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Review

Nanoparticle vaccines

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ABSTRACT

Nanotechnology increasingly plays a significant role in vaccine development. As vaccine development orientates toward less immunogenic "minimalist" compositions, formulations that boost antigen effectiveness are increasingly needed. The use of nanoparticles in vaccine formulations allows not only improved antigen stability and immunogenicity, but also targeted delivery and slow release. A number of nanoparticle vaccines varying in composition, size, shape, and surface properties have been approved for human use and the number of candidates is increasing. However, challenges remain due to a lack of fundamental understanding regarding the *in vivo* behavior of nanoparticles, which can operate as either a delivery system to enhance antigen processing and/or as an immunostimulant adjuvant to activate or enhance immunity. This review provides a broad overview of recent advances in prophylactic nanovaccinology. Types of nanoparticles used are outlined and their interaction with immune cells and the biosystem are discussed. Increased knowledge and fundamental understanding of nanoparticle mechanism of action in both immunostimulatory and delivery modes, and better understanding of *in vivo* biodistribution and fate, are urgently required, and will accelerate the rational design of nanoparticle-containing vaccines.

1. Introduction

Vaccine development has a proud history as one of the most successful public health interventions to date. Vaccine development is historically based on Louis Pasteur's "isolate, inactivate, inject" paradigm. As vaccine development moves increasingly to draw on modern concepts of rational design, the number of candidate vaccines is increasing [1,2]. Most candidate vaccines represent "minimalist" compositions [3], which typically exhibit lower immunogenicity. Adjuvants and novel delivery systems that boost immunogenicity are increasingly needed as we move toward the era of modern vaccines.

Nanotechnology offers the opportunity to design nanoparticles varying in composition, size, shape, and surface properties, for application in the field of medicine [4,5]. Nanoparticles, because of their size similarity to cellular components, can enter living cells using the cellular endocytosis mechanism, in particular pinocytosis [6]. These cutting-edge innovations underpinned a market worth US $6.8 billion in 2006 [7] and predicted to reach US $160 billion by 2015 [8]. Indeed, nanoparticles are revolutionizing the diagnosis of diseases as well as the delivery of biologically-active compounds for disease prevention and treatment. The emergence of virus-like particles (VLPs) and the resurgence of nanoparticles, such as quantum dots and magnetic nanoparticles, marks a convergence of protein biotechnology with inorganic nanotechnology that promises an era of significant progress for nanomedicine [9,10]. A number of approved nano-sized vaccine and drug delivery systems highlight the revolution in disease prevention and treatment that is occurring [4,11–13].

The use of nanotechnology in vaccinology, in particular, has been increasing exponentially in the past decade (Fig. 1), leading to the birth of "nanovaccinology" [3]. In both prophylactic and therapeutic approaches, nanoparticles are used as either a delivery system to enhance antigen processing and/or as an immunostimulant adjuvant to activate or enhance immunity. Therapeutic nanovaccinology is mostly applied for cancer treatment [14–16], and is increasingly explored to treat other diseases or conditions, such as Alzheimer's [17], hypertension [9], and nicotine addiction [11]. Prophylactic nanovaccinology, on the other hand, has been applied for the prevention of different diseases. A number of prophylactic nanovaccines have been approved

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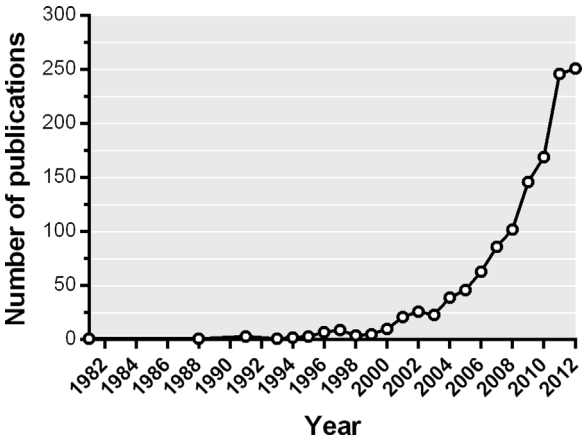

Fig. 1. Number of publications returned using the search terms "nanoparticle* and vaccin*" from Web of Science (http://apps.webofknowledge.com/; results for a search conducted on 29 July 2013).

for human use and more are in clinical or pre-clinical trials [13,18–20].

In this review, we provide an overview of recent advances in the broad area of nanovaccinology, but limit our review only to prophylactic vaccines. We first survey advances in the types of nanoparticles, which are defined as any particulate material with size 1–1000 nm [21], used for prophylactic vaccine design (Fig. 2). We then discuss the interaction of nanoparticles with the antigen of interest, differentiating the role of the nanoparticle as either delivery system and/or immunostimulant adjuvant. The interaction of nanoparticles with immune cells and the biosystem are also discussed to provide understanding of antigen and nanoparticle processing *in vivo*, as well as clearance. This latter aspect is of particular timeliness considering that there is limited history of safe use for non-VLP nanoparticles in humans. We then conclude with remarks about the further potential and future prospects for prophylactic nanovaccinology.

2. Types of nanoparticles

2.1. Polymeric nanoparticles

A great variety of synthetic polymers are used to prepare nanoparticles, such as poly(D,L-lactide-co-glycolide) (PLG) [22–24], poly(D,L-lactic-coglycolic acid)(PLGA) [22,25–30], poly(g-glutamic acid) (g-PGA) [31,32], poly(ethylene glycol) (PEG) [24], and polystyrene [33,34]. PLG and PLGA nanoparticles have been the most extensively investigated due to their excellent biocompatibility and biodegradability [35,36]. These polymeric nanoparticles entrap antigen for delivery to certain cells or sustain antigen release by virtue of their slow biodegradation rate [27–29,31,36]. PLGA has been used to carry antigen derived from various pathogens including *Plasmodium vivax* with mono-phosphoryl lipid A as adjuvant [37], hepatitis B virus (HBV) [22], *Bacillus anthracis* [29], and model antigens such as ovalbumin and tetanus toxoid [26,27]. g-PGA nanoparticles are comprised of amphiphilic poly(amino acid)s, which self-assemble into nano-micelles with a hydrophilic outer shell and a hydrophobic inner core [31,32]. g-PGA nanoparticles are generally used to encapsulate hydrophobic antigen [31,32]. Polystyrene nanoparticles can conjugate to a variety of antigens as they can be surface-modified with various functional groups [33,38].

Natural polymers based on polysaccharide have also been used to prepare nanoparticle adjuvants, such as pullulan [39,40], alginate [41], inulin [42,43], and chitosan [44–49]. In particular, chitosan-based nanoparticles have been widely studied due to their biocompatibility, biodegradability, nontoxic nature and their ability to be easily modified into desired shapes and sizes [31,50,51]. These nanoparticles have been used in the preparation of various vaccines including HBV vaccines [49], Newcastle disease vaccines [48], and DNA vaccines [44,46,47]. Inulin, a well-known activator of complement *via* the alternative pathway [52], is also a potent adjuvant. Nanoparticle adjuvants derived from inulin, such as Advax^{TM}, have shown enhancement of immune response in vaccines against various viruses including influenza [42] and hepatitis B [43].

Polymers, such as Poly(L-lactic acid) (PLA), PLGA, PEG, and natural polymers such as polysaccharides [41,53–55], have also been used to synthesize hydrogel nanoparticles, which are a type of nano-sized hydrophilic three-dimensional polymer network. Nanogels have favorable properties including flexible mesh size, large surface area for multivalent conjugation, high water content, and high loading capacity for antigens [55,56]. Chitosan nanogels have been widely used in antigen delivery, such as *Clostridium botulinum* type-A neurotoxin subunit antigen Hc for an adjuvant-free intranasal vaccine [57], and recombinant NcPDI antigen for *Neospora caninum* vaccination [58].

2.2. Inorganic nanoparticles

Many inorganic nanoparticles have been studied for their use in vaccines. Although these nanoparticles are mostly non-biodegradable, the advantage of them lies in their rigid structure and controllable synthesis [33]. Gold nanoparticles (AuNPs) are used in vaccine delivery [35], as they can be easily fabricated into different shapes (spherical, rod, cubic, *etc.*) [59] with a size range of 2–150 nm [60], and can be surface-modified with carbohydrates [61]. Gold nanorods have been used as a carrier for an antigen derived from respiratory syncytial virus by conjugating the antigen to the surface [62]. Other types of gold nanoparticles have been used as carriers for antigens derived from other viruses such as influenza [63] and foot-and-mouth disease [64], or as a DNA vaccine adjuvant for human immunodeficiency virus (HIV) [65].

Carbon nanoparticles are another commonly-studied composition for drug and vaccine delivery [60]. They are known for their good biocompatibility and can be synthesized into a variety of nanotubes and mesoporous spheres [66–68]. The diameter

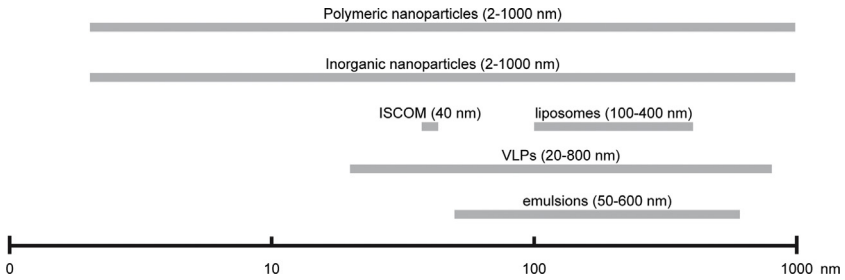

Fig. 2. The size range of nanoparticles used in nanovaccinology.

of carbon nanotubes (CNTs) used as carriers is generally 0.8–2 nm with a length of 100–1000 nm [69,70], while the size of mesoporous carbon spheres is around 500 nm [67]. Multiple copies of protein and peptide antigens can be conjugated on to CNTs for delivery and have enhanced the level of IgG response [67,69–71]. Mesoporous carbon nanoparticles have been studied for application as an oral vaccine adjuvant [67].

One of the most promising inorganic materials for nanovaccinology and delivery system design is silica. Silica-based nanoparticles (SiNPs) are biocompatible and have excellent properties as nanocarriers for various applications, such as selective tumor targeting [72], real-time multimodal imaging [73], and vaccine delivery. The SiNPs can be prepared with tunable structural parameters. By controlling the sol–gel chemistry, the particle size and shape of SiNPs can be adjusted to selectively alter their interaction with cells [74]. The abundant surface silanol groups are beneficial for further modification to introduce additional functionality, such as cell recognition, absorption of specific biomolecules, improvement of interaction with cells, and enhancement of cellular uptake [75–78]. In addition, porous SiNPs such as mesoporous silica nanoparticles (MSNs) and hollow SiNPs can be prepared by templating methods, which can be applied as a multifunctional platform to simultaneously deliver cargo molecules with various molecular weights [74]. MSNs with sizes in the range of 50–200 nm have been studied as both nano-carriers and adjuvants for delivery of effective antigens [79–81], such as those derived from porcine circovirus [82] and HIV [83]. MSNs can be used to control the release of antigens by controlling the shape, pore size and surface functionalization [79,84]. Compared to solid SiNPs, MSNs have higher loading capacity for their larger specific surface area, and better performance in delivery and controlled release due to the tunable hollow and mesoporous structure. In addition, MSNs can be degraded which can then be excreted in the urine [85–87]. With these properties, MSNs show potential to become high-efficiency, controlled-release nano-carriers in future vaccine formulations.

Calcium phosphate nanoparticles can be created by mixing calcium chloride, dibasic sodium phosphate and sodium citrate under specific conditions [88,89]. They are non-toxic and can be formed into a size of 50–100 nm [90]. These nanoparticles are useful adjuvants for DNA vaccines and mucosal immunity [79,88–90], and show excellent biocompatibility.

2.3. Liposomes

Liposomes are formed by biodegradable and nontoxic phospholipids. Liposomes can encapsulate antigen within the core for delivery [91] and incorporate viral envelope glycoproteins to form virosomes [92,93] including for influenza [94]. Combination of 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) modified cationic liposome and a cationic polymer (usually protamine) condensed DNA are called liposome-polycation-DNA nanoparticles (LPD), a commonly used adjuvant delivery system in DNA vaccine studies [95,96]. The components of LPD spontaneously rearrange into a nano-structure around 150 nm in size with condensed DNA located inside the liposome [96]. Liposomes modified with maleimide can be synthesized into interbilayer-crosslinked multilamellar vesicles (ICMVs) by cation driven fusion and crosslinking [97] enabling slowed release of entrapped antigen. A number of liposome systems have been established and approved for human use, such as Inflexal® V and Epaxal®, which have been discussed in other reviews [91,98].

2.4. Immunostimulating complex (ISCOM)

ISCOMs are cage like particles about 40 nm large in size, made of the saponin adjuvant Quil A, cholesterol, phospholipids, and protein antigen [35,92,99–101]. These spherical particles can trap the antigen by apolar interactions [35]. ISCOMATRIX comprises ISCOMs without antigen [35,92,100,102]. ISCOMATRIX can be mixed with antigen, enabling a more flexible application than is possible for ISCOMs, by removing the limitation of hydrophobic antigens [35]. Various antigens have been used to form ISCOMs, including antigens derived from influenza [103,104], herpes simplex virus [105], HIV [106], and Newcastle disease [99].

2.5. Virus-like particles

Virus-like particles (VLP) are self-assembling nanoparticles, lacking infectious nucleic acid, formed by self-assembly of biocompatible capsid proteins [107,108]. VLPs are the ideal nanovaccine system as they harness the power of evolved viral structure, which is naturally optimized for interaction with the immune system, but avoid the infectious components. VLPs take the good aspects of viruses and avoid the bad. The naturally-optimized nanoparticle size and repetitive structural order means that VLPs induce potent immune responses, even in the absence of adjuvant [109]. VLP based vaccines are the first nanoparticle class to reach market – the first VLP vaccine for hepatitis B virus was commercialized in 1986 [110] – and have become widely administered in healthy populations. In nanovaccinology, VLP nanoparticles have the strongest evidence base for safe use in healthy humans. Newer VLP vaccines for human papillomavirus [111] and hepatitis E [112] have been approved for use in humans in 2006 and 2011, respectively.

VLPs can be derived from a variety of viruses (Fig. 3) [107], with sizes ranging from 20 nm to 800 nm [13,113], and can be manufactured with a variety of process technologies [114]. The historical approach to VLP manufacture involves an *in vivo* route, where the assembly of capsid proteins into VLPs occurs inside the expression host. The assembled particle is then purified away from adherent and encapsulated contaminants. In some cases it becomes necessary to disassemble and then re-assemble the VLP to improve quality [114]; recently-approved VLP vaccines typically include some aspect of extracellular assembly within the processing regime. An emerging approach for VLP assembly is through cell-free *in vitro* processing [115–119]. This approach inverts the traditional assemble-then-purify paradigm; large-scale purification of the VLP building blocks from contaminants occurs first, then these are assembled *in vitro*, avoiding the need to disassemble VLP structures after assembly in a cell. Further review of VLP manufacturing approaches is available elsewhere [13,19,120,121].

VLPs commercialized to date are based on self-assembly of proteins derived from the target virus. However, VLPs can also act as a delivery platform where a target antigen from a virus unrelated to the VLP used is modularized on the surface of a VLP [20,122–125]. These modular VLPs exploit known benefits of VLPs (optimized particle size and molecular structure) to target disease in an engineered fashion. With many VLP vaccines currently in clinical or pre-clinical trials [13,19], an increase in the number of approved VLP-based vaccines can be expected.

2.6. Self-assembled proteins

Recognizing the power of the VLP approach, self-assembling systems that attempt to drive higher levels of protein quaternary structuring have emerged for the preparation of nanoparticle-based vaccines. Ferritin is a protein that can self-assemble into nearly-spherical 10 nm structure [126]. By genetically fusing influenza virus haemagglutinin (HA) to ferritin, the recombined protein spontaneously assembled into an octahedrally-symmetric particle and reformed 8 trimeric HA spikes [126] to give a higher immune response than trivalent inactivated influenza vaccine, which typically is processed to destroy rather than build viral

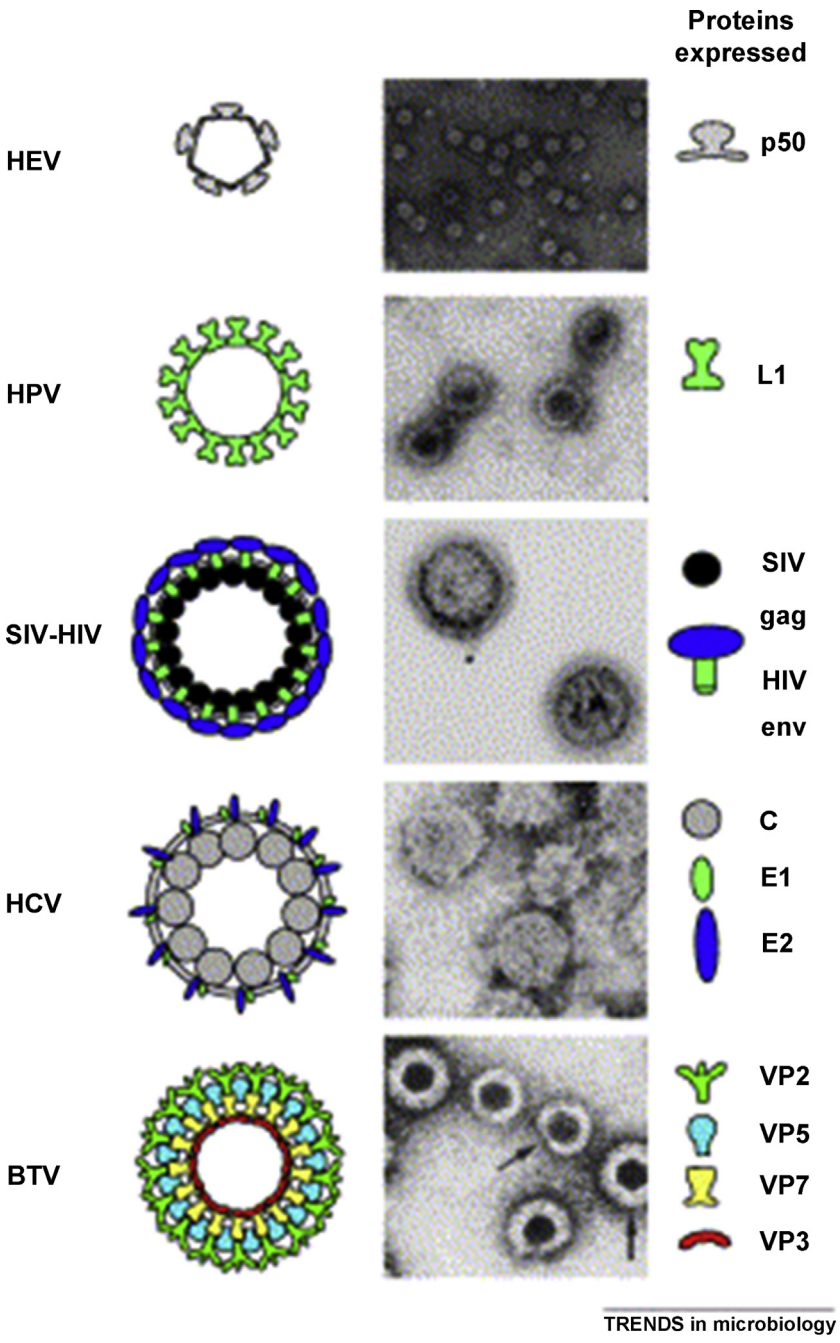

Fig. 3. Structure of virus-like particles. Virus-like particles can be derived from a variety of viruses. HEV, hepatitis E virus; HPV, human papillomavirus 16; SIV-HIV, hybrid VLP between simian immunodeficiency virus gag and human immunodeficiency virus *env*; HCV, hepatitis C virus; BTV, bluetongue virus.

Reprinted from Trends in Microbiology, Vol. 11, Issue 8, Rob Noad and Polly Roy, Virus-like particles as immunogens, Pages 438–444, Copyright (2003), with permission from Elsevier [107].

structure. This example highlights the importance of driving higher-order molecular structure in modern vaccines. The major vault protein (MVP) is another kind of self-assembling protein. Ninety-six units of MVP can self-assemble into a barrel-shaped vault nanoparticle, with a size of approximately 40 nm wide and 70 nm long [127]. Antigens that are genetically fused with a minimal interaction domain can be packaged inside vault nanoparticles by self-assembling process when mixed with MVPs [127]. Vault nanoparticles have been used to encapsulate the major outer membrane protein of *Chlamydia muridarum* for studies of mucosal immunity [127].

2.7. Emulsions

Another type of nanoparticles used as adjuvants in vaccines delivery is nano-sized emulsions [100,128,129]. These nanoparticles can exist as oil-in-water or water-in-oil forms, where the droplet size can vary from 50 nm to 600 nm [128]. Emulsions can carry antigens inside their core for efficient vaccine delivery [128] or can also be simply mixed with the antigen. One commonly-used emulsion is MF59^{TM}, an oil-in-water emulsion which has been licensed as a safe and potent vaccine adjuvant in over 20 countries [35,130]. It has been widely studied for use in influenza vaccines

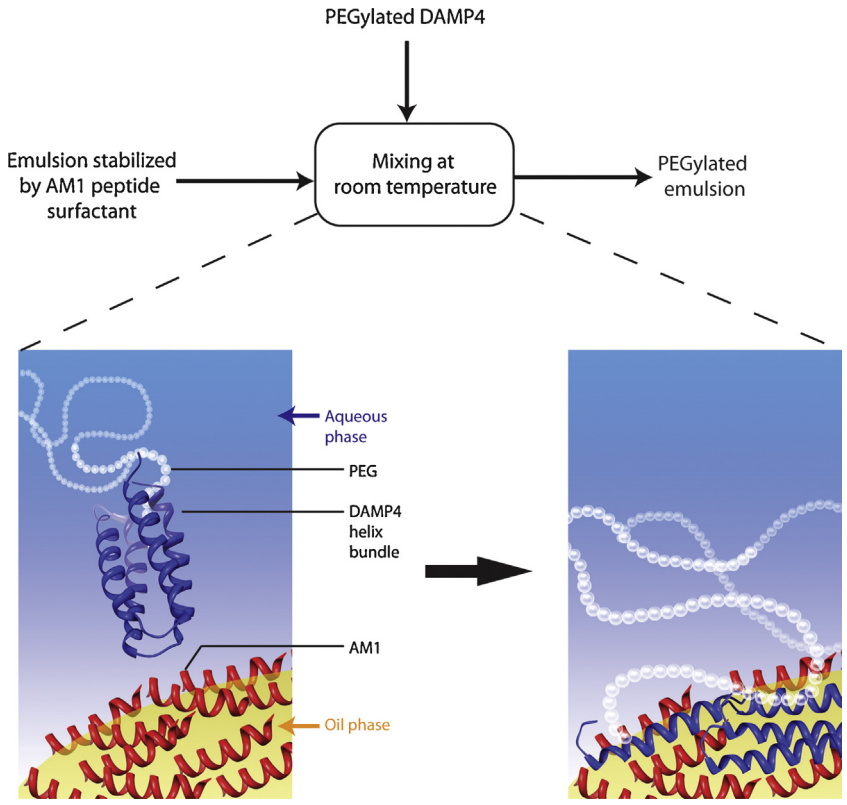

Fig. 4. Schematic representation of PEG (white) chemically conjugated to DAMP4 protein (dark blue) being introduced to a solution containing pre-formed nanoemulsion oil core (light yellow) stabilized by AM1 peptide (red), in aqueous buffer (light blue background). DAMP4 protein, which is chemically similar to AM1 peptide, is able to integrate into the oil-water interface formed between the core and the aqueous bulk. Prior conjugation of PEG to DAMP4 leads to its functional display at the interface through non-covalent molecular self-assembly. (For interpretation of the references to color in figure legend, the reader is referred to the web version of the article.)

Reprinted from Small, http://dx.doi.org/10.1002/smll.201300078, B.J. Zeng, Y.P. Chuan, B. O'Sullivan, I. Caminschi, M.H. Lahoud, R. Thomas, A.P.J. Middelberg, Receptor-Specific Delivery of Protein Antigen to Dendritic Cells by a Nanoemulsion Formed Using Top-Down Non-Covalent Click Self-Assembly, Copyright (2003), with permission from John Wiley and Sons [138].

[130–132]. Another is Montanide™, a large family of both oil-in-water and water-in-oil emulsions, including ISA 50 V, 51, 201, 206 and 720 [35,133]. Montanide ISA 51 and 720 have been used in Malaria vaccines [134,135], Montanide ISA 201 and 206 have been used in foot-and-mouth disease vaccines [136].

Recently, a tailorable nano-sized emulsion (TNE) platform technology has been developed using non-covalent click self-assembly for antigen and drug delivery [137,138]. An oil-in-water nanoemulsion is formed using designed biosurfactant peptides and proteins. Using a self-assembling peptide-protein system, immune-evading PEG and a receptor-specific antibody can be arrayed in a selectively proportioned fashion on the aqueous interface of a nano-sized oil-in-water emulsion (Fig. 4). Targeted delivery of protein antigen to dendritic cells was achieved [138]. This work demonstrates a new and simple way to make biocompatible designer nanoemulsions using non-covalent click self-assembly by sequential top-down reagent addition.

3. Nanoparticle interaction with antigen

Vaccine formulations comprising nanoparticles and antigens can be classified by nanoparticle action into those based on delivery system or immune potentiator approaches. As a delivery system, nanoparticles can deliver antigen to the cells of the immune system, *i.e.* the antigen and nanoparticle are co-ingested by the immune cell, or act as a transient delivery system, *i.e.* protect the antigen and then release it at the target location [79]. For nanoparticles to function as a delivery system, association of antigen and nanoparticle is typically necessary. For immune potentiator approaches, nanoparticles activate certain immune pathways which might then enhance antigen processing and improve immunogenicity.

Hard material nanoparticles, such as those based on silica, gold, and calcium phosphate, have predominantly been examined for use as a delivery system [139] and have thus been engineered to promote antigen attachment. Attachment of antigen has been achieved through simple physical adsorption or more complex methods, such as chemical conjugation or encapsulation (Fig. 5). Adsorption of antigen onto a nanoparticle is generally based simply on charge or hydrophobic interaction [79,140,141]. Therefore, the interaction between nanoparticle and antigen is relatively weak, which may lead to rapid disassociation of antigen and nanoparticle *in vivo*. Encapsulation and chemical conjugation provide for stronger interaction between nanoparticle and antigen. In encapsulation, antigens are mixed with nanoparticle precursors during synthesis, resulting in encapsulation of antigen when the precursors particulate into a nanoparticle [88]. Antigen is released only when the nanoparticle has been decomposed *in vivo* or inside the cell. On the other hand, for chemical conjugation, antigen is chemically cross-linked to the surface of a nanoparticle [142]. Antigen is taken up by the cell together with the nanoparticle and is then released inside the cell. In soft matter nanoparticle delivery system, such as those based on VLPs, ISCOM, ISCOMATRIX™, or liposomes, attachment of antigen is achieved through chemical conjugation, adsorption, encapsulation, or fusion at DNA level [91,94,101,102,123–125].

For nanoparticles to act as an immune potentiator, attachment or interaction between the nanoparticle and antigen is not

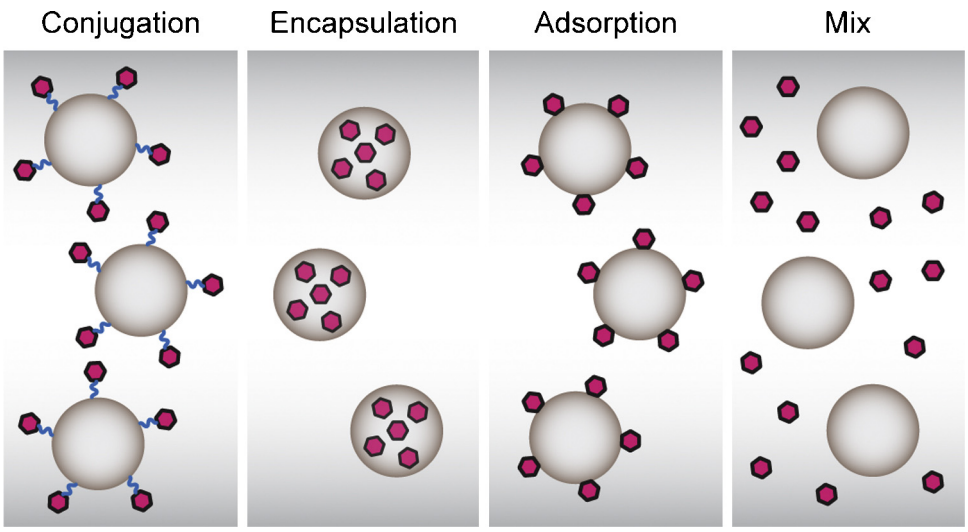

Fig. 5. Interaction of nanoparticle with antigen of interest. Formulation of nanoparticle and antigen of interest can be through attachment (*e.g.* conjugation, encapsulation, or adsorption) or simple mixing.

necessary, and may be undesirable in cases where modification of antigenic structure occurs at the nanoparticle interface. Soft-matter nanoparticles, such as emulsion-based adjuvants MF59™ and AS03™, have been shown to adjuvant a target antigen even when they are injected independently of, and before, the antigen [143,144]. Building on this idea, formulation of immune potentiator nanoparticles with a target antigen could be possible through simple mixing of nanoparticle and adjuvant, shortly prior to injection, with minimal association between nanoparticle and antigen needed. This approach has only recently been investigated for hard-material nanoparticle adjuvants, with results suggesting that nanoparticles may act as a size-dependent immune potentiator adjuvant even when not conjugated to the antigen [145]. This new finding is consistent with a number of other studies that have demonstrated induction of inflammatory immune responses after injection of hard material nanoparticles alone and without antigen [146,147]. Further studies into the use of nanoparticles as immune-potentiating adjuvants are clearly needed. As the interaction of nanoparticles with the immune system becomes more fully understood, we expect their impact to be broadened.

4. Nanoparticle interactions with antigen presenting cells

Incorporating antigenic components into nanoparticles has attracted extensive interest with a focus on how to deliver antigen more efficiently to antigen presenting cells (APCs) and subsequently induce their maturation and cross presentation of antigen for activation of a potent immune response [148–152]. As specialized APCs which efficiently uptake and process antigen, dendritic cells (DCs) and macrophages are often targeted in vaccine design. Good understanding of DC and macrophage uptake mechanisms and interactions of NPs with these cells is therefore very important for developing efficacious nanoparticle vaccines [153–155]. Studies have reported that size, charge and shape of nanoparticles play significant roles in antigen uptake.

Generally, nanoparticles having a comparable size to pathogens can be easily recognized and are consequently taken up efficiently by APCs for induction of immune response [156–162]. DCs preferentially uptake virus-sized particles (20–200 nm) while macrophages preferentially uptake larger particles (0.5–5 μm) [156]. In an *in vitro* study using polystyrene particles ranging from 0.04 μm to 15 μm, the optimum size for DC uptake was found to be smaller than 500 nm [163]. Similarly, 300 nm sized PLGA particles also showed higher internalization and activation of DCs in comparison to 17, 7 and 1 μm particles [164]. Higher uptake of smaller PLA particles (200–600 nm) in comparison to larger ones (2–8 μm) has also been reported for uptake by macrophages [165]. Different studies however, show discrepancies in optimum nanoparticle vaccine size. Amphiphilic poly(amino acid) (PAA) nanoparticles of 30 nm were shown to have a lower DC uptake than that of 200 nm nanoparticles [166]. Polyacrylamide hydrogel particles of 35 nm and 3.5 μm in size showed no difference in macrophages uptake [167]. These discrepancies may be related to the intrinsic differences in the material properties, with each material having an optimum size for induction of potent immune response [168].

In addition to particle size, surface charge also plays a significant role in the activation of immune response. Cationic nanoparticles have been shown to induce higher APC uptake due to electrostatic interactions with anionic cell membranes [163]. *In vitro* studies suggested that a cationic surface could significantly enhance the uptake of polystyrene particles of micron size (~1 μm) by macrophages and DCs in comparison with a neutral or negative surface [163,169,170], but not for the smaller nanoparticles (100 nm) [163]. However, other *in vivo* studies revealed that either positively [171] or negatively charged [172] liposomes could act as efficient adjuvants to induce cell-mediated immune response. Furthermore, due to their electrostatic interaction with anionic cell membranes, cationic particles are more likely to induce hemolysis and platelet aggregation than neutral or anionic particles [173].

Particle shape plays an equally important role in the interaction between nanoparticles and APCs. For big particles (>1 μm), particle shape plays a dominant role in phagocytosis by macrophages as the uptake of particles is strongly dependent on the local shape at the interface between particles and APCs [174]. Worm-like particles with high aspect ratios (>20) exhibited negligible phagocytosis compared to spherical particles [175]. On the other hand, spherical gold nanoparticles (AuNPs) (40 nm) were more effective in inducing antibody response than other shapes (cube and rod) or the 20 nm-sized AuNPs, even though the rods (40 nm × 10 nm) were more efficient in APC uptake than the spherical and cubic AuNPs [59].

A number of studies also reported the effect of hydrophobicity, showing higher immune response for hydrophobic particles than hydrophilic ones [176,177]. A number of other factors such as

surface modification (pegylation, targeting ligands) and vaccine cargo [45] have been shown to affect the interaction between nanoparticles and APCs as well.

5. Nanoparticle-biosystem interactions

Designing safe and efficacious nanoparticle vaccines requires a thorough understanding of the interaction of nanoparticles with biological systems which then determines the fate of nanoparticles *in vivo*. Physicochemical properties of nanoparticles including size, shape, surface charge, and hydrophobicity influence the interaction of nanoparticles with plasma proteins [178,179] and immune cells [176]. These interactions as well as morphology of vascular endothelium play an important role in distribution of nanoparticles in various organs and tissues of the body.

The lymph node (LN) is a target organ for vaccine delivery since cells of the immune system, in particular B and T cells, reside there. Ensuring delivery of antigen to LNs, by direct drainage [180,181] or by migration of well-armed peripheral APCs [182], for optimum induction of immune response is therefore an important aspect of nanoparticle vaccine design. Distribution of nanoparticles to the LN is mainly affected by size [183,184]. Nanoparticles with a size range of 10–100 nm can penetrate the extracellular matrix easily and travel to the LNs where they are taken up by resident DCs for activation of immune response [184–187]. Particles of larger size (>100 nm) linger at the administration point [181,186,188] and are subsequently scavenged by local APCs [181,187,189], while smaller particles (<10 nm) drain to the blood capillaries [184,189]. The route of administration and biological environment to which nanoparticles are exposed could also affect the draining of nanoparticles to the LN. It was reported that small PEG coated liposomes (80–90 nm) were significantly present in larger amounts in LNs after subcutaneous administration as compared to intravenous and intraperitoneal administration [190].

In addition to targeting lymphatic organ for efficient activation of immune response, design of nanoparticle vaccines also needs to consider nanoparticle clearance from the body. Adverse effects may occur when nanoparticles are not degraded or excreted from the body and hence, accumulate in different organs and tissues. Clearance of nanoparticles could be achieved through degradation by the immune system or by renal or biliary clearance.

Renal clearance through kidneys can excrete nanoparticles smaller than 8 nm [191,192]. Surface charge also plays an important role in determining renal clearance of nanoparticles. Few reports have suggested that for appropriate identically sized particles, based on surface charge, ease of renal clearance follows the order of positively-charged < neutral < negatively charged [193,194]. This may be attributed to the presence of negatively-charged membrane of glomerular capillary [195].

On the other hand, biliary clearance through liver allows excretion of nanoparticles larger than 200 nm [191,196]. Surface charge also plays role in biliary clearance with increase in surface charges showing increased distribution of nanoparticles in the liver [197]. Furthermore, a study reported shape dependent distribution of nanoparticles where short rod nanoparticles were predominantly found in liver, while long rods were found in spleen. Short rod nanoparticles were excreted at a faster rate than longer ones [198].

In order to aid understanding of interaction of nanoparticles with immune cells and the biosystem, many different *in vivo* molecular imaging techniques including magnetic resonance imaging (MRI), positron emission tomography (PET), fluorescence imaging, single photon emission computed tomography (SPECT), X-ray computed tomography (CT) and ultrasound imaging could be employed. Owing to its excellent soft tissue contrast and non-invasive nature, MRI imaging is extensively used for obtaining three-dimensional images *in vivo*. Superparamagnetic iron oxide nanoparticles (SPION) have been extensively used as contrast agents for morphological imaging [199,200]. PET usually employs an imaging device (PET scanner) and a radiotracer that is usually intravenously injected into the bloodstream. Due to high sensitivity of this technique, it is used to study the biodistribution of particles of interest. The only disadvantage of this technique is relatively low spatial resolution as compared to other techniques. PET imaging of ^{64}Cu radiolabelled shell-crosslinked nanoparticles has been demonstrated [201]. Fluorescence imaging facilitates imaging of nanoparticles using fluorescent tags. Dye-doped silica nanoparticles as contrast imaging agents for *in vivo* fluorescence imaging in small animals have been reported [202].

Nowadays, more attention is being paid to synergize two or more imaging techniques that complement each other and provide an opportunity to overcome shortcomings of individual techniques in terms of resolution or sensitivity. For instance, simultaneous PET-MRI imaging is a new emerging hybrid imaging system that combines the morphological imaging component of MRI with the functional imaging component of PET [203]. Multifunctionality of nanoparticles can be utilized for such hyphenated imaging.

6. Concluding remark

Nanoparticle-containing vaccines have attracted tremendous interest in recent years, and a wide variety of nanoparticles have been developed and employed as delivery vehicles or immune potentiators, allowing not only improvement of antigen stability and the enhancement of antigen processing and immunogenicity, but also the targeted delivery and slow release of antigens. In addition, nanoparticles have been increasingly used to deliver not only antigen of interest but also co-adjuvant, such as poly(I:C), CpG and MPL [188,204]. However, the application of nanoparticles in vaccine delivery as well as in drug delivery is still at an early stage of development. A number of challenges remain, including difficulty in reproducibly synthesizing non-aggregated nanoparticles having consistent and desirable properties, a lack of fundamental understanding of how the physical properties of nanoparticles affect their biodistribution and targeting, and how these properties influence their interactions with the biological system at all levels from cell through tissue and to whole body. Therefore, rational design in combination with the reproducible production of nanoparticles with desirable properties, functionalities and efficacy becomes increasingly important, and it is anticipated that the adoption of new technologies, for example microfluidics, for the controlled synthesis of nanoparticles will accelerate the development of suitable nanoparticles for pharmaceutical applications [205]. Furthermore, by integrating some other attractive properties, such as slow release, targeting and alternative administration methods and delivery pathways, novel vaccine systems for unmet needs including single-dose and needle-free delivery will become practical in the near future.

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