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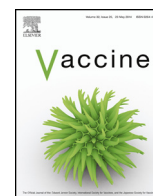
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## Particle-based platforms for malaria vaccines<sup>☆</sup>



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### ABSTRACT

Recombinant subunit vaccines in general are poor immunogens likely due to the small size of peptides and proteins, combined with the lack or reduced presentation of repetitive motifs and missing complementary signal(s) for optimal triggering of the immune response. Therefore, recombinant subunit vaccines require enhancement by vaccine delivery vehicles in order to attain adequate protective immunity. Particle-based delivery platforms, including particulate antigens and particulate adjuvants, are promising delivery vehicles for modifying the way in which immunogens are presented to both the innate and adaptive immune systems. These particle delivery platforms can also co-deliver non-specific immunostimulators as additional adjuvants. This paper reviews efforts and advances of the Particle-based delivery platforms in development of vaccines against malaria, a disease that claims over 600,000 lives per year, most of them are children under 5 years of age in sub-Saharan Africa.

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### 1. Introduction

Compared to inactivated or live-attenuated whole sporozoites and whole merozoites, malaria vaccine development strategies using recombinant subunits and/or synthetic peptides have the advantage of directing the immune responses toward specific targets. These strategies, in general, are amenable to large-scale controlled manufacturing processes leading to the production of a well-characterized antigen with acceptable purity at a low cost. Multiple malaria vaccine targets, most of which are antigens expressed on the surface of parasites in liver, blood, and mosquito stages, have been evaluated for their ability to induce protective immunity [1]. Promising protective immunity was often observed in initial proof-of-concept studies where animals were immunized with the soluble recombinant proteins formulated with Freund's adjuvants. However, the level of protection declined or was lost in clinical trials when these proteins were formulated with adjuvants suitable for human use [2,3]. The development of RTS,S, a recombinant subunit vaccine based on the *Plasmodium*

*falciparum* circumsporozoite protein (PfCSP) present on the surface of infectious sporozoites, has demonstrated the critical roles of adjuvants and delivery vehicle in order to achieve a significant efficacy. Despite mounting evidence that recombinant PfCSP was capable of inducing protective immunity against malaria infection, it was not until certain modifications to the formulation were made that the RTS,S vaccine demonstrated a significant level of protection against clinical malaria. These modifications include: (i) PfCSP was fused to and co-expressed with the hepatitis B Surface antigen (HBsAg) which then self-assembled to form a virus-like particle (VLP), with multiple copies of PfCSP presented on the particle surface; and (ii) the VLP was delivered in an oil-in-water emulsion or a liposomal formulation containing additional immunostimulatory molecules such as MPL and QS21 [3,4].

VLPs, stable emulsions, and liposomes belong to a category of nanoparticle-based delivery platforms that have gained increasing interests in recent years due to their ability to enhance the immunogenicity of recombinant subunit vaccines (Table 1). A nano-sized particle displaying high-density repetitive antigens or epitopes, devoid of invasive pathogenic materials yet permitting inclusion of desired immunostimulatory molecules, is an ideal representation that enables incorporation of quality-by-design concepts during vaccine development. There are multiple benefits to these platforms. First, a successful immune response, be it a pathogen or a vaccine, requires the ability of antigens to be taken up by antigen presenting cells (APCs), processed and presented to immune effectors. At a size comparable to pathogenic

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**Table 1**  
Particle-based delivery platforms.

	Type	Characteristics <sup>*</sup>	Reference
Particulate antigens	Virus-like particles (VLP)	Viral capsid protein self-assembled to form a stable core; exogenous target antigens are displayed repetitively on the surface by recombinant fusion or chemical conjugation to the viral capsid protein. A lipid membrane may be present for certain VLPs (e.g. retrovirus derived VLPs)	[5,64]
	Virosomes	Lipid-enveloped VLPs without viral capsid core; exogenous target antigens are displayed repetitively on the envelop surface. Antigens can be inserted by chemical conjugation to the lipids or through the natural transmembrane domain present on native proteins; co-delivery of additional immunostimulants possible	[13]
	Self-assembling polypeptide nanoparticles (SAPN)	Polypeptide core scaffold formed by multiple coiled-coils oligomerization domains; target antigens are present at the N- and C-termini of the scaffold polypeptide, and displayed repetitively on the core surface	[18]
	Antigen-carrier conjugates	Polypeptides, polysaccharides, or recombinant proteins are chemically conjugated to a protein carrier, and cross-linked to form a matrix	[34]
	BLPs	Micron-sized; peptidoglycan (PGN) matrices derived from food grade bacteria; target antigens are recombinantly tagged with a PGN-binding domain and displayed on the matrix surface by non-covalent interaction with PGN	[8,9]
Particulate Adjuvants	Liposomes	Lipid bilayer vesicles with an aqueous core; target antigen may be displayed on vesicle surface, intercalated in lipid bilayer, or entrapped in aqueous core; co-delivery of additional immunostimulants	[40,42]
	O/W emulsions	Nano-to-micron-sized, oil droplets are emulsified in bulk water phase; target antigens may interact with the oil droplets by hydrophobicity or electrostatic force; co-delivery of additional immunostimulants	[51,65]
	W/O emulsions	Nano-to-micron-sized, water droplets are emulsified in bulk oil phase; target antigens are entrapped in the water droplets	[66]
	Iscomatrix	A cage-like structure formed at interactions between phospholipids and saponin-cholesterol micelles; target antigens are entrapped in the void space of the cage	[67]
	Biodegradable polymers	Nano-to-micron-sized; bio-degradable synthetic polymers such as poly (lactic-co-glycolic) acid (PLGA); target antigens are entrapped within the polymer	[61]
	Aluminum salts	Micron-sized; porous aggregates of fibrous aluminum hydroxide or plate-like aluminum phosphate; target antigens are adsorbed to aluminum salts by electrostatic attractive force or trapped in void spaces within the aggregates	[68]

<sup>\*</sup> The particles are nano-sized unless otherwise indicated.

microorganisms, the antigen-bearing nanoparticles are more easily recognized and efficiently taken up by APCs at the site of vaccine injection [5]. Second, Pattern Recognition Receptors (PRRs) of the innate immune system are the first line of host defense against a pathogen. PRRs recognize specific Pathogen-Associated Molecular Patterns (PAMPs) that leads to the induction of a proinflammatory response responsible for the induction of both innate and subsequently adaptive immunity. Presence of high-density repetitive epitopes on particle surface facilitates B-cell activation through cell surface receptor oligomerization and the generation of specific antibodies with higher affinity [6,7]. Third, prolonged and controlled release of target antigens from the particle will result in a “depot” effect favoring the induction of lasting immune responses. Last but not least, the particle may allow co-delivery of antigen and immunostimulatory adjuvants such as toll-like receptor (TLR) agonists to the same APC, ensuring a specific APC activation.

This review aims to summarize efforts and advances in applying nanoparticle-based delivery platforms to enhance the immune response of malaria vaccines based on recombinant antigens or synthetic peptides. The focus is directed toward the works published from 2010 and onwards. Though they will not be discussed in this review, it is worth noting that aluminum salts and Bacterium-Like Particle (BLP) are also particulate in nature. However, they are micron-sized, and their modes of action in immune enhancement differ from those of nanoparticles. To date, aluminum salts do not appear to provide adequate adjuvanticity to recombinant malaria

vaccine candidates. On the other hand, BLPs have shown some promising immune enhancement that warrant further exploration [8,9]. RTS,S, a particulate antigen formulated in particulate adjuvant, is the subject of a separate review article in this issue [200].

## 2. Particulate antigens

### 2.1. Virus-like particles

There are two main types of VLPs: (i) the non-enveloped VLPs made of viral capsid proteins that self-assemble into stable structures. The vaccine target antigens can be recombinantly fused to the viral protein and displayed on the particle surface; and (ii) enveloped VLPs harboring a lipid membrane with the vaccine target antigens anchored at the surface. Among enveloped VLPs there are two categories: those having a core of proteins forming a capsid-like structure surrounded by a lipid membrane, and those devoid of a protein core and having only a lipid membrane with the target antigens embedded, such as the influenza virosomes. In the latter case, target antigens could be recombinant proteins or synthetic peptides that are conjugated to lipid tails for membrane anchoring, or it could be native proteins with their own transmembrane and cytoplasmic domain.

Utilizing recombinant expression in Tobacco plants as a platform technology, Jones et al. constructed an expression vector

encoding Pfs25, a *P. falciparum* ookinete surface protein and a transmission blocking vaccine candidate, fused to the coat protein (CP) of *Alfalfa* mosaic virus [10]. The purified recombinant product composed of the Pfs25–CP fusion protein and two truncated CP polypeptides, in planta co-assembled to form VLPs 20 nm in diameter. The requirement of the truncated viral coat protein in the assembly process is reminiscent of RTS,S, where assembly of PfCSP–HBsAg VLP also required truncated HBsAg [3]. Interestingly, mice immunized with Pfs25–CP VLPs formulated in Alhydrogel<sup>®</sup> elicited high-titer, long-lasting transmission blocking antibodies even with a single vaccination dose. A Phase 1 trial with Pfs25–CP VLP formulated with Alhydrogel<sup>®</sup> has been conducted in healthy adults in the USA though the results are not yet published [101].

Enhancement of antibody responses by VLP was also demonstrated in study in rhesus monkeys immunized with soluble and particulate versions of a vaccine based on *Plasmodium vivax* CSP, a.k.a CSV [11]. Four groups of rhesus monkeys were immunized with two doses each of a soluble form of the vaccine (VMP001) or the particulate version (CSV-S,S). The latter are VLPs that are formed by fusing VMP001 to hepatitis B Surface antigen (CVS-S) and co-expressing with free HBsAg to form CSV-S,S. While both vaccines, formulated with AS01 which consists of MPL, QS21 and liposomes, induced robust cellular and humoral responses, CSV-S,S generated higher antibody titers, particularly with regard to a potentially protective B cell epitope.

Another way to present target antigens on the surface of a VLP is by chemical conjugation of a recombinant antigen to the surface of a preassembled VLP. A well tested example of this principle is the VLP formed from the capsid protein of the bacteriophage Q $\beta$ . The amino group of the surface exposed Lysine residues are reacted with a bi-functional chemical cross-linker which is subsequently reacted with the target antigen engineered to contain a reactive Cysteine residue. The resulting VLP products present repetitive arrays of the target epitope on the particle surface. This platform has been evaluated extensively in therapeutic vaccines such as ones against nicotine dependence, hypertension, and Alzheimers diseases [12]. The Q $\beta$  VLP has also been used as a core to present multiple copies of recombinant Pfs25 on its surface. Immunizing mice with the Pfs25–Q $\beta$  VLP, formulated with or without Alhydrogel<sup>®</sup>, induced anti-Pfs25 antibody titers higher than that of Pfs25–EPA conjugates. The antibodies displayed transmission blocking activity, and stronger IgG2 and IgG3 titers indicating a Th1 response induced by Pfs25–Q $\beta$  VLP [201].

Virosomes, enveloped VLPs constituted of phospholipids and viral proteins, may incorporate the target antigens by coupling them to lipid components of the envelope. Although virosomes can be produced from a variety of enveloped viruses, the influenza virus has been the dominant substrate for producing virosome-based vaccines [13]. The virosomal delivery platform benefited from an extensive human safety profile, as demonstrated over the last 20 years in adults as well as children, elderlies, and immune-compromised populations [14,15]. Early efforts of conjugating virosomes with peptides derived from repeat region of PfCSP and an ectodomain of the *P. falciparum* apical membrane antigen-1 (PfAMA1), a micronemal protein involved in merozoite invasion led to several clinical trials. When evaluated individually, both PfCSP–virosome (PEV302T) and PfAMA1–virosome (PEV301T) formulations demonstrated excellent safety, tolerability and immunogenicity, though none of the participants were protected against blood stage infection in a challenge study [13]. However, when PEV302T and PEV301T were administered in combination, a reduction in the malaria incidence rate was observed in children aged 5–9 years old, indicating the value of a multivalent vaccine approach [16]. The virosome was also used to deliver GMZ2, a recombinant fusion protein consisting of the N-terminal portion of the *P. falciparum* Glutamate Rich Protein (PfGLURP)

and C-terminal fragment of the *P. falciparum* Merozoite Surface Protein 3 (PfMSP3). Although the antibody titer elicited by the unadjuvanted GMZ2–virosome conjugate was merely comparable to the titer by GMZ2 formulated with alum [17], the result seemed encouraging for 2 reasons: (i) In addition to small peptide focusing on key epitopes (also called mimotopes), the virosome is a versatile platform allowing also the delivery of small-to-large size (~2 to 100 kDa) recombinant antigens, some in dimeric or trimeric forms; and (ii) Virosome platform still has the potential to further enhance immunity if additional and relevant adjuvants are inserted into the lipid membrane or packaged within the aqueous core of the virosomes.

## 2.2. Self-assembling polypeptide nanoparticles (SAPNs)

Self-Assembling Polypeptide Nanoparticles (SAPN) are based on the alpha-helical coiled-coil oligomerization motifs present in protein sequences that allow them to self-assemble into well-defined ordered structures. The basic building block of the SAPN is a monomeric polypeptide chain containing one pentameric and one trimeric coiled-coil oligomerization domains. Driven by these domains, about 60 polypeptide chains self-assemble into an ordered structure with icosahedral symmetry, exposing both N- and C terminal ends on the surface [18]. Extending the polypeptide chain with target epitopes would allow presentation of the target epitopes in a repetitive array on the particle surface.

To test this novel concept for development of malaria vaccines, Kaba et al. designed and recombinantly produced a polypeptide chain based on the pentameric coiled-coil sequence of cartilage oligomeric matrix protein (COMP), a de novo-designed trimeric coiled-coil, and a B cell immunodominant repeat epitope of *Plasmodium berghei* CSP that assembled into ~25 nm particles [19]. Immunized mice generated CD4<sup>+</sup> T cell-dependent, high-titer, high-avidity long-lasting antibody responses that were capable of protecting mice challenged with *P. berghei* sporozoites. In order to develop the SAPN platform for human use, the pentameric coiled-coil forming sequence was switched from the COMP domain to a de novo designed Tryptophan-zipper to prevent possible cross reactivity [20,21]. Interestingly the replacement of the COMP sequence resulted in a loss of immunogenicity and protective efficacy in mice, which was restored by introducing a universal helper T cell epitope PADRE into the trimeric coiled-coil domain. These second generation constructs were designed to expose the immunodominant B cell epitope from the repeat region of *P. falciparum* CS at the surface of the SAPNs and also included three *P. falciparum* CD8<sup>+</sup> epitopes within the scaffold of the nanoparticle. The long-lasting antibodies elicited by the new SAPNs were capable of protecting mice challenged with Tg–Pb/PfCSP, a transgenic *P. berghei* parasite expressing PfCSP on its sporozoite surface [20]. Using SAPNs that contained heterologous B cell epitopes and/or lacked the CD8 epitopes, protection was demonstrated to be mediated via both antibodies and CD8 T cells. The process to produce these SAPNs is amenable to cGMP manufacturing [22] and they are currently being developed further for human testing.

## 2.3. Antigen-carrier conjugates

Development of chemical conjugate vaccines, in general, is based on early work using non-immunogenic targets or haptens chemically conjugated to a “carrier protein”. Active immunization with hapten-carriers enabled production of hapten specific antibodies due to appropriate communication between T cells and B cells [23]. These observations supported production of chemically conjugated polysaccharide vaccines that switch T cell-independent antigen presentation of polysaccharide moieties to T cell-dependent antigens for use in licensed vaccines for



predominately young children against *Neisseria meningitidis*, *Haemophilus influenzae* type b and *Streptococcus pneumoniae* [24]. Various carriers are used in these commercial vaccines including cross reactive material of diphtheria detoxified toxin bearing an amino acid substitution, identified as CRM<sub>197</sub>; Tetanus toxoid (TT), and meningococcal outer membrane protein complex (OMPC) [25].

Encouraging results were obtained by conjugating Pfs25 to OMPC: the conjugation significantly enhanced the antibody titers in mice, rabbits, and non-human primates. Chemical coupling did not seem to affect functional epitopes, as the correlation between the conjugate-induced antibody titres and their transmission reducing activities was similar to that induced by soluble Pfs25 formulated with Montanide® ISA51, a water-in-oil formulation. More interestingly, the antibody response induced by the conjugate was sustained for 2 years, and recalled by a booster dose of Pfs25 formulated with an aluminum adjuvant [26]. It was noted that OMPC differs from other carriers in that its proteins are present in a liposomal form containing lipids, including lipopolysaccharide, a natural adjuvant. The potential for development of a Pfs25-OMPC chemical conjugate vaccine is under further investigation.

A well-defined protein carrier, the recombinant *Pseudomonas aeruginosa* detoxified toxin, ExoProtein A (rEPA) produced in *Escherichia coli*, has also demonstrated the capacity of immune enhancement [27]. Although rEPA is not yet a component of any licensed vaccine, it appears to be safe in humans including infants [28–31]. Early preclinical studies evaluating the immunogenicity of chemically conjugated recombinant Pfs25 and Pfs28 to rEPA formulated on Alhydrogel® demonstrated an enhanced functional antibody response, which was further enhanced by adding CpG [27,32,33]. A pilot-scale production process was developed to manufacture a Pfs25-EPA conjugate in compliance with current good manufacturing practices [34], and a Pfs25-EPA/Alhydrogel® formulation was tested in Phase 1 trials in US and Mali adults [102,202]. Of particular interest, the Pfs25-EPA chemical conjugate appears as a nanoparticle in solution with a similar diameter to VLPs of the RTS,S vaccine [34]. A second Phase 1 trial in US and Mali adults is in progress testing a second generation of Pfs25-EPA conjugate vaccine, using a recombinant Pfs25 protein without any heterologous amino acids, alone and in combination with a recombinant subdomain of a *P. falciparum* gamete surface protein Pfs230 chemically conjugated to EPA [103,203]. Unfortunately, chemical conjugation of larger recombinant proteins may not provide a similar immunological benefit. For example, chemical conjugation of two recombinant proteins larger than 40 kDa to rEPA failed to provide any significant enhancement in immunogenicity when tested in small animals [35] [204].

Chemical conjugation of the nontoxic cholera toxin B subunit with recombinant Pvs25, a Pfs25 ortholog on *P. vivax* zygote surface, has been reported to increase the transmission reducing efficacy against *P. vivax* [36]. Miyata et al. also developed a novel strategy to increase immune responses by creating genetic fusion proteins to target specific APCs. The fusion complex was composed of three physically linked molecular entities: (i) A vaccine antigen, (ii) A multimeric alpha-helical coiled-coil core, and (iii) An APC-targeting ligand linked to the core via a flexible linker. Immunization of mice with the tricomponent complex fused to Pvs25 induced a robust antibody response and conferred substantial levels of *P. vivax* transmission blockade [37].

Epitope repetition within conjugates, in addition to particle formation is likely to be another mechanism of immune enhancement. Kubler-Kielb et al. demonstrated that polymerizing Pfs25 by chemical conjugation to itself improved Pfs25 immunogenicity and response longevity in mice [38]. The multimeric Pfs25 core, in addition to its own merit as a target antigen, was also used as a carrier for conjugation with the central repeats of PfCSP. The conjugation enhanced immunogenicity of the PfCSP repeats in mice [39],

likely due to the epitope repetition and the particulate nature of the conjugates.

### 3. Particulate adjuvants

#### 3.1. Liposomes

Liposomes are self-assembling spherical vesicles composed of a phospholipid bilayer enclosing an aqueous core. Liposomes can also be made as multilamellar in which multiple concentric lipid bilayers are separated by aqueous layers. Liposomes have versatile ways to deliver target antigens and additional immunomodulators as adjuvants. Depending upon chemical and biochemical properties, water-soluble target antigens can be entrapped within the aqueous phase, and lipophilic molecules can be intercalated within the lipid bilayer. Antigens and adjuvants can also be attached to the surface of liposomes by adsorption, covalent conjugation, and electrostatic interactions. Liposomes have been broadly applied to enhance T and B cell responses to subunit vaccines candidates, including several being evaluated in humans [4,40–42].

The most advanced application of the liposomal delivery system in malaria vaccine development is AS01, an Adjuvant System comprised of liposome, QS21, and MPL, a TLR4 agonist. In completion of a multi-center Phase 3 trial of RTS,S/AS01 involving >15,000 children including 6000 infants in malaria endemic regions, the adjuvant has an extensive safety profile [200]. In addition to RTS,S, several recombinant malaria antigens formulated with AS01 have been evaluated in humans. These include LSA-NRC, a recombinant protein comprised of N- and C-terminal regions and two of the central repeats of *P. falciparum* Liver Stage Antigen 1 and a candidate vaccine to block merozoite egress from hepatocytes, in a Phase 1 trial in US adults followed by a Phase 2a sporozoite challenge [43]; AMA1, a recombinant ectodomain of *P. falciparum* merozoite apical membrane antigen 1, in a Phase 1 trial in US adults followed by a Phase 2a sporozoite challenge [44]; and MSP1<sub>42</sub>, the 42 kDa N-terminal region of *P. falciparum* merozoite surface protein 1, in a Phase 1 trial in US adults followed by a second Phase 1 trial in Kyan adults [45]. All formulations demonstrated reassuring safety profiles. Despite the fact that none of these formulations showed any protection in challenge studies (for PfLSA-NRC and PfAMA1) or in growth inhibition assay (for PfMSP1<sub>42</sub>), all have elicited high titers of specific antibodies. In addition, PfLSA-NRC/AS01 formulation induced higher CD4<sup>+</sup> T cell response than did PfLSA-NRC formulated with AS02, another Adjuvant System and an oil-in-water emulsion also containing MPL and QS21 (see Section 3.2), indicating the role of liposomal formulation in induction of cellular responses [43]. However, no significant difference was found in CD4 responses induced by PfAMA1/AS01 or by PfAMA1/AS02 [44].

The PfAMA/AS01 and PfAMA1/AS02 were also evaluated in UK healthy adults, followed by a challenge with blood-stage parasites [104]. Also being evaluated in human trials were PfCelTOS/AS01 [105] and PvCSP/AS01 [106], two vaccine candidates to block sporozoite invasion of hepatocytes by *P. falciparum* and *P. vivax* parasites, respectively. In both studies the participants receiving the full intended dose were challenged with falciparum or vivax sporozoites. Anticipated analysis will shed light whether protective efficacies, if any, are associated with B cell or T cell responses, and the role of the adjuvant in these responses.

GLA-LSQ is a novel liposomal adjuvant consisting of liposome, QS21 and Glucopyranosyl Lipid A (GLA). Since GLA is a known TLR4 agonist [46], GLA-LSQ is expected to have similar adjuvanticity as AS01. With a recombinant full-length PfCSP produced in *Pseudomonas fluorescens* expression platform, NOE et al. evaluated the solubilized rPfCSP in various delivery systems including GLA-LSQ, GLA in stable emulsion (GLA-SE), and aluminum salts, with the

intention to select one for human use. Whereas the GLA-LSQ and GLA-SE formulations were comparable in antibody induction, in functional assays including *in vitro* inhibition of sporozoite invasion and *in vivo* protection against *Tg-Pb/PfCSP* sporozoite challenge, *rPfCSP*/GLA-LSQ formulation was a clear winner in induction of cellular and lasting antibody responses. Partial protection against sporozoite challenge was observed, and it would be interesting to know whether the protected animals had higher humoral and cellular responses [47].

While it is unclear how liposomes in ASO1 and GLA-LSQ interact with the target antigens, the cationic liposomal adjuvants clearly attract target antigens with opposite charges through electrostatic interaction. One of these is CAF01, a novel liposomal adjuvant composed of cationic lipid vesicles stabilized with a glycolipid immunostimulant TDB, a synthetic analogue of mycobacterial cell wall. A CAF01 formulated vaccine candidate against tuberculosis was shown to be safe and tolerable in humans, and induced strong lasting T-cell responses. However, there was no detectable antibody responses in humans, in contrast to observation in preclinical animal studies [42]. CAF01 has also been used to formulate *PyMSP1<sub>19</sub>* [48], a recombinant protein based on the 19 kDa C-terminal region of *Plasmodium yoelii* MSP1, and DBL4 $\epsilon$ -ID4, a recombinant subunit of *P. falciparum* VAR2CSA and a vaccine candidate to protect women against placental malaria [49]. *PyMSP1<sub>19</sub>*/CAF01 induced significantly higher IgG1 and IgG2 antibodies in mice than did the alum formulation, and conferred a stronger partial protection in mice from a blood stage parasite challenge. In the DBL4 $\epsilon$ -ID4/CAF01 study, the DBL4 $\epsilon$ -ID4 was also formulated with 2 other adjuvants: Alhydrogel and Montanide ISA720, a water-in-oil emulsion. Although antibody titers induced by all 3 formulations were similar, the DBL4 $\epsilon$ -ID4/CAF01 induced IgGs displayed a stronger inhibition of infected erythrocytes binding to chondroitin sulphate proteoglycans, and a stronger binding to the target antigen DBL4 $\epsilon$ -ID4.

Interbilayer-crosslinked multilamellar vesicle (ICMV) is another type of liposomal delivery system that shows promise. Moon et al. reported enhanced antigen-specific humoral responses when ICMVs were used to co-deliver a TLR4 agonist along with *PvCSP* that was either entrapped within the vesicle or entrapped as well as displayed on the surface of the vesicles ICMV [50]. The resulting ~180 nm particles elicited antibodies that were 9-fold higher in the formulation with entrapped and surface-displayed *PvCSP* compared to the group which did not have antigen on the surface. This vaccine delivery method allowed dose-sparing, generated a more balanced Th1/Th2 response with high avidity long-lasting antibodies. The response correlated with the induction of T<sub>H</sub> cells in germinal centers that formed adjacent to the vaccination site. These observations seem consistent with the mechanisms of immune enhancement by a particulate delivery system.

### 3.2. Stable emulsion

Emulsion-based nanoparticle adjuvants include oil-in-water (O/W) emulsions that interact with target antigens mainly by hydrophobic or electrostatic forces, and water-in-oil (W/O) emulsions that entrap target antigens in water droplets. They have been widely used in vaccine development, and the O/W emulsion MF59 and ASO3, and W/O emulsion Montanide<sup>®</sup> ISA51 are components of several licensed vaccines [51].

Following unsatisfactory outcomes in animal studies with MF59 adjuvanted malaria antigens including *P. falciparum* AMA1, MSP1, SERA, Pfs25, and *P. vivax* Duffy-binding protein, no further clinical development was justified with the adjuvant [52]. Despite superb immune-enhancement offered by Montanide<sup>®</sup> ISA51 and a similar W/O emulsion Montanide<sup>®</sup> ISA720, the adjuvants ceased to be the choice for clinical development due to high rate of local reactogenicity and unexpected systemic reactions in Phase 1 trials

[53,54]. On the other hand, ASO2, an O/W emulsion with additional immunostimulants MPL and QS21, has been shown in clinical trials to enhance immune responses of multiple malaria antigens including *P. falciparum* AMA1, MSP1 and LSA1, though the adjuvant did not increase their protective efficacy [43,44]. Although ASO2 was the initial adjuvant that delivered RTS,S with proven clinical efficacy, it was outperformed by RTS,S/ASO1 formulation for induction of humoral and cellular immunity, and protective efficacy [4,55].

GLA-SE, an O/W emulsion containing TLR4 agonist, was expected to have similar adjuvant activity as ASO1 due to their similar composition except for lacking QS21 in GLA-SE. Indeed, GLA-SE has a demonstrated capacity to elicit strong Th1 type immune responses. *PbCSP* and *PfCelTOS* formulated with GLA-SE induced higher level of IgG2 antibodies and more INF $\gamma$ -producing cells in mice [56,57]. GLA-SE also has a broad safety profile in humans: It has been injected in hundreds of humans in multiple Phase 1 trials evaluating vaccine candidates against various diseases. The completed or currently on-going Phase 1 trials with GLA-SE formulated recombinant malaria antigens include *PfCelTOS*, *PfAMA1*, and *PfP27A*, a synthetic polypeptide corresponding to the unstructured domain of *P. falciparum* Trophozoite exported protein 1 (Tex1) [107]. While results of these trials are not yet published, the attempt has been made to further increase the immune enhancing capacity of GLA-SE by inclusion of additional TLR7/8 agonists, such as R848. Unfortunately *PvSCP* formulated with GLA/R848-SE induced lower humoral and cellular responses in mice [58].

### 3.3. Others

Several other particulate delivery systems, including ISCOMs and biodegradable polymers, caught interest of malaria vaccine developers.

ISCOMs are cage-like particles composed of phospholipids, saponin-cholesterol micelles and the antigens incorporated into particles through hydrophobic interaction, which requires a hydrophobic tag if the target antigen is hydrophilic [59]. Subsequently, by using different lipids, Isomatrix<sup>®</sup> became a versatile adjuvant system that can entrap any type of target antigens, deliver the target antigens to APCs, and enhance both B cell and T cell responses in multiple species. Isomatrix<sup>®</sup> has been applied to over one thousand volunteers in Phase 1 and Phase 2 trials evaluating prophylactic and therapeutic vaccines, and was shown to be well tolerated. Regarding malaria antigens, Przysiecki et al. evaluated immunogenicity of (NANP)<sub>6</sub>-OMPC/MAA, an alum adsorbed, OMPC-conjugated peptide corresponding to the central repeats of *PfCSP*, with and without Isomatrix<sup>®</sup>. In in-bred mice, addition of Isomatrix<sup>®</sup> to the alum formulation did not have much effect on antibody titer, while in rhesus monkeys, addition of Isomatrix<sup>®</sup> in formulation induced a long-lived high-level anti-repeat antibodies compared to the animals immunized with the formulation without Isomatrix<sup>®</sup> [60]. The observations prompted cautions of using alum-formulation as a bench marker for adjuvant selection in mice, as the species and the adjuvant is widely used in initial proof-of-concept studies.

Biocompatible and biodegradable polymeric nanoparticles may represent a promising new technology for improved immune responses [61]. Their ability to sustain and control the release of the entrapped antigens and their delivery to APCs raised hope for eliciting long-lasting immune responses. Among a wide variety of synthetic polymers used to prepare nanoparticles, poly (lactic-co-glycolic) acid (PLGA) has been evaluated extensively and is already in several FDA-approved therapeutic delivery devices. By emulsifying PLGA and maleimide-activated lipids, Moon et al. created new PLGA particles enveloped with a lipid bilayer, which allowed covalent conjugation of thiolated *PvCSP* on the particle surface [62]. The resulting *PvCSP*-NP elicited durable antibody with significantly

higher titers and more balanced Th1/Th2 responses, compared to the PvCSP monomer. Antibody titers raised by PvCSP-NP were able to agglutinate *P. vivax* sporozoites, and exhibited higher avidity and affinity toward the recombinant protein. Using a derivative of PLGA, Dinglasan et al. prepared a nano-to-micron-sized particle incorporated with recombinant AnAPN1, an *Anopheles gambiae* aminopeptidase in mosquito midgut and a TBV candidate [63]. Immunization of the An-APN1 loaded particle induced functional antibodies, as measured by direct feeding of mosquitoes on immunized mice challenged with *P. berghei*, even 6 months after a single dose of vaccination.

#### 4. Reflection and conclusion

Since a strong safety profile in humans is paramount for development of prophylactic vaccines, a rather conservative approach has been taken in adjuvant/delivery selection for recombinant subunit-based malaria vaccine candidates. Nearly all clinical trials with recombinant malaria antigens started with aluminum salts as the adjuvant. Now it has become a consensus that recombinant subunit vaccines are unlikely to achieve desired protective efficacy without an appropriate delivery vehicle that includes not only adjuvant but also a proper antigen presentation format. Particulate delivery of vaccine antigens is advantageous as it can simulate a pathogen-mimicry by presenting the proteins as larger moieties, allowing more efficient uptake by APCs, stimulating innate immune responses through Pattern Recognition Receptors (PRRs), and providing depot effect for lasting responses. The licensure of particle-based vaccines such as HPVs and the accumulation of safety data in other vaccines in clinical trials has raised the hope of applying such delivery systems in malaria vaccine development without compromising the safety precaution.

Selection of a suitable delivery platform for a particular malaria antigen has been and will likely remain to be empirical. There are still technical hurdles in optimizing the delivery platforms, particularly with regard to particulate antigens such as VLPs, SAPNs, and antigen-carrier conjugates. There seems to be a size and density limit to the target antigens to be displayed on the particle surface. To remedy this we need a clear understanding as well as tools for characterization of the functional epitopes or subdomains within the target antigen, and their native conformation. The processes and conditions to preserve the native conformation during the synthetic or recombinant production of the target epitope or antigen have to be empirically determined. Other needs that have to be empirically determined are the processes and conditions to increase the antigen to core/carrier loading ratios. Finally during selection of the delivery platforms, the immune status of the target population needs to be considered. New born babies and young infants may have different adjuvant requirement as older children and adults. Vaccine must be “tailored” according to the immune status of subjects to be vaccinated.

While animal models are always used in initial proof-of-concept studies, the definitive evaluation of a delivery platform has to be trials conducted humans. It is an exciting time as multiple clinical trials have been conducted or are on-going to evaluate several particle-based delivery of subunit malaria antigens. Outcomes of these trials will not only help the selection of safe and potent delivery vehicles, but will also shed light on whether and/or how animal models predict the immune response and the protective efficacy in humans.

#### Disclaimer

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104	ClinicalTrials.gov identifier NCT02044198
105	ClinicalTrials.gov identifier: NCT02174978
106	ClinicalTrials.gov identifier: NCT01157897
107	ClinicalTrials.gov Identifiers: NCT01540474, NCT02014727, and NCT01949909

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