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Nano alum: A new solution to the new challenge

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ABSTRACT

Alum adjuvant has always been the first choice when designing a vaccine. Conventional aluminum adjuvant includes aluminum hydroxide, aluminum phosphate, and amorphous aluminum hydroxyphosphate (AAHS), which could effectively induce the humoral, and to a lesser extent, cellular immune responses. Their safety is widely accepted for a variety of vaccines. However, conventional alum adjuvant is not an ideal choice for a vaccine antigen with poor immunogenicity, especially the subunit vaccine in which cellular response is highly demanded. The outbreak of COVID-19 requires a delicately designed vaccine without the antibody-dependent enhancement (ADE) effect to ensure the safety. A sufficiently powerful adjuvant that can induce both Th_1 and Th_2 immune responses is necessary to reduce the risk of ADE. These circumstances all bring new challenges to the conventional alum adjuvant. However, turning conventional microscale alum adjuvant into nanoscale is a new solution to these problems. Nanoscale alum owns a higher surface volume ratio, can absorb much more antigens, and promote the ability to stimulate the antigen-presenting cells (APCs) via different mechanisms. In this review, the exceptional performance of nano alum adjuvant and their preparation methods will be discussed. The potential safety concern of nano alum is also addressed. Based on the different mechanisms, the potential application of nano alum will also be introduced.

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1. Introduction

Aluminum has been used as an adjuvant in the vaccine industry for decades due to its widely acceptable safety and reliability to enhance the high immune response of vaccine antigens.[1] In the last century, aluminum salts including aluminum oxyhydroxide and aluminum phosphate were the only FDA-approved adjuvants for the human vaccines.[2] Then, amorphous aluminum hydroxyphosphate (AAHS), developed by Merck Sharp & Dohme Corp, was also used as an adjuvant for human papilloma virus (HPV) vaccine. Both European Medicines Agency (EMA) and the WHO evaluate AAHS as high safety and efficacy.[3] Initially, scientists believe that alum adjuvants could provide a depot effect that allows antigens be slowly released from the surface of the microparticles at the injection site, while antigen-presenting cells (APC) could continually uptake and process the antigens.[4,5] Recent research has demonstrated that the mechanisms of action of the aluminum salts as adjuvants are far more complicated. For instance, the excision of the injection site post-injection does not influence the intensity of the immune response in animal models, indicating the flaws of the depot effect mechanism of alum adjuvant.[6] Part of the research describes that alum adjuvant may induce an immune response through targeting nucleotide-binding oligomerization domain (NOD) like receptor protein 3(NLRP3).[5,7] The activation of the NLRP3 inflammasome boosts the maturation and secretion of cytokines, such as IL-1β and IL-18, which induce the immune response to infection and injury.[8] Despite the mechanism of alum adjuvant is still controversial, alum is always the first choice of the adjuvant for vaccine development.

With the rapid development of the vaccine industry, aluminum salts are not necessarily suitable for all vaccines and occasionally lead to compromised immune responses. A well-known case is the inactivated Respiratory Syncytial Virus (RSV) vaccine with alum adjuvant which was developed in the 1960s.[9] Compared with the infants who did not receive the vaccine, infants who received injections not only failed to gain sufficient protection from the vaccine but also induced a higher infection rate and even death.[10] Meanwhile, for the outbreak of SARS (severe acute respiratory syndrome) and MERS (Middle East Respiratory Syndrome), studies have demonstrated the safety issue using vaccine adjuvanted with aluminum salts.[11–13] Thus, it is necessary to develop new adjuvants to solve these new challenges. In the past decades, the US FDA has approved several vaccines with novel adjuvants, such as the squalene oil-in-water emulsion MF59 and GSK adjuvant system (AS) 01–04,[14–16] marking a golden age for adjuvant discovery and development. Several new immune-stimulating molecules such as different TLR agonists (MPLA,[17] CpG,[18] flagellin,[19] etc.) efficiently improved the Th_1 immunity for vaccines against malaria and tuberculosis.[2,20] However, extreme care and sufficient safety data are usually required to obtain approval by regulatory authorities, especially when used in vaccine products for children, elderly, and immune-compromised patients.

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Besides these new "tricky" molecules, scientists are also making efforts to improve the conventional aluminum adjuvant to overcome the obstacles in the new scenarios. Aluminum salts nanoparticle (short as Nano Alum) has been designed to manipulate the immune response. Typically, aluminum oxyhydroxide or aluminum phosphate forms aggregated microparticles with varied sizes, ranging from ~.5–10 μm.[14] Nano alums are expected to be much smaller and could be easily sterilized via .2 μm filters.[21] The difference in dimension between nano alum and conventional alum adjuvants also leads to the difference in the intensity and type of immune response as well as the mechanisms, which will be discussed in detail in this review. Advantages of nano alum over conventional alum will be introduced, including different immune stimulation mechanisms and preparation methods. Potential safety concern on nano alum is also addressed. Furthermore, suggestions on the design of the nano alum and potential applications are provided.

2. Nano alum vs. conventional alum: not only dimension

Dimension is the very first difference between nano alum and conventional alum adjuvants, which brings nano alum some distinctive features. Typically, conventional alum adjuvant needs to be sterilized in the upstream process due to its relatively large size. However, nano alum could be easily sterilized via filtration.[21] Related studies have demonstrated that high-pressure sterilization (121℃, 30 min) does not affect the physical properties of nano alum.[22] Such features enable nano alum with high feasibility of preparation, antigen absorption, and other processes.[23] Another advantage for nano alum is its solubility in desorption buffers. Desorb antigens from adjuvant is the first step to evaluate the *in vitro* relative potency (IVRP) of the alum adjuvanted vaccines. Therefore, the waiting time to complete such a process can be greatly reduced due to the high surface/volume ratio of nano alum.

The second difference is the surface charge of the adjuvant. For conventional alum adjuvant, the feeding ratio of alum salts and sodium hydroxide could determine the surface charge profile. For nano alum, additional excipients such as surfactants or polymers are required to stabilize the nanoparticles. Thus, the surface charge profile of nano alum is more dependent on these stabilizers. Different stabilizers would be applied to fit the antigen protein with a different point of zero charges. In addition, the surface of nano alum could also be functionalized via conjugation and adsorption to change the surface charge profile. Regarding aluminum oxyhydroxide, surface functionalization is shown to play important roles in mediating adjuvant effect.[24] In addition to aluminum oxyhydroxide, aluminum hydroxyphosphate nano adjuvants with different surface charges have also been shown to affect their adjuvanticity.[25]

The most attractive differences between nano alum and conventional micro alum are the intensity of immune response and the types of immune stimulation. Recently, results of several experiments have demonstrated that nano alum is capable of enhancing immunogenicity. For instance, Hepatitis B vaccine with nano alum induced much higher antibody titers than that with conventional alum in hamsters.[26] Aluminum hydroxyphosphate nanoparticles were shown to have a more potent adjuvant activity than microparticles to stimulate specific antibody response in mice.[27] Protection effect of the anthrax vaccine using nano alum increased substantially, while the pro-inflammatory response at the injection site was inhibited.[28] In a rabies vaccine study, nano alum showed a better immune enhancement effect than several adjuvants, including conventional alum adjuvant, AS02, AS03, and MF59.[29]

There are three explanations for the better adjuvant effect of the nano alum. First, nano alum has all of the advantages of nanoparticles: easier uptake by cells, especially APC, and easier membrane penetrating/permeating. Secondly, its relative surface/volume ratio leads to higher antigen absorption capability.[30] The same amount of nano alum may absorb approximately 10–20 times more antigens than that of conventional alum adjuvant. Results of THP-1 cell stimulation experiments demonstrated that aluminum nanoparticles are more potent than microparticles in activating NLRP3 inflammasome due to the higher cellular accumulation of nano alum.[27] The structure of alum aggregates could determine the abundance of antigen internalization by APC.[31] All these features enable nano alum to absorb more antigen proteins or immune stimulating molecules (such as a TLR agonist), which is highly demanded in developing multivalent or conjugated vaccines. Thirdly, it is well known that Alhydrogel elicited the modest Th_2 immunity characterized by IgG1 antibodies. Recent reports demonstrate that nano alum adjuvant elicited a robust Th_1 immune response, which is characterized by antigen-specific CD4+ T cells expressing IFN-g and TNF-α, as well as high IgG2 titers.[2] Despite being controversial, a plausible explanation is that micro-scale particles intend to induce humoral response, while nanoscale particles easily induce cellular response.[27,32,33] For example, the HIV TAT protein adsorbed on cationic polymeric nanoparticles of 220 to 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.[33] It was hypothesized that the nanoparticles (200–600 nm) can be efficiently taken up by APC, such as macrophages, to induce cellular immune responses. On the contrary, the macrophages cannot take up the large microparticles. Instead, microparticles simply attach to the surface of the macrophages and release the entrapped antigens. When macrophages directly uptake antigens without alum particles, humoral response and specific antibodies are induced.

3. Mechanisms of nano alum function and their biodistribution

The conventional alum adjuvant usually renders the dimension between 1 and 10 μm. So APC can only be recruited to the injection site, uptake the antigens, and deliver it to the lymph node to induce immune responses. Non-human primate studies have showed that activation of antigen-specific T cells is restricted within draining lymph nodes.[34] Considering APC is pretty small in peripheral tissues, delivery of antigen into lymph node is limited, which explains why conventional alum adjuvant could only induce weak cellular response.[35] In contrast, the smaller size of nano alum may compensate for

such limitations. Jiang et al. prepared PEG decorated alum nanoparticle and absorb antigen proteins labeled with fluorescence to study the uptake process of APC and the biodistribution of alum nanoparticle post injection.[36] For *in vitro* experiments, more antigen proteins absorbed on alum nanoparticles were uptaken by dendritic cells than free antigen proteins. Animal study results showed that an 85 nm diameter alum nanoparticle penetrates the lymph node and maintains high accumulation for 24 h.[36] More importantly, antigens absorbed on the surface of nanoparticles were co-delivered into lymph nodes leading to high antigen content accumulation at 34.7- and 55.7-folds in dendritic cells and macrophage cells, respectively.[36] Such results well explained the advantage of nano alum over conventional alum adjuvant. Other scientists demonstrate the advantage of nano alum via finely tuning the morphology, crystalline, and surface hydroxyl content of nano alum.[30] According to that study, high surface hydroxyl content may lead to more active reactive oxygen species (ROS), initiating vigorous activation of NLRP3 inflammasome and IL-1β secretion.[30] In mouse model, aluminum hydroxide nanoparticle could induce high antibody titers, including antigen-specific IgG1 and IgE.[30] In conclusion, it is currently feasible to develop a potent alum-based adjuvant that induces both Th_1 and Th_2 immune responses through combining engineering design.

4. Preparation of nano alum

For all nanoparticles, there are two categories of preparation methods: top-down and bottom-up strategies. In the first strategy, nano alums are obtained via gradually decreasing the dimension of alum microparticles or hydrogel. For instance, Amit *et al.* reported using microscale alum salts and size agents to prepare aluminum nanoparticles via sonication and high-pressure homogenization.[37] In this work, polyacrylic acid (PAA) was used as a sizing agent to stabilize nano alum. Thus, PAA determined the electric property of those nano alum particles. Commonly used stabilize agents include poly(lactic acid) (PLA), poly(orthoesters), and the copolymer poly(lactic-co-glycolic acid) (PLGA), biodegradable polyethylene glycol, and polyphosphazene.[38] In the second strategy, nano alum is co-precipitated from aluminum salts with stabilizers and gradually grow to the desired sizes.[30,39,40] Generally, microemulsion was used as a strategy to tune the size of particles.[26,41] First, micro water in oil (W/O) emulsion is prepared and aluminum salts are dissolved in the water phase. By adding a precipitate enhancer, aluminum nanoparticles will gradually grow to the desired size.[42] Such a method is easy to conduct in lab conditions, but the organic residue needs to be removed during the purification process. Similarly, Sun *et al.*[24] synthesized a library of aluminum oxyhydroxide AlO(OH) nanorods with different shapes and crystalline properties. Diethylamine was added into an aluminum nitrate solution, forming a saturated aluminum solution. Aluminum crystal would form under high pressure and high temperature. In all, there are a variety of preparation methods, safety, and impurities that would determine whether such adjuvant could be available for clinical usage.

Interaction of nano alum adjuvant and antigens

Typically, antigens are absorbed on the surface of the alum via static electric interaction and hydrophobic interactions. By varying the feeding amount of sodium hydroxide and aluminum salt, it is easy to manipulate the point of zero charges (pzc). Therefore, alum own opposite electric properties to antigens could be obtained. Meanwhile, these microparticles are gradually precipitated in the static conditions, so the conventional alum adjuvant is always a sub-stable heterogeneous suspension. Currently, there are few studies on the interaction between nano alum and antigens. It is generally believed that antigens could still be attracted to nano alum adjuvants through these interactions, but the assembled form may depend on their relative dimension. For example, if the antigens are relatively small compared to 100–200 nm alum nanoparticles, they can still absorb on the surface of alum. These nanoparticles with attached antigens will suspend in the solution to form a relatively homogenous sub-stable system, despite precipitate after all. For some antigens more than 50 nm, such as virus-like particles (VLP), they tend to form heterogeneous aggregates owing to a similar dimension. Such interaction could be characterized using different methods, such as Fortebio, Surface Plasma Resonance (SPR), analytical centrifugation, and other fluorescence tagged microscopy.

Besides these non-covalent interactions, antigens are also covalently coupled with alum nanoparticles.[40,43] Conjugation to nanoparticles brings antigens better stability and a high possibility to be co-delivered into the lymph node. However, for most antigens, there are limited sites available for conjugation without compromised bioactivity, thus the feasibility of conjugating antigens with nanoparticles should be well evaluated, especially the relative potency post conjugation.

5. Safety of nano alum

Although alum adjuvant has been used for decades, few studies have been carried out to explore the safety profile, and the side effect of alum-based adjuvant is still controversial. Using isotope Al^{26} rather than natural Al,[27] scientists were able to track the injected aluminum adjuvant and study the potential *in vivo* distribution.[44] Most of the soluble aluminum (via intravenous injection) had left the bloodstream after 15 min and less than 1% remained in the bloodstream after 2 days.[45] Further studies revealed that most aluminum that enters the blood is excreted in urine within a few days or weeks, but some aluminum is retained in the body, which deposits in the skeleton or brain.[44,46,47] Several studies claimed that such deposition of aluminum in brain via vaccine injection or other administration is associated with behavior, neuropathological impairment.[48–50] Such side effects always depend on the dose and the type of alum adjuvant used.

As for nano alum, the relatively small size may reduce the retention at the local injection site, which induces unpredictable biodistribution. Whether the trace amount of nano alum could penetrate the blood-brain barrier is still unknown. One study compared the distribution of aluminum nanoparticle with different surface properties. Results showed that the toxicity of aluminum nanoparticles is associated with the *in vivo* stability of nanoparticles and the surface modification.[51] These two

properties determine how quickly nano alum would dissolve into Al^{3+} ions and be eliminated via urine. Another study's data suggest that the shape of nanoparticles represents a significant factor in determining the potential toxicity.[52] Therefore, a more comprehensive investigation on potential secondary problems of nano alum adjuvant, especially the biodistribution before full dissolution, is highly recommended. And sophisticated design should be applied to nano alum to reduce the secondary problems.

6. Conclusion and perspective

As the derivative of alum adjuvant, nano alum has attracted scientists to study its features beyond conventional alum adjuvant. The powerful immune enhancement effect owes to its relatively small size, enabling it to be an excellent replacement to solve new challenges. Although more and more new adjuvants have been developed, these are not only economically desirable but also require animal data and clinical safety data to obtain approval. Thus, there are two strategies in future vaccine design to take advantage of nano alum. First, some complex adjuvants, such as AS04 (TLR4 agonist MPLA is absorbed on aluminum microparticles), can use nano alum to provide more binding sites for MPLA. This is an obvious strategy to improve the performance of the current adjuvant system. Secondly, a highly efficient adjuvant is necessary to solve the stealth property of tumor-specific antigen and self-antigen recognition in the development of anti-tumor therapy. Conventional alum adjuvant is not suitable for the development of anti-tumor vaccines[53,54] due to its inability to induce a powerful cellular immune response necessary for the elimination of tumor cells. A variety of experiments have demonstrated that nano alum could induce high level of cellular immune responses. A recent report indicates that a nano alum-based vaccine is highly effective in inhibiting tumors in animal models.[39] Therefore, nano alum-based vaccine will show exceeding performance in anti-tumor vaccine design. With the emergence of new scenarios such as the outbreak of COVID-19 and therapeutic vaccines, more powerful and economic adjuvants are required. Thus, nano alum will be one of these new solutions to the new challenges. Meanwhile, the safety of nano alum should be carefully evaluated.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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