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Review

Nanoalum adjuvanted vaccines: small details make a big difference

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ABSTRACT

Purified vaccine antigens offer important safety and reactogenicity advantages compared with live attenuated or whole killed virus and bacterial vaccines. However, they require the addition of adjuvants to induce the magnitude, duration and quality of immune response required to achieve protective immunity.

Aluminium salts have been used as adjuvants in vaccines for almost a century. In the literature, they are often referred to as aluminium-based adjuvants (ABAs), or aluminium salt-containing adjuvants or more simply "alum". All these terms are used to group aluminium suspensions that are very different in terms of atomic composition, size, and shape. They differ also in stability, antigen-adsorption, and antigen-release kinetics. Critically, these parameters also have a profound effect on the character and magnitude of the immune response elicited. Recent findings suggest that, by reducing the size of aluminium from micro to nanometers, a more effective adjuvant is obtained, together with the ability to sterile filter the vaccine product. However, the behaviour of aluminium nanoparticles in vaccine formulations is different from microparticles, requiring specific formulation strategies, as well as a more detailed understanding of how formulation influences the immune response generated. Here we review the current state of art of aluminium nanoparticles as adjuvants, with a focus on their immunobiology, preparation methods, formulation optimisation and stabilisation.

1. Use of aluminium-based adjuvants

Adjuvants are key components of vaccines due to their ability to influence the magnitude, duration and quality of the antigen-specific immune response [1,2], improving the level and duration of protection afforded by vaccines [3]. Based on their immunological activity and chemical composition it is possible to group currently licensed adjuvants into four main categories: aluminium salt suspensions (*e.g.* aluminium hydroxide and aluminium phosphate), oil-in-water emulsions (*e.g.* MF59/AS03), liposomes (*e.g.* AS01) and Toll-Like Receptor agonists (TLRa) (*e.g.* MPLA, CpG 1018) [4]. The diverse range of agents with adjuvant activity and the complexity of some of these resulting formulations has made defining their mechanism(s) of action difficult [5]. Adjuvants enhance both T and B lymphocyte responses, resulting in the generation of an increased pool of memory lymphocytes and antibody secreting, long lived plasma cells [6]. These cells have increased frequency within the immune repertoire and together with better effector function and increasing intensity of response on re-exposure to specific antigen, form the basis of vaccine-induced immunity [7]. However, how adjuvants drive these responses is less clear.

TLRa are known to activate Dendritic Cells (DCs), the professional antigen presenting cells that can uniquely activate naïve T cells [8]. TLRa mimic pathogen derived molecular patterns (PAMPs) and therefore leverage this highly conserved pathway to achieve improved vaccine responses [9–11]. However, the mechanism of action of adjuvant formulations such as Aluminium salts, that do not contain PAMP molecules is much less well defined [12,13]. This is surprising, as Aluminium salts are the oldest [14], most widely used and safe adjuvants, in continuous human use for almost a century (Table 1).

Aluminium salts are particulate micron-sized suspensions that have inherent adjuvant activity as well as improving the availability of antigen and co-formulated adjuvants such as TLRa [15]. The adjuvant activity of aluminium salts seems to be mainly characterised by enhanced Th2 lymphocyte responses and IL-4 production, as well as increased B lymphocyte production of IgG1 and IgE isotypes of antibodies in mice [16]. *In vitro* human data suggest that more balanced Th1/Th2 profiles

Abbreviations: Al_2O_3, alumina; $AlCl_3$, aluminium chloride; AH, aluminium hydroxide; AP, aluminium hydroxyphospate; [Al(O)OH], aluminium oxyhydroxide; MPs, aluminium oxyhydroxide microparticles; NPs, aluminium oxyhydroxide nanoparticles; ABAs, aluminium-based adjuvants; IEP, isoelectric point; PZC, point of zero charge; PAA, poly(acrylic acid); PEG, polyethylene glycol; PVP, polyvinylpyrrolidone; $[AlK(SO_4)_2]$, potassium aluminium sulphate; NaOH, sodium hydroxide.

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Table 1
Vaccines in routine use that are adjuvanted with Aluminium salts.

Aluminium-adjuvanted Vaccines	Disease agent	Produced by
DTaP (Daptacel)	diphtheria, tetanus and pertussis	Sanofi Pasteur
DTaP (Infanrix)	diphtheria, tetanus and pertussis	GSK
DTaP-IPV (Kinrix)	diphtheria, tetanus, pertussis and poliovirus	GSK
DTaP-IPV (Quadracel)	diphtheria, tetanus, pertussis and poliovirus	Sanofi Pasteur
DTaP-HepB-IPV (Pediarix)	diphtheria, tetanus, pertussis, hepatitis B and poliovirus	GSK
DTaP–IPV/Hib (Pentacel)	diphtheria, tetanus, pertussis, poliovirus and Haemophilus influenzae type b	Sanofi Pasteur
Hep A (Havrix)	Hepatitis A	GSK
Hep A (Vaqta)	Hepatitis A	Merck Sharp & Dohme Corp
Hep B (Engerix-B)	Hepatitis B	GSK
Hep B (Recombivax)	Hepatitis B	Merck & Co
HepA/Hep B (Twinrix)	Hepatitis A and B	GSK
HIB (PedvaxHIB)	Haemophilus b Conjugate Vaccine,	Merck Sharp & Dohme Corp
HPV (Gardasil 9)	Human papilloma virus nine valent vaccine	Merck
Japanese encephalitis (Ixiaro)	Japanese encephalitis	Valneva
MenB (Bexsero)	Meningococcal B bacteria	GSK
MenB-FHbp (Trumenba)	Meningococcal B bacteria	GSK
Pneumococcal (Prevnar 13)	Pneumococcal bacteria	Pfizer
Td (Tenivac)	diphtheria and tetanus	Sanofi Pasteur
Tdap (Adacel)	diphtheria, tetanus and pertussis	Sanofi Pasteur
Tdap (Boostrix)	diphtheria, tetanus and pertussis	GSK

https://www.cdc.gov/vaccinesafety/concerns/adjuvants.html.

may result from the use of aluminium adjuvant, which can also be influenced by the immunising antigen used [17–19]. Despite this long history of use in vaccination, the exact mechanism of action of Aluminium salts has remained unclear [20]. It was first proposed that aluminium-containing adjuvants potentiated the immune response through the 'depot effect', where retention of antigen at the injection site, and local inflammation may favour immune recognition [21]. This was hypothesised to recruit and activate antigen presenting cells (APCs) thereby increasing migration to draining lymph nodes and enhancing antigen presentation to T cells [22]. However, the functional role of the depot effect in enhancing the adjuvant effect of Aluminium salts, has proven to be controversial, with studies demonstrating that the site of injection is dispensable for adjuvant activity as soon as 2 h after injection [23]. More recently, the NLRP3 inflammasome was proposed to play a role in the adjuvant activity of Aluminium adjuvants [24]. Aluminium salts have been proposed to activate this pathway *via* destabilisation of lysosomes after endocytosis [25], and the inflammasome then allows post-translational modification of inflammatory cytokines that may enhance T cell activation. However, Aluminium salts do not appear to stimulate cytokine production in the absence of PAMPs [26] and studies in caspase and NLRP3 gene knockout mice demonstrate that the inflammasome pathway is not required for adjuvant activity [27]. An important factor that may explain these divergent results in understanding the mechanism of action, is the use of different alum preparations with distinct physicochemical properties [21]. For example, properties such as particle size, point of zero charge (PZC) and surface area, greatly influence the interaction (later referred as adsorption) of vaccine antigens [28]. This is an important issue as both the degree of adsorption of antigen and the mechanisms of adsorption, are known to have significant effects on adjuvant activity [22] and the type of immune response elicited, as discussed below.

The vaccines shown in Table 1 elicit protective immunity through the production of high affinity antibodies that neutralise and opsonise pathogen, as well as block the pathological effects of toxins. These vaccines have clearly benefitted from the Th2-dominated, humoral immune response potentiated by Aluminium salts. However, protective immunity to intracellular pathogens generally requires a cellular immune response and Th1 cell responses [29]. As a result, research has focussed on alternatives to Aluminium salts or approaches to reformulate or augment Aluminium salt formulations to achieve the desired type of vaccine immune response [15].

2. Physicochemical aspects of aluminium adjuvants

Preparing aluminium salts and aluminium salt-adjuvanted vaccines in a consistent manner remains challenging [30]. A vaccine that is prepared by binding an antigen with an aluminium salt is physically a suspension of aluminium salt particles with antigens adsorbed on them [31]. Traditionally this was achieved by precipitation of the vaccine antigen with an aluminium containing suspension to form antigen aluminium complexes [32]. However, this method suffers from high batch-to-batch variation, and has largely been replaced by adsorption of antigens to preformed aluminium-containing gels [12]. In this second method, an antigen-containing solution is added to preformed aluminium salt gel, which can be composed of aluminium hydroxide, aluminium phosphate, aluminium hydroxide-aluminium phosphate mixture or alumina [33]. Alhydrogel, for example, is a clinically approved and widely used aqueous suspension of aluminium hydroxide gel adjuvant, the resultant preparations are called 'aluminium-adsorbed vaccines' [34].

As mentioned above, the physicochemical characteristics of aluminium salts can have a significant impact on the adsorption of vaccine antigens, and therefore can produce heterogeneous effects on the immune system. This is significant, as while the term aluminium salts sounds relatively simple, it encompasses a wide range of chemical species and physical states. In licensed vaccines, two types of aluminium adjuvants are used: aluminium hydroxide (AH) and aluminium hydroxyphospate (AP) (Fig. 1). AH and AP adjuvants are chemically referred to, respectively, as AlO(OH) and $Al(OH)_x(PO_4)_y$ [12,35–37]. AH can exist both in a dehydrated, crystalline form chemically referred to aluminium oxyhydroxide [AlO(OH)] and in a hydrated, aqueous phase where it acquires an additional water molecule to become aluminium trihydroxide [$Al(OH)_3$] [38]. AP is an amorphous aluminium hydroxyphosphate that can exist with a different degree of phosphate group substitution for hydroxyl on surface of aluminium hydroxide (Fig. 1) [12,34,36,39].

AH hydration state has an important impact on protein adsorption. The degree of AH hydration depends on the specific conditions under

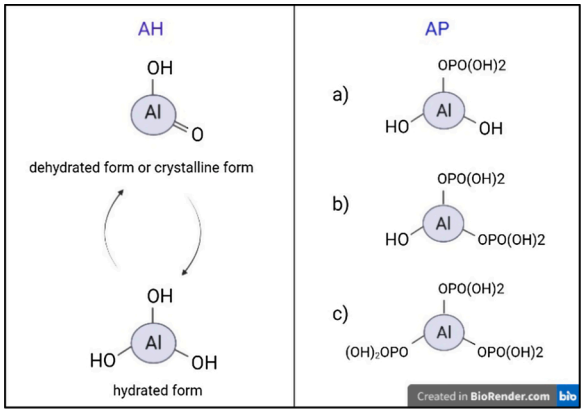

Fig. 1. AH and AP molecules. On the left, representation of the two forms of AH and, on the right, the different degree of phosphate group substitution in AP molecule. Created with BioRender.com.

which they are synthesised: lower temperatures and more acidic conditions induce the formation of small crystals containing an abundance of non-stoichiometric water molecules, which are present both at the surface and intercalated between octahedral layers [40]. Hydrated AH has 3 hydroxyl functional groups which role is fundamental for interaction with other molecules (*e.g.* antigen) in a pH-dependent manner. The point of zeta charge (PZC) of AH is about 11.4, and positively charged at neutral pH. AP particles have an amorphous nature that contain a high proportion of adsorbed water molecules at the interface [41]. In AP the ratio of surface hydroxyls and phosphate varies depending on the manufacturing conditions resulting in an PZC that varies between 4.5 and 5.5 and a negative charge at neutral pH [12].

The physical state of AH is mainly fibrous particles with dimensions of 4.5 × 2.2 × 10 nm, while AP is primarily a plate-like morphology, composed of particles with a diameter of 50 nm. Both AH and AP suspensions form loose aggregates of colloidal particles in aqueous solution ranging from 1 to 20 μm [39,42], and have a solubility minimum value in the range pH 5–7 [43,44]. Aggregates are defined as condensed structures of primary particles, which are held together by solid bridges, that generally emerge after various processes such as crystallisation, sintering, drying and wet granulation [45]. Several factors cause the particle aggregation, such as pH and the presence of salts [46], by surface and field forces (van der Waals, electrostatic and magnetic forces) at direct contact, and/or due to material bridges between particle surfaces (liquid and solid bridges, flocculants) and interlocking (by macromolecular and particle shape effects). Choice of buffer is therefore an important consideration in vaccine formulation as once large clusters are generated in the synthesis process, it can be very difficult to break them back into primary particles [45]. The solubility of AH increases rapidly below pH 5, whereas a sharp solubility increase for AP is observed at pH 6, as a consequence, pH value is the main driver both for the interactions of aluminium salts with other molecules (*e.g.* antigens) and for the aggregation with themselves. The solubility of aluminium salts and consequently the concentration of aluminium ions [Al^{3+}], decrease and increase respectively at higher or lower pH values (Fig. 2) [43,47].

When dispersed into physiological saline, AH shows the tendency to aggregate compared with dispersions of equivalent concentrations of Aluminium in ultrapure water [21,40]. The solubility of aluminium salts in biological fluids is broadly dependent upon their individual physicochemical properties and is likely to be affected by the formulated antigen [40], that can be adsorbed by electrostatic interaction or ligand exchange. Furthermore, aluminium adsorption capacity, which is influenced by many factors such as the charge and size of both the adjuvant and the antigen, and the presence of ions in formulation buffers, has an impact on the solubility and therefore particle size of aluminium salts.

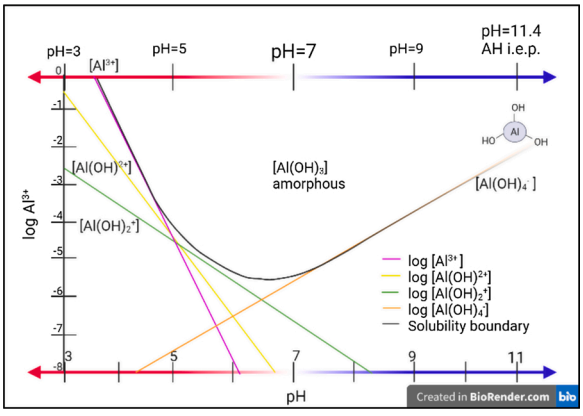

Fig. 2. AH solubility diagram. Representation of the variation in concentration of different ionic populations of aluminium hydroxide, expressed in logarithmic scale, as the pH of the solution varies. The solubility of aluminium hydroxide coincides with the lowest point of the curve, at pH 6.5. Created with BioRender.com.

3. Antigen adsorption

The degree of antigenic adsorption onto aluminum-containing adjuvant is generally recognized to be a crucial factor governing the potentiation of the immune response [48]. However, there has been mixed and controversial evidence regarding the requirement for antigen adsorption to aluminium salts to achieve adjuvant activity [49–54]. In some cases, the partial adsorption of antigens onto aluminium adjuvants has proved to be effective but the effect seems to be dependent by many factors, such as: antigen type and dosage, aluminium dosage and animal model used [50]. Other evidence suggests that a tight and strong adsorption of the antigen may interfere with antigen processing in antigen-presenting cells and result in a lower immune response [22]. However, aluminium adjuvants can also stimulate the immune response to non-adsorbed antigens, although this requires that the adjuvant and antigen are injected at the same site, and the need for adsorption appears to decrease with higher antigen doses [12,54].

In addition, there are many examples in the literature of antigens which have been destabilised through adsorption to aluminium [55], and it seems that the pH of the microenvironment of the aluminium surface can be different from the bulk formulation pH, due to attraction of ions, which can contribute to protein instability [56]. On the other hand antigen adsorption may reduce the possibility of antigen solution precipitation, antigen degradation and other sources of vaccine instability [49,57]. Therefore, while the importance of adsorption of antigen to aluminium for adjuvant effect is controversial, knowledge of the adsorption behaviour of proteins and peptides is crucial in the development of stable and reproducible vaccine formulations to ensure batch to batch consistency at a manufacturing level.

In formulation, the antigen adsorption depends on various factors, including physical and chemical characteristics of the antigens, the size and the type of aluminium used, charges on adjuvants and antigens, the pH of the formulation, the order of addition of reagents, and the speed of mixing [34]. The physical-chemical properties of the particles, such as size, charge, hydrophobicity, morphology, surface roughness, curvature, flexibility, influence the nature and extent of protein adsorption. Antigens can adsorb to aluminium adjuvants *via* hydrophobic and van der Waals forces, *via* electrostatic attraction and by ligand exchange [36,37,49]. The surface of AH is positively charged at neutral pH and strongly adsorb acidic antigens, whereas AP is negatively charged and attracts basic proteins. Antigens adsorbed *via* electrostatic mechanisms can be quickly released upon exposure to interstitial fluid or in presence of high ionic strength [40].

Ligand exchange is the strongest attractive force between antigens and aluminium adjuvants [36,37]. Aluminium has a higher affinity for phosphate than for hydroxyls and phosphates will displace surface hydroxyls on aluminium adjuvants. For this reason, molecules with multiple terminal phosphate groups have a very high affinity for AH as they adsorb *via* ligand exchange. The affinity can be modulated by pre-treatment of AH with phosphate buffer. Antigens adsorbed *via* the ligand exchange mechanism are only partially and very slowly released from the adjuvant following exposure to interstitial fluid compared with those adsorbed by electrostatic interactions [12,36]. Consequently ligand exchange binding of antigens to aluminium-containing adjuvants can be associated with reduced movement of antigen from the injection site to the draining lymph node resulting in weaker antibody responses [37,58].

4. Particle size reduction and impact on immuno-biology

Recently, a series of studies have demonstrated that reduction of the aluminium particle size from micron into the sub-micron range

(nanoalum), induces a more effective adjuvant effect. For example, the adjuvant activity of aluminium oxyhydroxide nanoparticles (NPs) of about ~110 nm is significantly stronger than that observed for aluminium oxyhydroxide microparticles (MPs) [39,58]. Li et al. [58], showed that the anti-Ovalbumin IgG level and anti-PA (Protective Antigen, component of the anthrax toxins) IgG level in mice that were immunised with OVA-adsorbed AH-NPs and PA-adsorbed AH-NPs was significantly higher and more durable than that in mice that were immunised with OVA-adsorbed AH-MPs and PA-adsorbed AH-MPs [58]. The adjuvant particle size also seems to influence the immune response produced not only quantitatively but also qualitatively. It is commonly accepted that conventional aluminium MPs stimulate predominantly Th2 responses *in vivo* in mice [16]. More recently a number of studies have demonstrated that aluminium NPs can stimulate Th1 responses that support cellular immunity (Fig. 3, Table 2) [1,35,39,58]. The ability to manipulate the phenotype of immune response is highly attractive in vaccine development to different pathogens, where Th1 or Th2 responses are required for immunity [59].

While the immunological basis of the improvement in response observed with nanoalum is not fully understood, several potential explanations can be suggested. For example using labelled antigens adsorbed to aluminium salts, NPs have been seen to increase antigen uptake by APCs compared with MPs [29]. Due to its smaller size, nanoalum can also disperse more easily after being injected, resulting in a milder local inflammation and cell recruitment compared with micro-sized aluminium salts [58]. In contrast, conventional aluminium MPs remain concentrated at the injection site (Fig. 4), recruiting innate immune cells, in particular neutrophils.

Importantly, some studies have suggested that the long term depot effect for MPs is a collateral effect and does not contribute to the overall immune response [23]. In support, it seems that a large amount of antigen rapidly desorbs from aluminium MPs, in response to the complex protein environment in tissue [61]. Thus, not only the size of the particle but also the nature of the interaction between antigen and aluminium particles are important factors in the performance of the adjuvant *In Vivo* [21,27].

Compared with larger particles, nanoalum particles have an increased surface area (number of surface atoms to volume ratio), which results in more metallic hydroxyl groups available and a more positive zeta potential compared with MPs and more binding sites for antigen adsorption [58]. Consequently, a lower amount of nanoalum is necessary to ensure complete antigen adsorption respect to MPs. When an antigen is adsorbed onto AH the size of the particle can slightly increase, while the zeta potential becomes respectively less positive or negative in case of NPs and MPs [58].

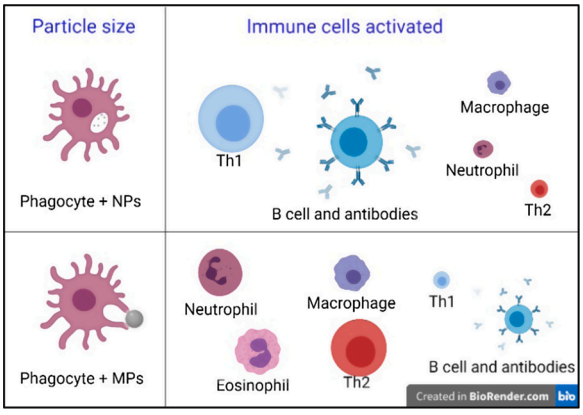

Fig. 3. NPs and MPs which, based on their size, activate different immune responses. The size of the immune cells that are activated represents the strength of the type of the immune response triggered. Created with BioRender.com.

Table 2
NPs and MPs which, based on their size, stimulate the production of different antibodies, cytokines and chemokines.

Particles	Antibodies	Cytokines
NPs	IgG2a*, IgG1	IL-1β*, IL-18*, IL-2*, IFNg*, IL-12, IL-4, IL-10
MPs	IgG1*, IgG2	IL-1α, IL-1β*, IL-2, IL-4*, IL-5*, IL-13, IL-17A, IL-18, IFNg and TNFα

*Mainly produced.

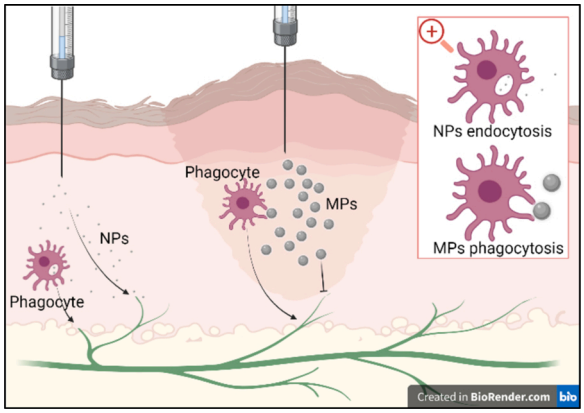

Fig. 4. Proposed behaviours of NPs and MPs at the injection site. NPs smaller than ~200 nm can be taken up by endocytosis and phagocytosis in skin, and then removed *via* cell migration. NPs can also directly access lymphatic vessels due to their capacity to enter junctions between lymphatic endothelial cells [60]. In contrast, larger MPs can only be removed by phagocytosis and therefore tend to reside at the injection site for long periods of time, forming a depot effect. Created with BioRender.com.

Particle size may also affect the ability of aluminium to leave the injection site *via* lymph drainage. In a similar manner to proteins and other soluble material, NPs can directly enter terminal lymphatic vessels through junctions between endothelial cells (Fig. 4) [60]. This allows them to rapidly leave the injection site and reach the lymphoid organs within few hours of injection. In contrast, larger particles require phagocytosis and migration of phagocytes such as DCs, taking approximately 12 h [62–64] to reach draining lymph nodes. Recruitment of phagocytes therefore plays an important part in the process of clearing MP aluminium salts from the injection site [62], contributing to tissue inflammation.

Both MPs and NPs can act as antigen carriers *In Vitro*, with NPs showing better enhancement of antigen uptake by APCs compared with MPs [29]. APCs transport both the antigen and adjuvant to the draining lymph nodes, process the antigen for presentation and activate T cells resulting in the initiation of adaptive immune responses. Particle size can affect transport and antigen presentation, with particles in the size-range of 20–200 nm, considered as small nanoparticles (<500 nm), taken up by APCs through endocytosis [59,65], while larger nanoparticles and microparticles (>500 nm), requiring phagocytosis and cellular transport from the injection site to the lymph nodes [59]. Small AH NPs are easily internalised by DCs, while larger AH MPs may adhere to the surface of DCs without being internalised [58].

In summary, compared with aluminium salt MPs, nanoalum offers significant advantages, including reduced local reactions following injection and the possibility to modulate the antibody and cellular immune responses as required [29].

5. Preparation of nanoalum formulations

Preparation of nanoalum introduces further complications to the already complex physicochemistry of aluminium salts. Reduction of

aluminium particle size can be achieved using different approaches which all result in an increase of the surface energy, hence nanoalum requires stabilisation with suitable compounds due to its tendency to aggregate [66]. The choice of stabilising compound depends upon the types of nanoalum produced and synthesis method used. Stabilisers that lead to surface modification (also termed as capping ligands) are used to achieve sufficient repulsive forces between nanoparticles, stabilising suspensions by preventing aggregation. The functional groups of these stabilisers react with the complementary aluminium groups forming stable monolayers by their adsorption. Consequently, the properties of the stabiliser and the polymer shell formed on nanoalum particles can significantly alter the original properties of the core [66]. Currently, there are several new and efficient techniques for the synthesis of nanoalum, but not all are applicable to generate nanoparticles as adjuvants.

HogenEsch *et al.* [67] reported the precipitation method to prepare aluminium oxyhydroxide [Al(O)OH] by mixing an aluminium solution, usually aluminium chloride ($AlCl_3$) or potassium aluminium sulphate [$AlK(SO_4)_2$], with sodium hydroxide (NaOH). The suspension is then dehydrated under hydrothermal conditions. Once AH particles have been generated, exposure to shear forces, for example sonication or ultrasonication cycles decreases the size of the aggregate adjuvant particles [34,67]. The ultrasonic dispersion mechanism involves acoustic cavitation (formation, growth and implosion of bubbles resulting in the rupture of agglomerates) and acoustic streaming–inducing chaotic mixing. Nanoalum may regroup back into several hundred nanometer clusters shortly after ultrasonication, if the suspension is not stabilised enough against re-agglomeration [45,68]. Approaches to avoid particle reaggregation are clearly important in achieving stable formulations and the interactions between the adjuvant and the surrounding formulation buffer can be considered a crucial mediator of systemic agglomeration in colloidal suspensions [40]. As the formulation buffers can alter the charge on the aluminium salt and antigens, according to their respective PZC and isoelectric point (i.e.p.), they can impact the adsorption degree. Therefore, the buffer ionic strength should be kept as low as possible, while pH and the presence and concentration of salts in formulation can cause aggregation [34,67]. For this reason, it is preferred to reduce/avoid presence of phosphate-based buffers in the formulation as this alters surface charge [34], and in general polyols and sugar alcohols are preferable to adjust the tonicity rather than salts.

Stabilisers can also be added to the formulation to maintain monodispersity of particle size distribution. The stabilisation may be carried out through electrostatic, steric and electrosteric effects [45], using substances that vary pH and ionic strength. These excipients and polyelectrolytes require optimisation to reduce the agglomeration tendency of aluminium adjuvants as adsorption of electrolyte ions on aluminium salts surface, especially at high electrolyte strengths, could lead to the bridging of particles or flocculation [45]. Harris et al. [68] suggest that, following the sonication, adding sufficient protein to produce saturation of the binding capacity of aluminium adjuvants can prevent subsequent aggregation produced by cross-linkage of the adjuvant particles [68].

As mentioned above, traditional aluminium-adjuvanted vaccine formulations are extremely heterogenous (*e.g.,* particle size, shape, PZC), so each formulation is unique depending on which excipients are used and the order of addition of formulation components [34]. Stabilising nanoalum brings further challenges due to its greater surface energy and the ease with which it interacts with the other formulation components that could result in aggregation. This brings an advantage in the great variety of nanoalum formulations that can be prepared using different materials and protocols. However, this variation in physicochemistry will require formulation optimisation on a case-by-case basis and also questions the generalisability of the *In Vivo* behaviour of one nanoalum formulation to another.

6. Alternative preparation methods and stabilisers for nanoalum adjuvants

Recent progress in the field of nanotechnology has identified new approaches for production of nanoalum with controlled size, shape, and surface properties. These include resuspension of nanopowder [29], microfluidisation [69] and laser ablation [70] in liquid phase.

Ruwona *et al.* [39], employed preformed AH Nanopowder/Nanoparticles from US Research Nano-materials to generate both AH-NPs and AH-MPs [58]. Briefly, AH Nanopowder was slowly resuspended into warm water while stirring: the suspension was repeatedly probe-sonicated and centrifuged to separate supernatant from the pellet containing AH-MPs. The resultant supernatant suspension was stabilised by adding polyvinylpyrrolidone (PVP) and used as AH-NPs. The majority of the AH-NPs were below 100 nm, whereas the median diameter of the AH-MPs was 5 mm. The AH-NPs were more potent than AH-MPs in activating NLRP3 inflammasome as they caused a higher level of IL-1b production and were more effectively taken up by APCs [39]. Both the results from Li et al. work where NPs were obtained by precipitation and MPs by AH dried gel [58], and those of Ruwona et al. work [39], agree that NPs are more effective adjuvants than MPs. However, it has not yet been investigated whether the starting materials and processes have produced nanoalum with different properties, nor whether PVP plays a role in affecting the resulting immune response.

Orr *et al.* [69] performed high-pressure microfluidisation to generate AH-NPs, starting from Alhydrogel. To prevent reaggregation they introduced a low molecular weight anionic polymer, 2 kDa poly(acrylic acid) (PAA), prior to the microfluidisation step, and managed to produce stable and monodispersed nanoalum of about 68 nm and a negative zeta potential of -18.0 $\pm$ 2.8 mV at pH 7.4. The same group also produced a second nanoalum formulation, replacing the PAA with PEG(5000)-DSPE as the stabilising polymer. This produced a PEG:nanoalum with similar particle size (70 nm), but a neutral net surface charge of -0.7 $\pm$ 0.3 mV. Comparing PAA:nanoalum and PEG:nanoalum they suggested that surface charge was the key determinant in antigen binding. Thus, coating the surface of the aluminium particles by the PAA polymers (negative zeta potential) prevented the electrostatic binding of negatively charged antigen while effective antigen adsorption was achieved using PEG. According to Orr et al. [69] the capacity to produce strong immune responses was due to the PAA:nanoalum combination, since they demonstrated that an equal molar amount of PAA alone and a physical mixture of PAA and Alhydrogel were not sufficient to promote Th1 immunity. Furthermore, use of PEG:nanoalum did not result in the Th1 response seen with PAA:nanoalum. These studies highlight the important role that stabilisers (PAA *vs* PEG) play in adjuvant activity, but only in the presence of nanoalum. They also underline the unpredictability of the behaviour of these formulations *In Vivo* [69].

High energy, pulsed Nd-YAG lasers have been employed to generate nanoparticles from solutions of metal oxides such as alumina (Al_2O_3) [71]. This technique, called laser ablation, generates nanoparticles with low polydispersity and a negative zeta potential (e.g.−31.81 mV) [70] indicating a good colloidal stability. While the adjuvant activity of nanoparticles produced by laser ablation has not been tested, the starting material, alumina appears to be much more stable than AH [72] but less effective as adjuvants [39]. Investigating the effects of changing the starting material, and the stability of resulting nanoparticle suspensions will be important steps in developing this interesting approach to vaccine adjuvant production.

While there are several approaches to prepare nanoalum, it seems clear that that stabilisers are almost always needed to maintain the physicochemistry of the formulation. The different processes and materials used lead to particles of the desired size, however, may cause unpredictable differences in physical or biological behaviour *In Vivo*. They also preclude analysis of the specific effects of nano- or microparticle size on immunological performance.

7. Antigen adsorption, storage and stability of nanoalum

As for micro-sized aluminium, the interaction between nanoalum and proteins can be dynamic or static and different degrees of protein structural alterations have been reported upon adsorption of proteins on the nanoparticle surface [70]. The previous discussion of protein adsorption to aluminium hydroxide is probably more significant with nanoalum-based formulations, as the increased surface area provides a greater adsorption surface than micro-sized aluminium adjuvants. This means the amount of nanoalum needed for the complete adsorption of antigens is smaller compared to micro-sized aluminium adjuvants. However, it also means that formulation optimisation on a case-by-case basis is required.

Another important issue from a manufacturing perspective is how to store vaccines and how long they are stable, but very little is known in the literature regarding vaccines with nanoalum as an adjuvant. Aluminium-based adjuvants are not only sensitive to various manufacturing-related stresses, but also supply chain-related stresses [73]. The stability of nanoalum vaccines in the supply chain are therefore likely to be a significant issue in their development. Freeze-thaw stress on aluminium adjuvants has been reported both to result in significant aggregation and reduction in immunogenicity: as a consequence, vaccines can be impacted by flocculation and extent of sedimentation [73]. Vaccines adversely affected by freezing lose their physical, chemical, and immunological properties, and the loss of potency can never be restored [74], and can result in increase of adverse local reactions at an injection site, such as sterile abscesses or anaphylactic shock [74]. It is equally true that most aluminium adjuvanted vaccines are highly sensitive to heat, thus requiring expensive refrigeration facilities to maintain the potency [75].

Nanoalum formulations may help to overcome temperature stability issues with vaccines. Zhou *et al.* [75] demonstrated that by using nanoalum for vaccine encapsulation, the thermostability and the efficacy of vaccines stored at 25 °C could be kept for more than two weeks [75]. Addition of PAA helps to heat stabilise nanoalum, as indicated by a lack of particle size growth over 3 months when stored at 25 or 37 °C and over 1 year at 5 °C. Moreover, PAA:nanoalum formulations remained stable even after multiple freeze-thaw cycles.

In conclusion, while the stability and storage requirements for nanoalum adjuvanted vaccines are still being explored, there is evidence that this approach may offer advantages over conventional vaccine in terms of storage. However, the increased surface area of nanoalum makes this formulation highly interactive which therefore demands that care is taken in preparation and characterisation [73].

8. Conclusion

Experimental studies have clearly demonstrated the immunological advantages of reducing the size of aluminium particles to obtain more effective adjuvants that stimulate both cellular and humoral immune responses. The mechanistic basis of this improvement in immune performance is currently unclear, however drawing from the behaviour of other nanoparticles in biological systems *In Vivo* [76], delivery effects such as improved distribution to the draining lymph node, increased antigen uptake and improved magnitude and duration of antigen presentation are likely to play a significant role. However, as nanoalum formulations come in different charge, size and shape, they also show diversity in terms of stability, antigen-loading, and antigen-release kinetics and these parameters can have profound effects on the quality of immune response elicited. As a consequence of the variety of nanoalum preparations that can be produced it might be inappropriate or simplistic to group them within the generic aluminium-based vaccines (ABAs) definition.

Due to the heterogeneity of vaccine preparation conditions and formulation components, nanoalum behaviour in vaccine formulations is difficult to be predictable. This requires a detailed knowledge of the physical and chemical properties, including the impact of vaccine antigens, excipients such as salts, buffers, and tonicity modifiers on formulation parameters. This information will allow more systematic mechanism of action determination, and at the same time generate robust and reproducible vaccine formulations.Therefore, defining the smallest details will make big differences to progress in nanoalum adjuvant development.

Declaration of Competing Interest

Arianna Raponi's PhD studentship was partly supported by GlaxoSmithKline Biologicals SA through the University of Glasgow industrial partnership PhD Program. Donatello Laera is an employee of the GSK group of companies. James Brewer and Paul Garside do not have any conflict of interest to declare.

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