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REVIEW

Immunological basis for enhanced immunity of nanoparticle vaccines

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ABSTRACT

Introduction: Immunization has been a remarkably successful public health intervention; however, new approaches to vaccine design are essential to counter existing and emerging infectious diseases which have defied traditional vaccination efforts to date. Nanoparticles (ordered structures with dimensions in the range of 1–1000 nm) have great potential to supplement traditional vaccines based upon pathogen subunits, or killed or attenuated microorganisms, as exemplified by the successful licensure of virus-like particle vaccines for human papillomavirus and hepatitis B. However, the immunological mechanisms that underpin the potent immunity of nanoparticle vaccines are poorly defined.

Areas covered: Here, we review the immunity of nanoparticle immunization. The display of antigen in a repetitive, ordered array mimics the surface of a pathogen, as does their nanoscale size. These properties facilitate enhanced innate immune activation, improved drainage and retention in lymph nodes, stronger engagement with B cell receptors, and augmented T cell help in driving B cell activation.

Expert opinion: In the near future, increasingly complex nanoparticle vaccines displaying multiple antigens and/or co-delivered adjuvants will reach clinical trials. An improved mechanistic understanding of nanoparticle vaccination will ultimately facilitate the rational design of improved vaccines for human health.

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1. Introduction

From a crude extract of cowpox pus to a highly tunable, precision-engineered nanoparticle, vaccine concepts have evolved dramatically over the past 200 years. The global deployment of vaccines capable of limiting or preventing infectious diseases has been a highly successful public health initiative, preventing 2–3 million deaths each year [1]. The first commonly used inoculations contained whole organisms killed by heat or chemical treatment, or alternatively, live-attenuated strains that could cross-protect against virulent organisms. While potently immunogenic, due to dense antigen arrays and the inclusion of pathogenic 'danger signals', such vaccines can be highly reactogenic, potentially triggering some illness in their own right [2,3]. For some pathogens, a difficulty in culturing the organism makes it impossible to reliably prepare attenuated vaccines. Despite these limitations, live-attenuated and/or inactivated viral vaccines are still widely used to prevent serious viral diseases including yellow fever, measles, and polio. In a move to limit excessive reactogenicity, subunit vaccines comprising a well-defined purified protein, polysaccharide or other pathogen component were subsequently developed. While demonstrating an excellent safety profile, these vaccines often suffer from poor immunogenicity and require co-formulation with adjuvants and booster injections to maintain immunity [4]. Over the past 50 years, subunit vaccines have been remarkably effective in preventing childhood illnesses including whooping cough and meningococcal disease. But despite the overwhelming success of immunization, both whole organism and subunit vaccine have demonstrably failed to induce adequate immunity for many globally important diseases. Communicable diseases still account for approximately one in five of all deaths worldwide [5]. Protection against tuberculosis, malaria, and HIV have defied traditional empirical approaches to vaccine development. Furthermore, vaccines for influenza, another leading cause of global mortality, are hampered by limited global production capacity, the evolution of escape variants during production and in the field and rapidly waning protective efficacy. Alongside the major causes of communicable disease-mortality detailed above, emerging infections in developing countries are also a challenge in vaccine development with multiple vaccines currently in clinical trials for both Zika and Ebola. All of these important human pathogens require novel vaccines that ideally would be rapid to produce, low cost, easy to transport, formulate and administer, heat stable, safe, highly immunogenic, and provide life-long immunity from a single dose [6]. Modern nanoparticle-based vaccine development efforts, combining a suite of newly available informatics, materials science, genetics, and molecular tools, offers a chance to specifically engineer maximally protective immunity against tenacious pathogens.

2. Nanoparticle vaccines

The term 'nanoparticle' has wide usage in the literature and could delineate a number of vaccine modalities. Here, we use the term to broadly apply to non-replicative, organized

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Article Highlight

- New vaccine technologies are needed for important global pathogens.
- Small (<1000 nm) particle-based vaccine technologies – either virus-like particles or synthetic nanoparticles – offer great potential for improved vaccination strategies.
- Components of the immune system – including complement and IgM – bind to repetitive surfaces that can be mimicked by the presentation of vaccine antigens on nanoparticles.
- Nanoparticle vaccines can be finely tuned to express adjuvants and targeting motifs.
- Trafficking of small nanoparticles to regional lymph nodes can be achieved to facilitate enhanced immunogenicity.

structures on a nanoscale of 1–1000 nm produced by recombinant expression [7] or chemical synthesis [8]. The composition of materials used for nanoparticle generation is extremely diverse. Virus-like particles (VLP) take advantage of the self-assembling properties of viral antigens to form non-infectious particles lacking genetic material. VLPs can be antigens in their own right, or alternatively, harnessed as a scaffold for the delivery of heterologous antigens. Virosomes are ~150 nm vesicles consisting of phospholipids combined with integrated viral envelope proteins and further combined with vaccine antigens from viral or non-viral pathogens. For bacterial and parasitic pathogens, as well as viruses lacking self-assembling capsid proteins, VLPs, and other self-assembling proteins can be utilized as scaffolds to display antigen. While biological nanoparticles have thus far predominated nanovaccine trials, inorganic and chemically synthesized nanoparticles including metals [9] and synthetic polymers [10] are being explored in preclinical studies. Nanoparticles have potential advantages over classical vaccines, making them attractive platforms for vaccine development. In this review, we describe how the material composition of nanoparticles and their physicochemical properties, in addition to the display of antigen and adjuvanting molecules, all contribute to the resulting immunogenicity of a nanoparticle vaccine.

2.1. Virus-like particle vaccines

The first manufactured VLP vaccines to reach the market in the late 1980s were Recombivax-HB and Engerix-B which both comprise self-assembling particles formed by recombinant expression of the hepatitis B virus (HBV) surface antigen (HBsAg). A difficulty in culturing HBV made a whole organism vaccine unfeasible. HBsAg particles are produced in excess during natural infection and assemble in the blood, mimicking the size (22 nm), shape, and repetitive display of HBV but lacking any genetic material. Unsuccessful attempts to express immunogenic HBsAg particles in E. coli demonstrated the need to use an expression host delivering the appropriate glycosylation [11]. The demonstrable success of the HBsAg-VLP approach drove the development of investigational conjugate vaccines where antigens from other pathogens were loaded onto the HBV platform. For example, the only vaccine to demonstrate partial protection against malarial infection, RTS,S/AS01, is a VLP consisting of 18 copies of a portion of the major circumsporozoite protein of Plasmodium falciparum (PfCSP) fused to HBsAg combined with a liposomal adjuvant [12].

Other widely used VLP vaccines are those used to prevent human papillomavirus (HPV) infection. As of 2017, 71 countries include these vaccines in their national immunization program [13]. The efficacy rate at preventing infection with the HPV strains included in the vaccines is very close to 100% and the safety record is excellent [13]. The HPV L1 major capsid protein self-assembles into hollow 55 nm VLPs displaying 72 pentameric antigens. The lack of immunogenicity from vaccinating with denatured L1 protein subunits illustrates the requirement for correct assembly and display of antigenic epitopes [14]. HBV and HPV VLP vaccines are both self-assembling nanoscale displays of multivalent antigens. However, HBV VLPs require multiple vaccination boosts to consistently achieve seroconversion [15] and unlike HPV VLPs, lack the induction of long-lived antibody-producing plasma cells [16]. The differences between HPV and HBV in antigen valency (72 pentamers versus 24 protrusions) and particle size (55 nm versus 22 nm) may result in divergent patterns of lymphatic drainage, innate immune interactions, and cell uptake [16]. Unlike inactivated or whole cell vaccines, VLPs do not require inactivation, which may crosslink antigens and disrupt the native conformation of B cell epitopes. Furthermore, their composition is highly defined and production is easily scalable. While beyond the scope of this review, differences in VLP production systems are reviewed in detail elsewhere [17]. In addition to HPV, HBV, and malaria, VLP vaccines have been applied for wide-ranging pathogens including Hepatitis E [18] and West Nile virus [19]. The advantages and disadvantages of VLP vaccines over other particulate vaccine platforms are compared in Table 1.

2.2. Non-VLP nanoparticle vaccines

In addition to VLPs, nanoparticle vaccines for human immunization could consist of virosomes and self-assembling recombinant proteins. In common with VLPs, virosomes have a particulate structure which mimics a virus and are unable to replicate. Unlike VLPs, virosomes are assembled in vitro, not

Table 1. Advantages and disadvantages of VLP-based nanovaccines compared to non-VLP nanovaccines.

	VLP-nanovaccine	Non-VLP nanovaccine
Licensed Examples	• Hepatitis B virus VLP • Human Papillomavirus VLP	• Influenza virosome • Hepatitis A virosome
Advantages	• Takes advantage of natural assembly using viral proteins • Composed almost entirely of viral antigen • Models virus structure accurately	• Not confined to specific structure • Can be finely tuned for size/shape/charge • Can alter valency and include diverge antigens • Can more readily co-formulate with adjuvants
Disadvantages	• May need multiple VLPs to cover pathogenic strains • Commonly manufactured using cell lines	• Manufacture may be more challenging • Immune responses against scaffold can limit efficacy • Must be biocompatible

Abbreviation: VLP, virus-like particle.

by a genetically engineered host cell, allowing greater control over particle assembly. While virosomes containing envelope proteins from a wide range of viruses including Rabies, Hepatitis B and HIV have been generated, only virosomes incorporating haemagglutinin (HA) and neuraminidase proteins from the influenza viral envelope have been licensed as human vaccines. Pre-existing immunity to influenza can augment the immune response making virosomes both a carrier and an adjuvant system. This rational has been applied to produce vaccines for Influenza (Inflexal) [20] and Hepatitis A (Epaxal) [21].

Self-assembling proteins can also be used as antigen scaffolds. For example, fusing the gene encoding the iron storage protein ferritin from Helicobacter pylori bacteria to the influenza virus HA protein resulted in the presentation of eight trimeric HA molecules in their native confirmation around a spherical ferritin core [7]. This vaccine displayed superior protective efficacy against influenza challenge in animal models and in currently entering human clinical trials. Examples of other nanoparticle vaccines currently in clinical trials are outlined in Table 2. Notably, all the vaccines that have achieved licensure so far have been organic in nature, composed of protein and/or lipid, likely indicative of a more straightforward path to regulatory approval compared to many non-biological materials. Nonetheless, a broad range of nanoparticle vaccines for infectious diseases (including synthetic platforms) are in pre-clinical development.

Table 2. Examples of particle-based vaccines for infectious diseases that have been licensed or are in clinical development.

Disease	Material/Platform	Clinical Phase	Trial Number/ Trade name	Company
Hepatitis B	VLP	Licensed	Recombivax HB	Merck
			Engerix-B	GSK
HPV	VLP	Licensed	Gardasil	Merck
			Cervarix	GSK
Hepatitis E	VLP	Licensed	Hecolin	Innovax
Influenza	Virosome	Licensed	Inflexal	Crucell
Hepatitis A	Virosome	Licensed	Epaxal	Crucell
Malaria	VLP	Phase 3	NCT00866619	GSK
Influenza	VLP	Phase 3	NCT03301051	Medicago
RSV	VLP	Phase 3	NCT02624947	Novavax
Norovirus	VLP	Phase 2	NCT03039790	Takeda
HSV	Liposome	Phase 2	NCT02837575	Vical
Tuberculosis	Liposome	Phase 2	NCT01755598	GSK
HSV	Nanoemulsion adjuvant	Phase 2	NCT00453401	NanoBio
Influenza	VLP	Phase 1/2	NCT02078674	Novavax
HIV	Liposome	Phase 1/2	NCT03122223	GSK
HSV	Matrix M adjuvant	Phase 1/2	NCT01667341	Genocea
Influenza	Matrix M adjuvant	Phase 1/2	NCT03293498	Novavax
Influenza	Self-assembling Ferritin	Phase 1	NCT03186781	NIAID
Tuberculosis	Self-assembling IMX313	Phase 1	NCT01879163	Imaxio
Malaria	Self-assembling IMX313	Phase 1	NCT02532049	Jenner Institute
Dengue	Liposome	Phase 1	NCT01502358	Vical
RSV	BLP	Phase 1	NCT02958540	Mucosis BV
Candidiasis	Virosome	Phase 1	NCT01057131	Pevion
Hepatitis C	Virosome	Phase 1	NCT00445419	Pevion
Ebola	Matrix M adjuvant	Phase 1	NCT02370589	Novavax
Malaria	Matrix M adjuvant	Phase 1	NCT01669512	Genocea
Influenza	Matrix M adjuvant	Phase 1	NCT01444482	Isconova AB
Dengue	ISCOMATRIX adjuvant	Phase 1	NCT01477580	Merck
HPV	VLP+ISCOMATRIX adjuvant	Phase 1	NCT00851643	Merck
Influenza	ISCOMATRIX adjuvant	Phase 1	NCT00851266	Merck
Influenza	Nanoemulsion adjuvant	Phase 1	NCT01333462	NanoBio
Influenza	GelVac (gelling polysaccharide)	Phase 1	NCT01258062	Ology Bioservices

Abbreviations: BLP, bacterium-like particle; HA, haemagglutinin; HPV, human papilloma virus; HSV, herpes simplex virus; ISCOM, immune stimulating complexes; NA, neuraminidase; RSV, respiratory syncytial virus; VLP, virus-like particle.

2.3. Design of nanoparticle vaccine scaffold

By controlling nanoparticle composition, their physico-chemical properties can be fine-tuned to deliver enhanced immune responses. In particular, modifying particle size, shape, charge and surface chemistry has been shown to impact innate immune cell interactions, lymphatic drainage, degradation and their biological fouling profile, explored extensively in other reviews [22–24]. The effect of particle size on cellular interactions and immune responses is probably the most well-studied characteristic. Following mouse footpad administration, polystyrene particles of 500 nm-2μm associated with CD205+langerhans dendritic cells (DC) at the site of injection [25]. In contrast, 20–-200 nm particles and 30 nm VLPs were found in association with lymph node (LN)-resident CD8+DC and macrophages within 2 h of injection indicative of rapid, cell-free drainage to the LN [25]. OVA-conjugated gold nanoparticles of 22 and 33 nm displayed greater LN delivery and OVA-specific T cell responses compared to 10 nm particles following mouse footpad administration [9]. On a larger size scale, OVA-conjugated lecithin/glyceryl monostearate-in-water emulsion nanoparticles of 230 nm displayed greater LN delivery, as well as humoral and cellular immune responses compared to 708 nm particles following murine subcutaneous administration [26]. Altering the shape of particles also affects cellular uptake. Vaccination of mice with BSA-conjugated spherical nanoparticles of 15 and 50 nm resulted in higher antibody titers than vaccination with rods or shells [27]. The 300 x 18 nm rod-shaped nanoparticles formed from coat proteins from tobacco mosaic virus were cleared from tissues more slowly than ~54 nm thermally denatured spherical counterparts following in vivo administration of mice [28]. Similarly, surface charge can modulate immunogenicity. For example, pulmonary immunization of mice with OVA-conjugated cationic hydrogel nanoparticles resulted in enhanced systemic and lung antibody titers compared with their anionic counterparts [29]. In another study, also using pulmonary administration, nanoparticles of different charges showed similar patterns of LN drainage but cationic nanoparticles had enhanced association with DCs in the lung [30]. In vitro uptake of polystyrene particles of varying sizes by dendritic cells was greatest with particles 500 nm and smaller [31]. In the same study, uptake of microparticles could be enhanced by making the particle surface positively charged. Increasing the hydrophobicity of gold nanoparticles correlated with increased expression of pro-inflammatory cytokines following in vitro incubation with splenocytes as well as following intravenous administration in mice [32]. It should be emphasized

that in comparing nanoparticles of varying shapes and sizes, it is not only the diameter of the particles that changes but also the surface area-to-volume ratio, potentially resulting in increased interaction of surface displayed antigen at the biological interface. If, instead, the total antigen load is kept consistent between different sized particles, the result is a variation in antigen density. In order to accurately assess the differences in biological interaction of multiple nanoparticles and between multiple studies, it is essential that detailed information about the physicochemical properties of the nanoparticles are provided [33]. Taken altogether, nanoparticles mimicking virion size and morphology of roughly spherical and 20–200 nm appears preferentially recognized by the immune system and drive maximal humoral immune responses [34,35].

2.4. Loading of antigen and adjuvant

Control of antigen loading is a key advantage of nanoparticle vaccines, as it can be optimized to deliver a concentrated and sustained dose of antigen. Vaccine antigens can be attached to nanoparticles in a variety of ways including co-expression [36], chemical conjugation [37], covalent [38], adsorption [39], or encapsulation [40]. These different approaches will affect antigen display and valency and thus, immunogenicity. Methods of malarial antigen Pfs25 conjugation were compared using genetic fusion to a ~ 189 kDa IMX313 heptamer, chemical crosslinking to a ~ 35 nm bacteriophage Qβ VLP and covalent, plug-and-display SpyTag/SpyCatcher loading on a ~ 35 nm AP205-VLP [37]. While vaccination of mice with the chemically conjugated VLP resulted in the highest quantity anti-Pfs25 serum antibody titers, the SpyTag conjugation approach resulted in the higher quality anti-Pfs25 antibodies at preventing malarial transmission upon mosquito feeding. The reason for the discrepancy between antibody quantity and quality can be explained in several ways. Comparison of the three methods of conjugation required three different types of scaffold nanoparticles. Chemical conjugation may result in some unfolding of protein antigen, resulting in antibody production against functionally irrelevant epitopes. Heptameric IXM313 only displays seven molecules of Pfs25 while SpyTag method resulted in much higher valency. By mimicking the organized, repetitive antigen display on the surface of pathogens, immune detection and interaction with B cells are enhanced [41]. While organized antigen display may be beneficial, nanoclusters composed entirely of cross-linked peptide antigen demonstrate enhanced immunogenicity over peptide alone, illustrating that, in some cases, concentrated antigen dose may be the critical factor [42]. Nanoparticle vaccines can be further functionalized by the addition of targeting moieties such as antibodies [43] or the direct incorporation of molecular adjuvants. For example, the addition of toll-like receptor (TLR) [40] or Nod-like receptor (NLR) [44] ligands.

2.5. Nanoparticle vaccine safety

In addition to potent immunogenicity relative to subunit vaccines, nanoparticle vaccines (like subunit vaccines) can exhibit improved safety profiles compared to live, attenuated vaccines. Combining an adjuvant on the same entity as the antigen focuses the immune response, leading to minimal systemic reactions or side effects. In addition, unlike whole organism vaccines, nanoparticles contain no genetic/replicative material, limiting the chance of uncontrolled infection or adverse immunological responses as seen in those with weakened immune systems. In contrast to VLPs, the toxicological effects of synthetic nanoparticle vaccines in biological systems are unclear [45]. Particle material, size, and shape as well as the dose and route of administration appear to influence toxicity. For example, following pulmonary administration of silver particles of varying sizes, nanoparticles were able to deposit deeper into airways than microparticles [46] and 15 nm nanoparticles resulted in increased toxicity and release of inflammatory cytokines than 410 nm nanoparticles [47]. Assuming safety can be established, the controlled composition of nanoparticle vaccines using chemical or recombinant biological synthesis is a highly attractive strategy for the generation of highly scalable immunogens with limited reactogenicity (at least in animal model studies to date) that can be honed for optimal effects.

3. The immunity of nanoparticle vaccines

There is accumulating evidence that nanoparticle vaccines offer a mechanism to enhance immunogenicity over subunit immunization approaches. Below we review five intrinsic and tunable properties of nanoparticle vaccines that result in qualitative improvement of vaccine-induced immunity. These features are summarized in Figure 1.

3.1. Interaction of nanoparticles with the innate immune system

The majority of currently licensed vaccines are administered intramuscularly. Soon after injection, nanoparticles are coated in a corona of interstitial fluid proteins. The composition and mass of this corona are influenced by the physico-chemical properties of the nanoparticle [48] including size and surface chemistry [49] and may result in enhanced or diminished cellular interactions [50] and recognition by the immune system. While studies of protein corona formation following intramuscular nanoparticle administration are lacking, intravenous and in vitro studies provide some indication. Proteolytic analyses of coronas formed on nanoparticles following intravenous administration or serum incubation consistently identify opsonins including fibrinogen, complement and immunoglobulin proteins [51–53].

3.1.1. Complement system

The complement system is a proteolytic cascade of >30 proteins functioning to identify, neutralize and remove foreign objects and potential pathogens. Alternative activation of the complement cascade is continuously initiated at a low level, then amplified following deposition of C3b on foreign surfaces. Nanoparticle surfaces displaying free amino groups are more amenable to C3b binding [54] and alternative activation of complement has been observed for multiple nanoparticle systems [32,55]. The most abundant protein identified in the corona formed on OVA-conjugated, dextran-coated ferrous nanoparticles following incubation in mouse serum was complement protein C3 [51]. Compared to vaccination with soluble OVA, administration of the nanoparticles resulted in stronger splenic B cell binding as well as

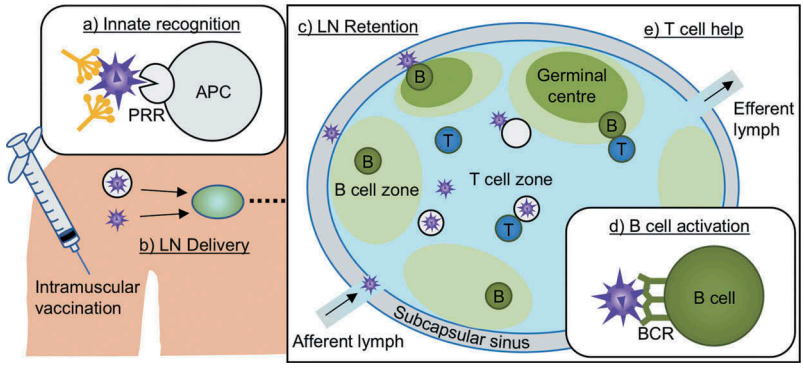

Figure 1. Mechanisms driving enhanced immunity of nanoparticle vaccines. (a) Most vaccines are administered intramuscularly. Nanoparticles are quickly coated in soluble factors including complement and immunoglobulins. This marks nanoparticles for recognition and uptake by local innate immune cells such as neutrophils, monocytes, macrophages and dendritic cells, a subset of antigen presenting cells (APC). This uptake can be enhanced by pathogen recognition receptors (PRRs) including Toll-like receptors (TLRs) located either on the cell surface, within the endosome or in the cytoplasm of the APC. These PRRs recognize pathogen-associated molecular patterns (PAMPs) on the nanoparticle. (b) The kinetics of particle trafficking to the lymph node (LN) are largely dependent on nanoparticle size. Smaller particles are able to drain freely to the LN while larger particles require cellular transport from APCs. (c) As well as enhanced LN drainage, nanoparticles are retained in the LN for longer than soluble antigens, allowing greater opportunities for interactions with immune cells. (d) On the nanoparticle surface, a repetitive display of optimally spaced antigen facilitates crosslinking of B cell receptors (BCRs), resulting in B cell activation. (e) T cell activation is also enhanced following nanoparticle vaccine administration. A portion of activated CD4 + T cells become T follicular helper (TFH) cells, and migrate to the B cell follicle. Here, TFH cells drive sustained germinal center reactions, driving the selection of B cell clones with high antigen affinity into long-lasting memory B cells and/or antibody-producing plasma cells.

higher OVA-specific antibody titers. This differential was no longer observed if the anti-CD21/35 antibody was used to block the complement receptors (CRs) in vivo, suggesting the augmented immunogenicity of the nanoparticle was complement-mediated. Activation of complement via the classical pathway requires C1q binding either IgM or multimers of IgG in an immune complex (IC) with an antigen [56]. This method of complement activation has also been detected for multiple nanoparticle systems [53,57].

Highly repetitive surfaces constitute a pathogen-associated geometric pattern [58]. C1q and IgM along with other innate immune components (such as pentraxins, ficolins, and collectins) are multimeric, allowing higher avidity interactions with repetitive surfaces, even if, in the case of IgM, the interaction is of low affinity. This allows nanoparticles to induce complement activation more readily than a subunit vaccine. CR1, 3, and 4 are present on antigen presenting cells (APC) such as monocytes/macrophages, neutrophils, and DCs and mediate phagocytosis while CR2 functions as a co-receptor on B cells [59]. This allows enhanced complement deposition to facilitate the transfer of nanoparticles between innate and adaptive immune cells.

3.1.2. Innate cells

In addition to interstitial fluid proteins at the site of injection, nanoparticles will also immediately encounter muscle-resident innate immune cells, with localized inflammation facilitating infiltration of APCs including DCs. Internalization of antigen drives the maturation of DCs, allowing trafficking to the draining LN. Once in the LN, DCs will present processed nanoparticle-derived peptides to CD4 + T cells, which in turn, provide help to activated B cells, driving class switching and the generation of long-lived antibody responses. In the absence of T cell help, B cell responses remain short-lived. Therefore, enhanced phagocytosis of nanoparticle vaccines by DCs results in both enhanced humoral and cellular responses and is more readily achieved by nanoparticles than by microparticles [60] or by soluble antigens [61].

3.2. Co-delivery of molecular adjuvants and/or targeting motifs

The innate immune system has evolved to recognize a series of pathogen-associated molecular patterns (PAMPs). These molecular motifs provide 'danger signals' that are recognized by pattern recognition receptors (PRRs) on host cells. PRRs include opsonizing soluble proteins such as mannose-binding lectin. Cytoplasmic NLRs and RIG-like receptors (RLRs) allow the detection of intracellular pathogens. Unlike their addition to subunit vaccines, assembly of PRR agonists directly onto antigen-displaying nanoparticles reduces off-target effects and toxicity.

3.2.1. Toll-like receptors

An important group of transmembrane PRRs is TLRs found on B cells and APCs. TLRs recognize microbial lipoproteins, proteins and nucleic acids. Natural and synthetic agonists for many TLRs have been investigated for their potential as vaccine adjuvants to enhance the pathogen-like qualities of nanoparticle vaccines and improve targeting to B cells and DCs. For example, addition of TLR7/8 agonist Resiquimod (R848) or TLR9 agonist CpG oligodeoxynucleotide (CpG-ODN) to a 130 nm poly(lactic-co-glycolic acid) (PLGA)-based synthetic nanoparticle displaying malarial antigen Pfs25 resulted in greater innate cell activation and cytokine production in a non-human primate model [40]. Furthermore, this greater induction of innate immunity resulted in augmented germinal center (GC) responses, expanded antigen-specific B cells, higher avidity antibodies with longer half-life, maintenance of long-lived plasma cells in the bone marrow and enhanced induction of antigen-specific T follicular helper (TFH) cells. In humans, CpG-ODN has been added to a VLP displaying a melanoma-specific peptide resulting in enhancement of ex-vivo detectable, multifunctional CD8 + T cells [62].

Combination of multiple TLR agonists within one nanoparticle can elicit synergistic improvement of vaccine-induced immunity. Vaccines to prevent nicotine addiction rely on indu-

cing an immune response to a poorly immunogenic chemical. Nicotine-protein conjugates were conjugated to a lipid PLGA hybrid nanoparticle (100–200 nm) containing multiple agonists for TLRs 4, 7/8, and 9 (monophosphoryl lipid A (MPLA), R848 and CpG ODN 1826) on the surface as well as the core of the particle [63]. The incorporation of any single TLR agonist increased nicotine-specific antibody responses in mice. Moreover, the combination of multiple agonists proved the most effective at lowering nicotine levels in the brain and inducing a shift in IgG subclass from predominant IgG1 to the higher effector function IgG2a. Similarly, combining TLR ligands MPL and Imiquimod (R837) within a synthetic 300 nm PLGA nanoparticle together with model antigens, augmented GC reactions (peaking at 4 weeks post-vaccination) and resulted in antibody plasma cells that persisted in the lymph nodes for >1.5 years post-vaccination [64].

The presence or absence of a direct physical linkage between TLR ligand and the nanoparticle affects the resulting immune response. Soluble OVA mixed with or conjugated to CpG ODN 1826 required signaling through TLRs on DCs but not B cells for augmented antibody titers [65]. In contrast, when the TLR ligand was directly conjugated to a bacteriophage Qβ VLP, signaling was mediated through TLRs on B cells resulting in augmented GC formation and anti-Qβ antibody class switching. In another study, mice were vaccinated in the footpad with two different 30 nm bacteriophage Qβ VLPs, one containing p33 peptide antigen, the other CpG [66]. Both particles reached the LN simultaneously and were taken up by the same individual APCs. No difference in cytotoxic T cell (CTL) activity was observed whether the TLR agonist was contained within the same VLP as the antigen or in an additional VLP. In a further assessment of antigen and TLR linkage, HPV oncoprotein E7 was either chemically linked or mixed with bacteriophage Qβ VLPs loaded with TLR9 ligand CpGs [67]. When E7 was chemically linked to the VLP, antigen-specific IgG responses were enhanced while T cell responses were comparable for the linked and mixed formulations. Taken together, these studies suggest direct linkage of the TLR ligand to the antigen-displaying nanoparticle is integral to the use of TLR ligands as adjuvants of humoral responses, but not for CTL responses.

3.2.2. Nod-like receptors

In addition to TLRs, NLRs are another class of innate immune receptors which trigger pro-inflammatory responses, recognizing bacterial peptidoglycan and flagellin. Targeting of NLRs has been exploited to enhance nanoparticle vaccine immunogenicity through the addition of ligands including peptidoglycan. Nod1 ligand CL235 and Nod2 ligand CL365 encapsulated inside poly(lactic acid)(PLA) nanoparticles were more efficiently taken up by DC in vitro and induced a strong release of pro-inflammatory cytokines [44]. In the same study, HIV-1 Gag p24 antigen was also encapsulated inside PLA nanoparticles. Subcutaneous co-administration of both nanoparticles resulted in 100-fold higher antibody titers compared to antigen adsorbed on alum adjuvant. NLRs are expressed by APCs including DC and macrophages in both humans and mice but not by B or T cells.

While the addition of PRR ligands to nanoparticle vaccines offers many opportunities to enhance immunity to the poorly immunogenic antigen, there are additional factors to consider. Differential expression of TLRs between species makes investigating optimal TLR ligands in small animal models more complicated. For example, in mice, CpG-recognizing TLR9 is expressed on all DC subsets, while in humans TLR9 expression is restricted to B cells and LN-resident plasmacytoid DC, necessitating the design of nanoparticles that freely drain to the LN if TLR9 is targeted [68]. While TLR7/8 agonists have shown an ability to activate B cells in mice, in human trials they have displayed high toxicity [69]. More in vivo data, particularly in models that more closely mimic humans is necessary to accurately assess the safety as well as the immunogenicity of using individual and synergistic PRR agonists as nanoparticle vaccine adjuvants.

3.3. Trafficking to the lymph node

Lymph nodes (LN) are anatomically strategic organs designed to sample antigens draining from peripheral tissues and facilitate adaptive immune responses, namely naïve B and T cell activation [70]. The kinetics of nanoparticle drainage to LNs depends on particle size. Two hundred nm pores in the walls of the lymphatic vessels allow the direct diffusion of smaller nanoparticles (with 10–80 nm being optimal) while particles exceeding this cut-off are more likely to require active transport by APCs [10,25,71]. As particle size increases to a micro scale (1μm+), cellular uptake becomes biologically impaired and trafficking to the LN is slower and of lower incidence [35]. LN-trafficking of nanoparticles is generally sustained for longer [35] and is more efficient overall [57] than for soluble antigen. Within the sub-200 nm size range, there are further differences in LN drainage kinetics. Following injection into the skin, poly(propylene sulfide) particles of 20 and 45 nm drained more readily than 100 nm particles and displayed higher association with DCs [72].

3.3.1. Trafficking within the lymph node

The induction of strong humoral immune responses depends not only on the efficiency of antigen trafficking but also on the persistence of antigen in the LN, which is modulated by innate immune cells. Incoming cells and particles move around the subcapsular sinus (SCS) surrounding the LN. Here, specialized macrophages capture nanoparticles in ICs of antigen and antibody. The antigen-loaded macrophages are able to penetrate follicles and transfer the antigen to B cells via CR2 (CD21) [59]. ICs crosslink B cell receptors (BCRs) with the CR2 complex, lowering the affinity threshold for B cell activation [73,74]. The antigen is then transferred to a follicular dendritic cell (FDC) via CR1 (CD35). FDCs play an important role in the development of humoral responses by secreting cytokines which attract activated B cells into GCs initiating BCR somatic hypermutation, and by retaining antigen for weeks enabling persistent GC reactions. Sustained GCs result in long-lasting plasma cells producing higher affinity antibodies. To study whether nanoparticles facilitate extended trapping of antigen in lymphoid organs, LN drainage of a VLP displaying an anti-angiotensin protein was compared to the corresponding soluble protein dimer. By 15 min post-mouse footpad administration, both antigens could be detected associating with CD169+SCS macrophages in the draining LN [61]. However, by 8 h, the VLP were mostly co-localized

with IgD+ follicular B cells and CD21/35+FDCs, lasting for at least 4 days after which GCs started to form. In contrast, the soluble antigen displayed only weak co-localization with B cells and FDC and failed to induce GCs. In the same study, clearance of SCS macrophages with clondronate treatment did not decrease the association of VLPs with FDCs, suggesting shuttling of the IC from the SCS to the follicle was mediated by follicular B cells. Direct interactions between nanoparticles and FDCs and follicular B cells upon primary vaccination have been observed in multiple nanoparticle vaccines studies [35,57]. Using VLPs, rapid delivery to FDC was shown to be mediated by natural IgM and complement [57,59]. In contrast, soluble antigen requires prior immunity and circulating antigen-specific IgG for delivery to FDC. By pre-opsonizing soluble antigen with vaccine-induced IgM or IgG serum, delivery to FDCs was improved [57]. Greater retention of nanoparticle-derived antigen by FDCs results in greatly augmented GC reactions in comparison to vaccination with soluble antigen [57] or with inactivated virus [75].

Particles entering the LN via active DC transport enter the T cell zone and present processed peptide fragments to naïve T cells with antigen-cognate T cell receptors (TCR), driving their activation. At the border between the B and T cell zones, activated B cells present antigen-derived peptides to activated T cells in the context of MHC II molecules. This interaction leads to the provision of T cell 'help' to B cells, sustaining their activation and driving the formation of GCs [76]. Optimal humoral immune responses may be achieved using nanoparticles designed for cellular interactions with both DC and B cells.

3.4. Nanoparticle interaction with B cells

The precise arrangement of multiple copies of an antigen on the surface of a nanoparticle vaccine can modulate the resulting immunogenicity and protective immunity. Repetitive, organized displays of one or a few proteins are found on the surface of viruses and bacteria. Responding to this geometric pathogen-associated molecular pattern has likely evolved to avoid eliciting immune responses against predominantly non-repetitive self-antigen. B cells recognize cognate antigen via the membrane-bound BCR. On resting B cells, BCRs exist as closed oligomers. Upon antigen binding, this structure shifts to a monomeric state, driving signaling [77]. While soluble antigens are largely limited to engagement with a single BCR, multivalent antigens can engage multiple BCRs on the same cell simultaneously, holding them apart in the monomeric state or interacting with multiple BCR oligomers, and thereby eliciting potent B cell activation [78]. Multimeric activation can overcome the low affinity of BCR for antigen [79]. The degree of engagement of an antigen-displaying nanoparticle with BCRs depends on a number of factors: the affinity of the BCR for the antigen, antigen valency, antigen density, and steric arrangement. Nanoparticles may allow fine control of these aspects of antigen delivery.

3.4.1. Antigen valency

While both monovalent and multivalent antigen may be able to trigger the BCR, multivalent antigen can promote 100-fold more efficient BCR activation [80], internalization of antigen [81] and T cell activation [76]. BCR crosslinking also reduces the need for T cell-derived co-stimulation [82]. In the context of multivalent displays of antigen on nanoparticles, biodegradable, calcium phosphate nanoparticles covalently linked to multiple HEL model antigens were specifically bound and internalized by HEL-specific B cells through crosslinking of BCRs [80]. Importantly, a multivalent display of HIV antigen was able to overcome a weak BCR avidity for antigen, even in the context of very few antigen-specific BCRs in germline sequences [83]. This resulted in enhanced GC responses in mice compared to vaccination with monomeric antigen.

3.4.2. Antigen spacing

In addition to valency, the distance between each antigen is critical for maximal BCR engagement. IgG is a bivalent molecule with two identical Fab arms 15 nm apart. This structure allows it to crosslink soluble antigens, recognize repetitive epitopes, and increase binding avidity. While most viruses have extremely dense surface antigen (for example, hemagglutinin trimers on the surface of the influenza viral membrane are ~14 nm apart [84]), HIV-1 viral envelope glycoprotein gp120/gp41 trimers are naturally sparse (~23 nm) [85], facilitating immune evasion by limiting BCR crosslinking potential. To combat this, a liposomal nanoparticle was engineered to display HIV-1 Env trimers 12–14 nm apart. This nanoparticle was highly immunogenic, activating B cells in vitro and augmenting GC responses in vivo [41]. Similarly, vaccinating mice with polystyrene particles coated with relatively sparse Env or HA antigen (17 nm apart) resulted in higher antibody titers and greater GC responses than vaccination with more densely coated antigen (9 nm apart) [86]. As well as displaying whole proteins, individual peptides can be displayed in repetitive arrays. An HIV-1 gp41-derived peptide is a target of broadly neutralizing antibodies but is poorly immunogenic [87]. By displaying a multivalent array of the peptide on the surface of a liposome in addition to TLR ligand MPLA, strong IgG responses in mice could be elicited. The optimal peptide density was found to be a distance of 10–15 nm on the liposome surface. Taken together, these studies point to an optimal antigen spacing of between 10 and 17 nm which can be lacking in some native viruses but can be readily engineered on nanoparticles [87].

3.4.3. Antigen presentation

B cells largely recognize conformationally defined protein epitopes (surfaces), making maintenance of the conformational integrity of the immunogen key for the elicitation of effective humoral immunity by immunization. Nanoparticles can facilitate the presentation of antigens in a pathogen-like context, while simultaneously providing an opportunity to fine-tune the presentation of immunogens to B cells. For example, shielding of poorly neutralizing epitopes while increasing access to previously obscured but highly neutralizing epitopes. HIV-1 antigen gp41 was truncated and stabilized to remove the immuno-dominant region and expose targets of broadly neutralizing antibodies [88]. When displayed upon VLPs, favorable antigenic structures drove the elicitation of

cross-clade serum neutralizing activity following vaccination of rabbits. In another example, the Cap model antigen was conjugated to gold nanoparticles through a linkage that resulted in the exposure of neutralizing epitopes on the outer surface while immunodominant but non-neutralizing epitopes were obscured within the inner surface [89]. Immunization of mice elicited higher antibody titers against the immunologically subdominant regions, as well as enhanced T cell activation.

Controlled assembly or fabrication can be harnessed to further enhance the immunogenicity and cross-protective potential of a vaccine. One way this can be achieved is through the combination of multiple antigens in one nanoparticle. Exploiting the geometric symmetry of self-assembling ferritin protein, a novel nanoparticle was designed to display four trimers per particle of influenza HA and HIV-1 envelope antigens [90]. Vaccination of mice elicited neutralizing antibodies against both pathogens.

3.4.4. B cell memory

An effective vaccine will elicit memory B cells and long-lived plasma cells (LLPC) to extend vaccine protection for years or decades following vaccination. In contrast to short-lived plasma cells, LLPCs have undergone affinity maturation and produce higher avidity antibodies. Memory B cells do not secrete antibodies but can replenish the LLPC pool upon reactivation. Numerous studies point toward nanoparticle vaccines eliciting superior serum antibody responses following immunization. However, quantification of serum antibody and infectious challenge are generally performed soon following immunization and limited studies have assessed the elicitation and persistence of memory B cell populations. In one study performed in rhesus macaques, TLR agonist-adjuvanted PLGA synthetic particles encapsulating malarial antigen Pfs25 elicited increased numbers of Pfs25-specific memory B cells compared to vaccination with the clinically tested Pfs25-EPA protein-conjugate vaccine [40]. The memory B cells expanded with subsequent vaccine boosts and correlated with antibody titer. In an additional study performed in nonhuman primates, vaccination with lipid nanoparticles encapsulating mRNA encoding Influenza HA generated circulating HA-specific memory B cells detectable two weeks after the prime immunization [91]. Memory B cell numbers expanded following subsequent boost vaccinations followed by a gradual decline. Importantly, the generation of a substantial memory response cannot always be predicted by serum antibody titers. While HBV vaccination only elicits a rapidly waning antibody response, B cell memory is prolonged and provides long term protection [92].

3.5. Nanoparticle induction of T cell immunity

While almost all currently licensed vaccines rely on humoral immunity, for some intracellular pathogens the elicitation of cell-mediated responses is desirable. Antigen-specific cytotoxic responses are generally mediated by activated CD8 + T cells following recognition of antigen presented on MHC I. While DCs generally present peptide derived from exogenous antigen upon MHC II, a subset of DCs are able to translocate digested peptides onto MHC I, thereby entering the endogenous pathway, termed cross-presentation. This mechanism likely evolved to deal with pathogens, but can also be taken advantage of in the design of nanoparticle vaccines. Vaccination of mice with iron oxide nanoparticles displaying mycobacterial antigens increased numbers of cross-presenting DC compared to a subunit vaccine [93] as did a VLP vaccine displaying Lassa virus antigens [94]. Cross-presentation can be enhanced by engineering pH-responsive particles for easier degradation inside acidic endosomes of DCs. OVA-conjugated onto pH-responsive, 25 nm polymer micelles resulted in improved intracellular antigen accumulation in DC in vitro [8]. Upon in vivo administration, antigen delivery to APC in the LN as well as expansion of CD8 + T cells were enhanced. To ensure simultaneous delivery of antigen and adjuvant to DCs, and to further boost immune responses, TLR ligands can be added to the nanoparticle. A 25 nm self-assembling E2 protein nanoparticle encapsulating an MHC I-restricted peptide showed stronger activation of DCs in vitro when an acid-releasable CpG TLR ligand was included compared to the addition of free CpG in solution [95]. The same particles also resulted in an increased display of peptide on MHC I and prolonged CD8 + T cell activation in vitro. TLR ligands have also been included in nanoparticle vaccines tested in human clinical trials. A VLP vaccine displaying melanoma peptides alongside CpG elicited a strong CD8 + T response in humans with multifunctional T cells detected ex vivo [62]. CD8 + T cell responses can also be increased by the presentation of antigen both on the surface and encapsulated within a nanoparticle. Intramuscular vaccination of mice with 200 nm cationic lipid PLGA nanoparticles with OVA adsorbed on the surface and encapsulated inside resulted in more efficient cross-presentation of OVA and longer antigen persistence at the injection site [96].

3.5.1. T follicular helper cells

Other than direct cytotoxic effects, a major role for antigen-specific T cells is the provision of co-stimulatory 'help' to B cells to enable and maintain effective humoral immune responses. The GC is a T cell-dependent, organized structure that forms within the draining lymph node following vaccination. GC reactions, where B cells mature into high-affinity antibody-producing plasma cells, are driven by T follicular helper (TFH) cells. These CD4+antigen-specific cells drive the formation and maintenance of GCs, selection of GC B cells with increased affinity for antigen through TCR recognition of peptide-MHC II complexes, costimulatory ligand-receptor pairs, and cytokine release. Multiple classes of nanoparticles have demonstrated an ability to expand antigen-specific TFH responses, resulting in the persistence of GCs in the draining LNs. For example, vaccination with mRNA encoding antigens from HIV, Influenza, and Zika viruses encapsulated in 80 nm lipid nanoparticles expanded antigen-specific TFH cells in both mice and nonhuman primates [97]. The antigen-specificity of TFH cells induced following vaccination with OVA encapsulated in interbilayer-crosslinked multilamellar vesicles (ICMV) was confirmed using a TCR-transgenic mouse model [98]. The precise properties of antigen driving or limiting TFH responses have yet to be determined.

3.5.2. Anti-scaffold responses

Some small moieties (termed 'haptens') are not immunogenic due to a lack of CD4 response and require conjugation to a carrier (usually protein). TFH cells specific for the carrier protein can provide 'help' to drive GC reactions of hapten-specific B cells. This is especially relevant for bacterial polysaccharides, which

require protein conjugates for vaccine efficiency, such as those used in medically important Haemophilus influenzae type B, meningococcal or pneumococcal vaccines. Nanoparticles can directly substitute for the protein carrier or scaffold in some cases to increase the immunogenicity of haptens [99]. For example, using a lipid polymeric hybrid nanoparticle as a carrier, high antibody titers against the normally non-immunogenic nicotine molecule could be achieved, with higher titers observed for 100 nm v 500 nm carrier particles [34]. Pre-existing anti-carrier immunity can both help or hinder the immune response to heterologous vaccination. For example, pre-existing influenza immunity can provide increased T cell help to B cells when an antigen is displayed on influenza-based virosomes [100,101]. Alternatively, anti-carrier responses can suppress the response to vaccination by driving the removal of vaccine antigens in vivo [102]. Using salmonella D2 peptide conjugated to bacteriophage VLPs, pre-existing VLP immunity could be overcome by high antigen density, multiple administration, or by using an increased vaccine dose [103]. The inconsistent effects of pre-existing immunity upon vaccine immunogenicity or protection [104] highlight the need for a greater understanding of the interplay between carrier and vaccine antigen immunogenicity in vivo.

4. Expert opinion

Traditional approaches in vaccine development, whilst responsible for the discovery of vaccines that have greatly improved human health globally, have failed to deliver long-lasting protection against globally significant pathogens such as HIV-1, malaria, tuberculosis, and influenza. Barriers to efficacious vaccine design include a lack of correlates of protection, weakly immunogenic antigens, and high rates of antigenic variation in the target pathogen. These barriers necessitate more advanced approaches for vaccine design and development. Nanoparticle-based vaccine technology may be able to surmount these challenges. As demonstrated by the VLP-based Hep B and HPV vaccines, nanoparticles offer highly tunable, rational vaccine platforms. We highlight three key areas where further research will greatly benefit human nanoparticle vaccine development. First, an improved mechanistic understanding of particle-based vaccine immunogenicity is required. Specifically, knowledge of the in vivo outcome of nanoparticle vaccination including their trafficking, toxicity, and induction of immunity. There is a poor public tolerance to side effects of prophylactic vaccines, so a guarantee that enhanced immunogenicity has not been traded for a reduction in safety is imperative. Increased testing in non-human primate models as well as human clinical trials will further confirm whether the findings of novel nanoparticle technologies in small animal models translate to improved efficacy. Second, an improved grasp on the rational design of nanoparticle vaccines is required. Is there an 'ultimate design' for superior immunity in terms of size, shape, material, and antigenic display? Or does each pathogen require a bespoke approach? This question can be answered by greater multidisciplinary interaction between immunologists and materials scientists performing larger, systematic testing of sets of particles. To date, the only nanoparticle vaccines to have made it to market have been protein-based. Inorganic particles, particularly gold and iron oxide, may have found a niche in drug delivery, cancer treatment, and imaging technologies, but have failed to bear fruit in vaccine development. This is likely due to the superior biocompatibility and easier regulatory approval of protein and lipid-based particles, as well as their intrinsic ability to better mimic pathogens and initiate immune responses. Lastly, given their complex design, the scalability of vaccine production should be considered early in vaccine development. This is of particular relevance as vaccine particles become increasingly functionalized with molecular adjuvants and targeting molecules. Research in the aforementioned areas will facilitate the improved rational design of next-generation nanoparticle vaccines.

5. Five-year view

There exists a tremendous diversity of novel nanoparticle vaccines, including those with increasingly complex designs, described in the literature. Excitingly, several candidate vaccines are entering early-stage human clinical trials (Table 2), which will clarify whether the demonstrable superiority of nanoparticle-based immunization platforms in pre-clinical animal models translates into vastly improved vaccines for human use. Within the next 5 years, in addition to clinical assessment, there will be some consensus around optimal nanoparticle design. In particular, the valency and spacing of antigen and the benefit of direct adjuvant attachment. It will also become more apparent whether synthetic nanoparticles have a place in vaccine design. It is clear they afford advantages in allowing large-scale, finely tuned production, but their advancement from in vitro cell-based assays to biological testing is poor. In addition to prophylactic vaccines for infectious diseases, therapeutic nanoparticle vaccines for cancer or chronic infection will start to deliver improved efficacy. With a strong foundation in fundamental immunology and materials chemistry, the theoretical and demonstrable potential of nanoparticle vaccines offers the opportunity to win the fight against frustratingly persistent human diseases.

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