIV-1 or MenB from *Neisseria*

meningitidis) adsorbed onto anionic microparticles (~1 μm) and nanoparticles (110 nm) prepared with PLGA [39]. Others, however, suggested that there may be an optimal particle size in order to induce the strongest immune response (Table 1). For example, Fifis *et al.* conjugated ovalbumin (OVA) as a model antigen onto solid polystyrene beads of different size (i.e., 20, 40, 100 and 500 nm, and 1 and 2 μm) and reported that the particles of 40 nm were ideal in inducing both antibody and cellular immune responses when intradermally administered to mice [55]. Therefore, it was concluded that particles of 40–50 nm were ideal as immune adjuvants. Even for microparticles, there are reports showing that after a single-point intramuscular immunization with TT entrapped in different-sized microparticles prepared with PLA [34], antibody titers from particles in the size range of 2–8 μm were the highest, whereas 50–150-μm particles and particles with a size less than 2 μm generated weaker antibody titers [34]. Finally, data from other studies reported considerable antibody responses even from large microparticles of 10–90- and 15–60-μm size ranges [34,64,65].

To add another layer of complexity, it is becoming evident that the size of the particulate adjuvants may have different effects on the type of immune responses induced. There are data showing that microparticles promote humoral immune responses, whereas nanoparticles may favor the induction of cellular immune responses [7,63,66]. For example, data from Caputo *et al.*'s study showed that HIV TAT protein adsorbed on cationic polymeric nanoparticles of 220 or 630 nm induced a stronger TAT-specific cellular immune response and a weaker anti-TAT antibody response than the same TAT protein adsorbed on large microparticles (>2 μm) prepared with the same materials [66]. Similarly, using PLA particles with HBsAg entrapped inside, Kanchan and Panda showed that a single-point immunization with nanoparticles (200–600 nm) induced a lower antibody titer in comparison to microparticles (2–8 μm) [63]. Immunization with the 200–600-nm particles favored T-helper (Th) type 1 immune responses, whereas immunization with the 2–8-μm particles favored Th2 responses [63]. It was reasoned, with supporting data, that the nanoparticles (200–600 nm) can be efficiently taken up by APCs, such as macrophages, to induce cellular immune responses, whereas the macrophages cannot take up the large microparticles. Instead, microparticles simply attach to the surface of the macrophages and release the entrapped antigens. The macrophages then take up the antigens directly. On the contrary, data from Mann *et al.*'s recent study using oral bilosomes with influenza A antigens showed that the larger bilosome particles (400–2000 nm) elicited an immune response that was significantly biased towards Th1 rather than the smaller bilosomes (10–100 nm) [67]. However, data from Gutierro *et al.* using BSA entrapped into PLGA particles of 200, 500 and 1000 nm indicated that differences in the total serum IgG response induced by particles of different sizes do not result in differences in the IgG1- or IgG2a-type immune responses [36]. Overall, with the conflicting data, it is difficult to achieve an accurate prediction of particle size ranges that will dictate a Th1 or a mixed Th1/Th2 immune response outcome.

Biological & immunological parameters that may be responsible for the effect of particle size on the resultant immune responses

The influence of particle size on the type, level and quality of the immune response may be ascribed to differences in pathways and mechanisms for cell uptake, and antigen presentation and processing [24,56,68,69]. The publication by Xiang *et al.* is a good starting point for initial discussion, where it was reported that virus-sized particles in the size range of 20 to 200 nm are usually taken up by endocytosis, resulting in a cellular-based immune response. It was also explained that particles with sizes between 500 nm and 5 micron are mainly taken up by phagocytosis and/or macro-pinocytosis and are more likely to promote a humoral immune response [62].

Investigators that are in support of a potential superiority of small-sized particles over large-size particles have offered a number of explanations, which include:

- The expectation that antigen delivery across the mucosal surface will warrant permeability via biological barriers and nano-sized particles will be the most effective [70];
- Particles in the submicron size range are expected to be taken up efficiently by the APCs [24];
- The smaller the particle size, the larger the surface area for antigen loading [35].

Conversely, there was also outstanding evidence that demonstrated that large-sized particles are much more effective. One viewpoint was that the increased uptake of small-sized particles into APCs could be negated if the uptake is closely followed by extensive exocytosis [71], although the process of exocytosis is applicable to particles having sizes in both submicron and micron ranges. Another view point in favor of large particles as immune adjuvants was that large particles are preferentially attached to the macrophage surfaces, thus serving as an effective depot system for continuous antigen release [63,72]. Effective interaction with APCs may not be feasible in cases where the dimensions of the particles are much larger than a typical APC, such as in cases of particle sizes ranging from 50 to 100 μm [34]. Taken together, it is apparent that many factors that are involved in achieving strong and lasting immune response are interconnected, encompassing parameters relating to vaccine formulation, process of preparation, route of administration, antigen presentation and antigen-processing mechanisms.

Many investigators have shown that the entrapment of antigen in particles alters its acquisition and processing by APCs [26]. Targeting the APCs as a means to amplify, control and mediate the immunological consequences of prophylactic and/or therapeutic vaccines has been strongly propelled by encouraging results from *ex vivo* loading of DCs with antigen [61,73]. APCs are of critical importance to the transport of antigen from the periphery to local organized lymphoid tissues [74]. It is largely believed that the manner in which antigen reaches the lymph organs is crucial to the induction of the immune response [75]. Considering antigen presentation and processing, particulate adjuvants will influence the resultant immune responses in many ways, itemized as:

- Particulate form of antigen will enhance antigen uptake by APCs and subsequently the delivery of the antigen to lymphoid organs [24,68];
- Based on their size, particles taken up by APCs could rapidly escape from the endolysosomal compartment to the cytosol, thereby supporting the generation of $CD8^+$ T-cell responses [55,76];
- Antigen-loaded particles could serve as a depot for constant antigen release to the APCs. This function will be dependent on the type of particulates (whether they are erodible or not), surface properties, biocompatibility (to facilitate interaction with APCs), size (in comparison to the size of typical APCs), antigen stability and release kinetics [26,63].

It has been reported that particles with a diameter of 500 nm or less are optimal for uptake by DCs or macrophages [77]. Particles of 20–200 nm are generally taken up via endocytosis with subsequent inducement of CD4 and CD8, and Th1-type immune responses [26]. For particles with a dimension greater than 500 nm, uptake is via phagocytosis or micropinocytosis, leading to a humoral immune response [77]. A number of reports have shown that biodegradable microparticles whose sizes allow them to be phagocytosed (size <10 μm) achieved prolonged antigen presentation to APCs [78,79]. Using polystyrene particles, Sharp *et al.* showed that particles of 430 nm and 1 μm in size were efficiently taken up by DCs, whereas there was limited uptake of 10-μm particles, and no uptake of particles of 32 μm [56]. In addition, Balasse

et al. demonstrated phagocytosis of hyroxyethyl starch particles of 4–15-μm size range (average size 8.3 μm) loaded with BSA [79]. In addition to facilitating antigen uptake by APCs, the ability of particulate adjuvant to drain freely into the lymph nodes is highly desirable [80]. In this respect, it was shown that particles with small sizes of 20–200 nm can freely drain to the lymph nodes for antigen presentation, whereas large-sized particles (0.5–2 μm) made of the same materials were mostly dependent on DCs for transport to the lymph nodes [81].

Possible formulation factors that contributed to the lack of consistency in the relationship between the adjuvant activity of particles & their size in literature

In addition to the biological and immunological parameters mentioned previously, various other formulation parameters are expected to affect the relationship between particle size and the adjuvant activity of the particulates. The formulation parameters can be in multiple folds, which may include, but are not limited to, materials used to prepare the particulates, nature of the antigens, method of antigen loading, particle size uniformity and distribution, and route of vaccine administration. It is important to emphasize that the effects of all aforementioned parameters are closely linked and should be viewed and evaluated in combination.

Materials used to prepare particulates

The material used to prepare particulate adjuvants is an important factor to consider in achieving reproducible immune responses. This is particularly important for materials that have adjuvant activity themselves. For example, ionic polyphosphazene was shown to have immune adjuvant activity [49]. The adjuvant activity of polyphosphazene appeared to be linked to its ability to form water-soluble noncovalent complexes with the antigen, which may enhance their interaction with APCs [49,82]. Moreover, recent evidence suggested that polyphosphazene activated innate immune cells to secrete IL-4 and IL-12 [82], and the cytokines may mediate the initiation of adaptive immune responses. Therefore, similar-sized particles prepared with polyphosphazene or other polymers, such as PLGA, may have different adjuvant activities.

Antigen & antigen dose

Another potential source of discrepancy is the variability that could arise from the nature of antigens, dose of the antigen and dosing frequency used in different studies even if other parameters pertaining to the immune adjuvants were kept constant. Considering plasmid DNA vaccines, parameters that may affect the performance may include the antigens encoded by the plasmids, the size of the plasmids, the amount of CpG motifs on the plasmids and the purity of the plasmids. There are even more variables that may be introduced by different protein antigens, which include the intrinsic immunogenicity of the proteins, size of the proteins, purity of the proteins and level of endotoxin. Many researchers used model antigens such as OVA, BSA, HBsAg, TT and HIV Tat to evaluate the adjuvant activity of the nanoparticles or microparticles. It is important that the level of endo-toxin in the antigen preparations is determined. Moreover, the intrinsic immunogenicity of the antigens used to evaluate the adjuvant activity of the nano-microparticles should be taken into consideration. For example, data in our previous studies showed that OVA as an antigen conjugated onto the surface of lecithin-based solid lipid nanoparticles induced stronger anti-OVA IgG responses in mice than when the OVA was adjuvanted with aluminum hydroxide [46]. However, when the OVA was replaced with the highly immunogenic *Bacillus anthracis* protective antigen (PA) protein, the PA-conjugated nanoparticles and the PA adjuvanted with aluminum hydroxide induced similar levels of anti-PA IgG in mouse serum samples after three doses [46]. It was interesting that when measured after a single injection, anti-PA IgG antibodies were detectable only in mice that received the PA-conjugated nanoparticles, but not in mice that received the PA adjuvanted

with aluminum hydroxide [46]. Therefore, it is possible that when different antigens are used, one may draw different conclusions regarding the effect of particle size on the adjuvant activity of nano-microparticles. Also, it can be inferred from the aforementioned study that if a highly immunogenic antigen is dosed multiple times, one may find that within a certain range, particle size does not have any detectable effect on the adjuvant activity of particulate adjuvant [46].

Methods of antigen loading

At least three different methods have been used to load antigens of interest onto particulates. These include: entrapment, surface chemical conjugation and surface physical adsorption. In many studies, the antigens were entrapped into the particles [34,63,70]. However, antigens can also be chemically conjugated onto the surface of preformed particulates [46,55] or simply adsorbed onto the particle surface [37]. Entrapment of the antigens into the particulates could protect antigens from degradation. However, the release of antigens from the particulates could be problematic if not controlled well. In addition, the entrapment process could potentially cause chemical or physical damage to the antigens to be entrapped. Surface chemical conjugation is likely to provide less protection and the extra step of conjugation may not be ideal, and chemical conjugation may make certain critical epitopes on the antigens unavailable for presentation. The attractiveness of the physical adsorption of antigens onto particles is that it requires simpler processing. Similar to the use of alum suspension as an adjuvant, it will be convenient if the antigen of interest can be simply mixed with the particles before being given to the host. In fact, depending on the antigen and the particulate system used, there were data suggesting that plasmid DNA vaccine adsorbed on PLGA particles was more effective than when it was entrapped into the PLGA particles [83,84]. In addition, there were data showing that when a protein antigen was conjugated onto the surface of the particulates, it induced a much stronger immune response than when the same antigen was simply physically mixed with the same particulates [55]. Using OVA conjugated onto *N*-trimethyl chitosan (TMC–OVA), Slütter *et al.* recently showed that surface conjugation of antigen to particles will ensure that both the antigen and particles reach the APCs at the same time [28]. Mice immunized with TMC–OVA conjugate produced 1000-fold higher OVA-specific IgG titers than mice immunized with the physical mixture of TMC and OVA. It is important to note that antigens on the surface of the particles could stabilize or destabilize an otherwise unstable or stable particle suspension. For instance, particle aggregates and/or agglomerates after antigen conjugation were reported by Kalkanidis *et al.* [11]. Overall, the sizes of the particles before and after the antigen conjugation or adsorption should be monitored and reported.

Particle size distribution &/or uniformity

The wide size range of particulate adjuvants used in various studies could also be a potential source of variability. Just to list a few examples, Tabata *et al.* used OVA-loaded PLGA particles with average diameters of 600 nm and 1, 4, 7, 11, 15, 21 and 26 microns [70]. When given orally, the 4-micron particles induced the best serum antibody response. Katare *et al.* used TT-entrapped PLA particles of four different groups, less than 2 microns, 2–8 microns, 10–70 microns and 50–150 microns, and showed that the 2–8-micron particulates induced the strongest serum antibody response after a single-point intramuscular injection [34]. In another study from the same group, Kanchan and Panda used HBsAg-entrapped PLA particles of 200–600 nm and 2–8 microns, and reported that the 2–8-micron particulates induced a stronger antibody response, whereas the 0.2–0.6-microns particulates induced a stronger cellular immune response [63]. Mann *et al.* used biolosomes of 400–2000 nm [67]. Borges *et al.* reported that high polydispersity index is one of the major drawbacks in the application of chitosan particles generated by precipitation with sodium sulfate [85]. It is understandable that it is not easy to prepare particulates with high size uniformity, but it is possible that the immune system will not be able to differentiate particles of 1-, 4- and 7-micron sizes. Since particles of sizes 200 and 600 nm may have different adjuvant activities, it is likely that the size range

in the aforementioned studies [63,67] of 400–2000 nm as well as 200–600 nm is too wide. To substantiate these points, it was shown that particulates of 20–200 nm can freely drain to local draining lymph nodes, whereas DCs were required for the transport of particles larger than 500 nm (0.5–2 microns) from the site of injection to the lymph nodes [81]. Moreover, it was reported that particulates of 500 nm or less were optimal for uptake by APCs [86]. Therefore, it is logical for one to reason that 500 nm should be used as a potential cutting point, with particles smaller than 500 nm and larger than 500 nm having totally different vaccine adjuvant activities. Fifis *et al.* used commercially available polystyrene beads of 20, 40, 100, 200 and 500 nm, and 1 and 2 microns [55]. OVA as a model antigen was chemically conjugated onto the beads and intradermally injected into mice. Their data showed that the 40-nm beads induced the strongest immune responses, suggesting that within the size range of 20 nm to 2 microns, the 40-nm size was optimal. For many reasons, we agree with the choice of particles and size ranges used by these investigators [55]. Firstly, the polystyrene beads were quite uniform in size (Polysciences Inc., Eppelheim, Germany). Secondly, beads of smaller than and larger than 500 nm were evaluated independently and, more importantly, the inclusion of 20-, 40- and 100-nm beads allowed the evaluation of adjuvant activities of particles with a diameter less than 100 nm. Unfortunately, in a subsequent paper from the same group of investigators, it was reported that the size of the OVA-conjugated 40–50-nm polystyrene beads was actually 232 nm with a polydispersity index of 0.384 when measured using a dynamic light scattering (DLS) particle sizer [11]. Therefore, it was the 232-nm OVA–polystyrene beads that actually induced the strongest immune responses [55]. Since it captures the hydrodynamic size of particles, the diameter measured from the DLS particle sizer may more closely resemble the size of the OVA–polystyrene beads that the immune cells have encountered. In that sense, it is encouraged that the size measured by DLS for particles dispersed in medium containing normal saline or serum proteins should be reported in all future studies.

Moreover, potential particle instability that can alter particle adjuvanticity could arise at different stages during particle preparation and application, such as during antigen loading, particle storage and upon particle contact with biological fluids. In addition to affecting antigen integrity, loading efficiency and release kinetics, particle instability could lead to changes in particle sizes involving an increase in sizes (as in particle aggregation/agglomeration and fusion) or decrease in sizes (as in polymer degradation). Kalkanidis *et al.* showed that vaccine formulations can undergo various degrees of aggregation during storage depending on the pH of the dispersing medium [11]. Therefore, it is important for investigators in the field to include studies on potential particle instability that can occur after storage and when dispersed in biologically relevant conditions. In other words, the physical stability or instability of the particles as an adjuvant before and after administration should be seriously considered when designing particle-based vaccine adjuvants.

Route of administration

It is known that the route of vaccine administration plays a significant role in shaping the induction of immune responses. In., p.o., intramuscular and subcutaneous (sc.) antigen delivery may encounter different subsets of DCs based on localization that may dictate the type, quantity and quality of immune responses [79]. For example, Newman *et al.* demonstrated that particles of the same size when dosed via different routes were taken up by different APCs, leading to different immune response [69]. Specifically, it was observed that intraperitoneal (ip.) immunization was associated with a predominant uptake of microparticles by macrophages in the peritoneal cavity, while intradermal immunization resulted in the uptake of microparticles by DCs [69]. In general, immunization by injection is most popular. However, considerable progress has been made with mucosal immunization over the years [87]. The following examples illustrate that the route of administration plays a significant role in the observed effect of the size of the particles on the resultant immune responses.

Using particles prepared with sulfobutylated poly(vinyl alcohol)-graft-PLGA with TT adsorbed, Jung *et al.* compared the serum anti-TT IgG titers induced by the particles of approximately 100, 450 and 1500 nm after p.o. or in. administration [37]. They reported that for the 450-nm particles, the in. route induced a stronger serum anti-TT IgG response than the p.o. route. However, for the 100-nm particles, the in. route was only slightly better than the p.o. route. Finally, for the 1.5-micron particles, the p.o. and in. routes did not differ, and only a very weak anti-TT IgG titer was observed as compared with that induced by the 100- and 400-nm particles.

In a study by Nakaoka *et al.*, PLA microparticles containing OVA were prepared [88]. The resultant microspheres were fractionated into six different sizes ranging from 3.4 ± 2.2 to 50 μm. Microparticles were applied intraperitoneally or subcutaneously. It was observed that ip. immunization was much more influenced by the microsphere size, and higher serum levels of anti-OVA antibodies were induced when microparticles of small sizes were used. By contrast, sc. immunization showed comparable levels of serum antibodies with microparticles at all size ranges, indicating that the sc. route was not affected (or less likely affected) by changes in size. The investigators explained that there was the likelihood that particle instability occurred with sc. immunization [88]. Potential particle instability after injection leading to particle aggregation and/or agglomeration will most likely negate the influence of administering particles at various size ranges, further supporting the need for investigators to monitor the stability of the particulate vaccine formulations in simulated biological medium before injecting them in animals.

In a study by Gutierro *et al.*, BSA was entrapped in particles of different sizes (200, 500 and 1000 nm) prepared from PLGA [36]. The particles were given either intranasally, orally or subcutaneously to BALB/c mice, and the serum IgG response elicited was compared. It was shown that the 1000-nm particles elicited a stronger serum IgG response than the 500- or 200-nm-sized nanoparticles. However, the immune response for 500-nm particles was similar to that obtained with the 200 nm administered by the sc. and the p.o. routes, but higher than the 200-nm particles by the in. route.

Secondary formulation factors that may affect the size of particles & the resultant immune responses

It is important to note that the development of any vaccine delivery system from bench to clinics will involve secondary processes such as sterilization and lyophilization. It is recommended that at the early stages of any vaccine formulation design, the potential impact of secondary processes should be investigated, since these processes can influence the stability of vaccine formulations, particle sizes and size distribution, and thus the resultant immune responses.

Sterilization

All vaccine delivery systems for parenteral administration require sterilization. It is important to state that the aim of sterilization is to destroy or eliminate unwanted living microorganism contamination that may be present in the vaccine product [89]. This is to ascertain that the product is free of unwanted health hazards. Sterilization can be carried out by aseptic method/manufacture, filtration, γ-irradiation, heating, gassing with ethylene oxide and hydrostatic pressure [90]. Irrespective of the method of sterilization, it is desirable to ensure that the process of sterilization does not negatively affect the stability of the materials used in making nano-microparticles as well as particle sizes and size distribution. Furthermore, the sterilization process should preserve the integrity of the antigen and maintain antigen-loading efficiency in the particles. Among all the methods, the process of aseptic (sterile) filtration is simple and

does not lead to direct degradation of materials used in preparing the particles [91–93]. It is important to note that sterile filtration will not be appropriate for particles with sizes larger than 200 nm [90,92]. Special attention should be paid to selecting suitable sterile filters to safeguard against antigen degradation or adsorption onto filters. The choice of γ-irradiation should be based on the chemical stability of components of the particles. Free radicals that are formed during γ-sterilization can initiate chemical modification of the materials used in making the particulate adjuvants [94]. Sterilization by heat has also been reported [89,90,95]. A major concern in the application of heat is the risk of triggering temperature-related changes resulting in: the degradation of antigens, chemical degradation of materials used to make the particulate adjuvants, and physical disruption of the integrity of antigens and particles. For instance, after moist heat sterilization of nanoparticles, an increase in size from 200 to 500 nm was reported where Miglyol was used as the oil phase [96].

Lyophilization (freeze-drying)

Vaccine formulations that will progress from bench to clinic are required to be stable during storage. While the primary consideration should be to maintain the immunogenicity from the time of preparation to the time of application, we believe that the stability of particle size and size distribution should also be evaluated. Instability during storage could lead to physical and chemical changes, antigen degradation or leakage from particulate adjuvants and particle size instability (aggregation or precipitation) [85,97,98]. One effective way to ensure storage stability is to lyophilize the vaccine formulation into a dry powder [89]. Freeze-drying is a well-known process used to produce stable proteins and polypeptides that are prone to instability in aqueous solutions [99]. Essentially, lyophilization is achieved in two major steps of freezing the sample and evaporation of water under vacuum. These two steps can potentially bring about a series of instabilities that are worth investigating while developing new vaccine formulations. Sizes of particles could significantly increase during the process of lyophilization [89,97]. A well-designed, freeze-drying cycle will ensure the physical and chemical stability of a product as well as increase the efficiency of the manufacturing process. Data obtained from lyophilization of vaccine formulations will be relevant to large-scale manufacturing, where it is necessary to convert particle-based vaccine formulations from suspensions to powders so as to reduce the bulk volume and to improve storage stability. The resultant lyophilized powder of the particulate adjuvants can be reconstituted to regenerate the original particle sizes using suitable cryoprotectants [89,97]. Examples of cryoprotectors that can be used are sorbitol, mannose, trehalose, sucrose, man-nitol, carboxymethylcellulose and polyvinylpyrrolidone. The type and amounts of cryoprotectants should be optimized for a given formulation.

Finally, due to the high cost associated with the storage and transfer of vaccines in cold chain and, in some situations, the lack of electricity to power freezers or refrigerators, the ability to store and transfer vaccines in ambient conditions is increasingly being incorporated into the early steps of vaccine formulations [100,101]. We recommend that future development of particulate adjuvant vaccine formulations should take into consideration the feasibility of preserving the particle size and, more importantly, the immunogenicity of the vaccine candidates while avoiding the cold chain at the early stage of formulation development.

Expert commentary

Nanoparticles and/or microparticles are promising vaccine delivery systems with potential adjuvant activity. It is generally agreed that the adjuvanticity of nano-microparticles is affected by particle sizes, which in turn affect the type of immune responses (humoral or cellular) induced by antigens carried by particles. The desired translation from bench to clinics will be greatly hampered by the conflicting results from different studies in correlating the adjuvant

activity and particle sizes. It is our belief that in future studies (attempting to correlate particle size and the adjuvant activity of the particles), there is a need to comprehensively evaluate and compare both humoral and cellular immune responses induced by very narrowly distributed particles prepared with the same materials, loaded with the same antigens by the proper method, and dosed via different routes to the same strain of animals. It is important to note that particle size as a parameter of interest herein cannot be separated from other parameters, such as particle surface properties or material type. Ultimately, it is possible that one may find the optimal particle size that favors strong humoral and cellular immune responses. In addition, one may have to mix particles of specific size ranges favoring either strong humoral responses or strong cellular immune responses in order to generate a desirable and balanced immune response. Another point to consider is the preservation of immunogenicity of antigens carried by particles while avoiding the cold chain in vaccine storage and transportation.

Five-year view

Over the next few years, there is a need for comprehensive studies to define and understand the relationship between sizes of particulate adjuvants and the resultant immune responses. We expect more confirmative studies supporting the adjuvanticity of particle-based vaccine delivery systems. Importantly, more studies are warranted that will generate mechanistic data to clearly explain how and why the size of particle-based vaccine delivery systems significantly affects their adjuvant activities.

Key issues

- Particulates prepared with biocompatible materials hold great potential as vaccine delivery system with potent adjuvant activity.
- The adjuvant activities of different particulates are influenced by factors such as particle size, surface charges, method of antigen loading, route of administration, and soon.
- There were numerous attempts to correlate the size of particulate adjuvants and the resultant immune response, but the findings were conflicting and inconsistent.
- The size of the particulate adjuvants is not only related to the strength of the immune responses induced, but also the type of immune responses.
- Pharmaceutical formulation parameters can potentially affect the size of the particulate adjuvants and, thus, the resultant immune response.
- There is a critical need for comprehensive studies to identify the effect of the size of the particulate adjuvants on their adjuvant activity.

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Table 1

Representative list of studies summarizing the effects of sizes of particulate adjuvants and the resultant immune responses.

Materials	Particle size (nm)	Route of administration	Immune responses measured	Comments	Ref.
Observations that small-sized particles were better immune adjuvants than large-sized particles					
Polystyrene	40–49/93–123	id.	IFN-γ, IL-4, IgG1	Production of IgG1 was observed across all size ranges. IFN-γ was significantly higher with particles of 40–49 nm than 93–123-nm particles, but particles of 93–123 nm gave a higher IL-4 response	[77]
PVA-grafted PLG	100/500/1500	p.o.; ip.	IgG, IgA	Antibody titers were higher with particles of 100-nm size when compared with those of 500 nm. Particles of 1500 nm did not induce antibody titers	[37]
PLG	450–600/1000–3000/6000–32,000	ip.; sc.	$CD8^+$	Particles of sizes less than 450–600 nm induced the strongest immune response	[102]
Chitosan	700–3000	in.; ip.	IgA	Particles of 400 and 1000 nm induced significantly higher IgA responses than particles of 3000 nm	[103]
PLA	7500–50,000	ip.	IgG	Particles of sizes 7500–15,000 nm gave higher antibody titers than particles of sizes 50,000 nm	[88]
PLGA	1000/5000	p.o.	IgG	Particles of 1000 nm were observed to give better immune response than particles of 5000 nm in less than 5 weeks of immunization	[104]
Observations that small-sized particles and large-sized particles were comparable immune adjuvants					
PLG	110/800–900	ip.; im.	IgG, IgG1,IgG2a	Comparable immune responses were observed from particles of 110 and 998 nm	[39]
PLGA	200/500	p.o.; sc.	IgG, IgG1, IgG2a	The 200- and 500-nm particles elicited similar immune responses	[36]
Chitosan	700–3000	in.; ip.	IgG	No difference in IgG production between particles of different size groups	[103]
PVA-grafted PLG	100/500/1500	in.	IgG, IgA	Particles of 100- and 500-nm were observed as equal in the levels of induced immune responses	[37]
Observations that large-sized particles were more effective immune adjuvants than small-sized particles					
PLGA	200/500/1000	in.; p.o.; sc.	IgG, IgG1, IgG2a	The immune responses elicited by 1000-nm particles were stronger than those induced by particles of 500 and 200 nm	[36]
PLGA	200/500	in.	IgG, IgG1, IgG2a	The 500-nm particles elicited a stronger immune response than the 200-nm particles	[36]
PLA	10,000–70,000/50,000–150,000	im.	IgG	The particles of 10,000–70,000 generated stronger immune responses than particles of 50,000–150,000 nm	[34]
PLA	200–600/2000–8000	im.	IgG, IFN-γ, IL-4,	Particles of 2000–8000 nm showed higher antibody titers than those of 200–600 nm. Particles of sizes 200–	[63]

Materials	Particle size (nm)	Route of administration	Immune responses measured	Comments	Ref.
				600 nm promoted IFN-γ production, while particles of sizes 2–8 μm promoted IL-4 secretion	
Lipid vesicles	10–100/60–350/400–2500	p.o.	IgG1, IgG2a, IFN-γ	Particles of sizes 60–350 and 400–2500 nm generated significantly higher IgG2a titers and IFN-γ responses than particles of sizes 10–100 nm	[67]
Reports on the possibility of an optimum particle size range for immune responses					
Polystyrene	20/40/100/500/1000/2000	id.	IFN-γ, IgG	Beads of 40 nm with OVA conjugated onto their surface induced the strongest immune responses	[55]
PLA	<2000/2000–8000/10,000–70,000/50,000–150,000	im.	IgG	At a particle size of 2000–8000 nm, the immune response was higher than for particles in other size ranges studied: <20,000, 10,000–70,000 and 50,000–150,000 nm	[34]
PLA	600–26,000	p.o.	IgG	The optimum particle size for induction of an IgG response was 4000 nm. Particles that were larger or smaller than 4000 nm could not enhance antibody production	[88]

id.: Intradermal; im.: Intramuscular; in.: Intranasal; ip.: Intraperitoneal; OVA: Ovalbumin; PLA: Poly(lactic acid); PLG: Poly(D,L-lactide-co-glycolide); PLGA: Poly(lactic-co-glycolic acid); p.o.: Oral; PVA: Polyvinyl alcohol; sc.: Subcutaneous.