

Nanoparticle Vaccines Adopting Virus-like Features for Enhanced Immune Potentiation

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Abstract

Synthetic nanoparticles play an increasingly significant role in vaccine design and development as many nanoparticle vaccines show improved safety and efficacy over conventional formulations. These nanoformulations are structurally similar to viruses, which are nanoscale pathogenic organisms that have served as a key selective pressure driving the evolution of our immune system. As a result, mechanisms behind the benefits of nanoparticle vaccines can often find analogue to the interaction dynamics between the immune system and viruses. This review covers the advances in vaccine nanotechnology with a perspective on the advantages of virus mimicry towards immune potentiation. It provides an overview to the different types of nanomaterials utilized for nanoparticle vaccine development, including functionalization strategies that bestow nanoparticles with virus-like features. As understanding of human immunity and vaccine mechanisms continue to evolve, recognizing the fundamental semblance between synthetic nanoparticles and viruses may offer an explanation for the superiority of nanoparticle vaccines over conventional vaccines and may spur new design rationales for future vaccine research. These nanoformulations are poised to provide solutions towards pressing and emerging human diseases.

Key words: Nanoparticle vaccine, lymphatic delivery, repetitive antigen display, vaccine nanotechnology, cellular immunity, anticancer vaccine.

Introduction

Vaccination is a process of introducing antigenic material to activate an individual's immune system to develop adaptive immunity to a pathogen. It has proven to be the most successful and cost-effective prophylactic measure against infectious diseases. Vaccines have been responsible for eradicating or effectively managing many major diseases, including smallpox, measles, mumps, rubella, diphtheria, tetanus, pertussis, polio, and yellow fever [1]. Despite the many examples of successful vaccines, many disease threats, such as human immunodeficiency virus (HIV), tuberculosis, dengue, and malaria, lack an effective prophylactic measure. Thus, development of new vaccine formulations and technology remains an ongoing quest [2]. Conventionally, vaccine formulations are comprised of biological materials in the form of attenuated viruses, killed pathogens, or

subunit protein antigens. Each platform has its distinct advantages and shortcomings, frequently presenting a trade-off between safety and efficacy. For example, live attenuated vaccines are excellent at inducing long lasting protective immunity and strong immune response, but their "live" nature poses safety concerns, especially to individuals who may be immunocompromised. On the other hand, subunit vaccines are safer to administer, but they are less immunogenic and less effective at eliciting cellular immunity for disease protection (Fig. 1). Emerging technology and formulations to combine the advantages of live attenuated and subunit vaccines thus continue to be developed with the aim of maximizing vaccine safety and potency.

In the last decade, advances in materials engineering have opened up new avenues for

innovative vaccine designs. In particular, synthetic nanoparticles have been widely adopted for vaccine development [3, 4]. These particles, typically 25 to 200 nm in diameter, have shown effective immune potentiation *in vivo*, capable of inducing strong humoral and cellular immune responses against antigen targets. Compared to live attenuated viral vaccines, synthetic nanoparticles promise better safety profiles because of their non-replicating nature. They are also readily amenable to different infectious pathogens [3]. From a holistic view, many advantages of nanoparticle vaccines may be attributed to their intrinsic semblance to natural viruses (Fig. 1). Many viral features, such as nanoscale morphology, repetitive multivalent antigen display and controlled antigen/adjuvant delivery are conducive to immune processing on both physiological and cellular levels. As our immune system has evolved to effectively respond to infectious viral nanoparticles, it should not come as a surprise that nanoformulations adopting virus-like features can be more potent than conventional subunit formulations.

To this date, numerous nanoparticle-based platforms have been examined for vaccine applications, and many have demonstrated encouraging efficacy against many pressing infectious threats, including malaria, influenza, Ebola, and HIV [5-8]. Also worth noting is the rapidly expanding field of nanoparticle-based anticancer vaccines, which exploits nanotechnology for enhanced induction of antigen-specific cellular immune responses against oncologic malignancies [9-12]. Many design considerations in nanoparticle vaccines can be traced to the fundamental interaction dynamics between viruses and the immune system [13]. Upon close scrutiny, virus-like mimicry in terms of size, geometry, antigen display, and adjuvanticity can be commonly observed among vaccine nanoformulations.

In light of the emerging landscape of nanoparticle vaccine research, this review highlights some of the underlying principles behind the advantages of nanoparticle vaccines. In the first section, commonly used biomaterials such as lipids, polymers and inorganic compounds are reviewed in the context of vaccine applications. In the sections that ensue, various nanoparticle design aspects are explored from a virus-mimetic perspective including lymphatic delivery, antigen display, and interaction with immune cells. Lastly, we highlight conjugation strategies that couple subunit protein antigens with nanocarriers. This review also summarizes the various strategies for conjugating antigens/adjuvants with nanocarriers. The review aims to provide a holistic view of the recent advances for the next generation of immunomodulatory vaccines.

Nanoparticles for vaccination

Towards mimicking viral features, preparing nanoparticulates in the range of 20 to 200 nm in size is the first step prior to subsequent vaccine development. It is therefore imperative to have an understanding of the various materials and their properties for nanoparticle preparations. The diverse range of nanomaterials for biomedical research encompasses a wide range of organic and inorganic matters, such as phospholipids, polymers, metal, silica, and carbon (Fig. 2). These nanomaterials can be modified to provide a functionalized and stable interface for different biomedical applications. In the context of vaccination, many nanocarriers have been modified to trigger specific immune responses analogous to natural defense mechanisms against viral invasion. The following section summarizes the different synthetic nanoparticles used in vaccine applications with an overview on their functionalizability and immunological adjuvanticity.

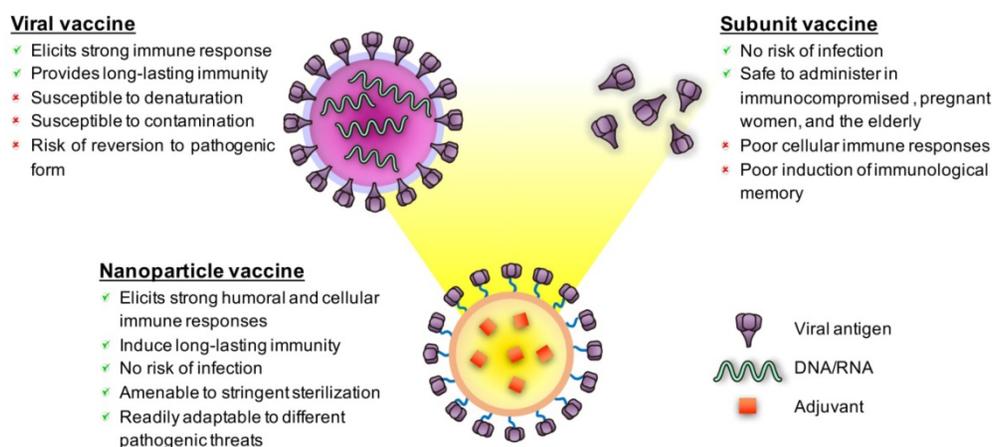


Figure 1. Schematics of a viral vaccine, a subunit vaccine, and a representative nanoparticle vaccine highlighting the strengths and shortcomings of each platform. Nanoparticle vaccines can be engineered to mimic viruses in terms of morphology, antigen display, and adjuvanticity.

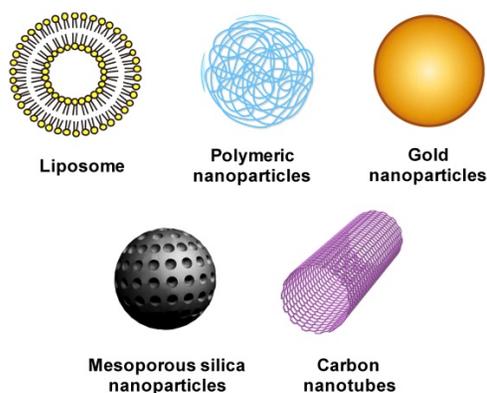


Figure 2. Synthetic nanoparticles for vaccine delivery.

Liposomes and lipid-based nanoparticles

Liposomes are self-assembled, spherical vesicles consisting of a phospholipid bilayer and an aqueous inner core [14, 15]. They are prepared from phospholipids with fatty-acid chains of defined length and saturation. The choice of phospholipids and the addition of cholesterol influence liposomal stability and performance [16-18]. The vesicles can have a unilamellar structure with one layer of phospholipids or a multi-lamellar structure with several concentric phospholipid shells. This platform is highly versatile for cargoes delivery and allows for incorporation of hydrophilic molecules in the inner core and hydrophobic molecules within the phospholipid bilayers. Antigens and adjuvants may thus be incorporated into liposomes based on their lipophilicity [19, 20].

The lipid bilayered structure of liposomes is structurally analogous to enveloped viruses, which are formed from budding of infected cells and are wrapped in pieces of cell's plasma membrane. The inherent semblance to viral particles may help explain the innate adjuvanticity of liposomes upon incorporation with protein and peptide antigens [21]. Numerous antigen targets, ranging from toxoid, viral antigens, and bacterial antigens, have been observed to elicit enhanced humoral responses following liposomal incorporations [22]. Mechanistic studies have shed light on the liposomes' adjuvanticity. For instance, liposomes have been shown to be capable of modulating CD8⁺ T cell mediated immune responses [23], reflecting facilitation of antigen processing via the classical MHC I and MHC II pathways. Liposomes have also been reported to promote the development of T cell-independent B cell immune responses [24] and have potential to promote long-term immunity through the development of T-cell memory [25]. In addition to their innate adjuvanticity, liposomes have been incorporated with a plethora of adjuvants ranging from small molecules [26, 27], glycolipids

[28-31], oligodeoxynucleotides [32-35], to cyclic dinucleotides [6, 36].

The structural fluidity of liposomes offers flexibility in platform modification towards virus mimicry. For example, the development of virosomes, which are fusion vesicles prepared from viral particles and synthetic liposomes, is an adaptation that highlights the fundamental similarity between liposomes and viruses. Derived from the membrane vesicles of viruses, virosomes consist of liposome-like lipid vesicles and viral envelope glycoproteins. The fusion vesicles retain some viral characteristics (i.e. epitope presentation) and have been applied for both drug delivery and vaccination purposes [37, 38]. The applicability of this platform as a vaccine candidate has been demonstrated extensively using influenza virus-derived virosomes [39-42]. In studies that apply virosomes for anti-influenza vaccination, derivatized influenza virosomes were shown to be non-infective as their genetic materials were removed. Upon *in vivo* administration, these virosomes were rapidly uptaken by antigen presenting cells and in turn activated numerous other immune cells [43-46]. Immunization with virosomes was reported to reactivate influenza-specific memory CD4⁺ T cells that subsequently supported the proliferation of antigen-specific effector cells [46], leading to enhanced anti-influenza immune responses [47]. Besides influenza-based virosomes, induction of cytotoxic T lymphocyte responses has been demonstrated with a Sendai virus-based carrier system loaded with ovalbumin (OVA). It was demonstrated that Sendai virosomes fused with OVA elicited stronger CTL responses against the model antigen [48].

Liposomes may also be modified to enhance the stability of carriers in a manner analogous to how viruses employ viral matrix proteins to stabilize their lipid envelope [49]. In a study by Moon et. al., interbilayer-crosslinked multilamellar vesicles have been prepared by covalently crosslinking multiple layers of liposomes via thiol chemistry [50]. These multi-lamellar liposomes showed added stability owing to short covalent bonds that crosslinked adjacent lipid layers within the vesicle walls. This modification served to address some of the shortcomings of liposomes, facilitating enhanced antigen encapsulation and increased particle stability.

Also belonging to the class of lipid-based nanoparticles are an emerging class of lipoplexes, which consist of cationic lipid derivatives for the complexation with nucleic acids [10, 51, 52]. Immunostimulatory RNAs or mRNAs encoding specific antigen targets have been formulated into lipoplexes to trigger immune responses. The function

of these lipoplexes can be likened to the immune response induction by RNA viruses [53]. As RNAs are delivered intracellularly by lipoplexes, they activate innate immune receptors, leading to an upregulation of type I interferons, which may further trigger a multitude of downstream immunological pathways [54]. Concurrently, these RNAs are translated into antigens of interest, thereby promoting an antigen-specific immune response. Lipoplexes carrying the mRNA of target antigens have recently been shown in a Phase I clinical trial to induce strong cellular responses against tumor antigens in humans [10].

Polymeric nanoparticles

A wide variety of polymers have been applied to the development of nanoparticle vaccines. Synthetic polymeric nanoparticles are typically solid particles between 10 nm to 200 nm. They have been an attractive platform for vaccine delivery as antigens and adjuvants can be either surface attached to or interior loaded inside these nanoparticles [55-59]. In particular, controlled release of biomolecules is one of the strongest advantage of polymeric nanoparticles, the release kinetics of which can be regulated by tuning of the copolymer composition and molecular weight [55]. Typically, polymeric nanoparticles are formed via self-assembly of amphiphilic copolymers under an emulsion or nanoprecipitation process [60-62]. Notable polymeric nanoparticles in vaccine development are as follows.

Poly(lactic-co-glycolic acid)(PLGA) is one of the most commonly used polymers for biomedical applications [63]. PLGA-based nanoparticles are known to be biodegradable, non-toxic and non-immunogenic. Upon administration, the polymer is degraded into lactic acids and glycolic acids *in vivo* to be safely metabolised in the body. In vaccine applications, PLGA nanoparticles provide a robust platform for antigen functionalization and have been used to carry antigen derived from various pathogens. Through surface conjugation or interior encapsulation, antigens including those derived from *Plasmodium vivax* [64], hepatitis B virus (HBV) [65], *Bacillus anthracis* [66], tetanus toxin [67], model antigens such as ovalbumin [68] have been associated with PLGA-based delivery systems for enhanced immune potentiation. Adjuvants have also been encapsulated or chemically attached to the PLGA polymer backbone for controlled delivery to enhance immune responses [69-72].

Natural polymers based on polysaccharide, such as pullulan [73-75], alginate [76], and chitosan [77-82], have been explored for nanoparticle vaccine preparations. In particular, chitosan-based

nanoparticles have been widely studied due to their biocompatibility, biodegradability, non-toxic nature and their ability to be easily modified into desired shapes and sizes [83-85]. More intriguing is the recent discovery of chitosan's immune potentiating mechanism via the DNA sensing cGAS-STING pathway [86]. The STING pathway is triggered in the host cells by many viral pathogens and plays a major role in both the innate and adaptive immunity [87]. Upon interaction with dendritic cells, chitosan induces type I interferons in a cGAS and STING-dependent fashion, mediating cellular maturation and the promotion of Th1 responses. Chitosan-based nanoformulations have been widely adopted for vaccine development, examples of which include vaccines against *Clostridium botulinum* type-A neurotoxin [56], *Neospora caninum* [88], HBV [77], and Newcastle disease [81]. The polysaccharide polymer has also been applied to enhance the potency of DNA vaccines against viral, bacterial, and parasite infections [78, 80, 82].

Other polymeric nanoparticle platforms include poly(γ -glutamic acids)(γ -PGA) nanoparticles [83, 89], polystyrene nanoparticles [90, 91], and poly-alkyl acrylate based nanoparticles [92, 93]. γ -PGA are comprised of amphiphilic poly(amino acid)s, which self-assemble into nano-micelles with a hydrophilic outer shell and a hydrophobic inner core. They are generally used to encapsulate hydrophobic antigens. Polystyrene nanoparticles are solid particles consisting of polymerized styrene monomers that can be conjugated to a variety of antigens. Poly-alkyl acrylate based nanoformulations have been prepared with poly (methylmethacrylate) (PMMA), poly (ethylacrylic acid) (PEAA), poly (propylacrylic acid) (PPAA) and poly (butylacrylic acid) (PBAA). Studies have shown that polyacrylate-based nanoparticles show an inherent adjuvanticity with several model antigens [4, 94, 95]. The type of polymer used in the nano-formulation strongly affects the structure, properties and applications of the particles.

Gold nanoparticles

As gold is chemically inert, gold nanoparticles (AuNPs) have been studied extensively for biomedical applications. AuNPs can be synthesized reproducibly with a high level of precision, offering an ultrastable metallic core for further modifications. Capable of being modified with a plethora of chemical functional groups such as thiols, phosphines, amines, and by extension protein antigens, AuNPs have been used broadly in many vaccination studies. A multitude of biomolecules, ranging from toxin, viral antigens, bacterial antigens, parasite proteins, and tumor antigens, have been coupled with gold

nanoparticles to enhance immune responses. AuNPs vaccines have been explored in clinical trials for hepatitis B and malaria vaccinations [96, 97], and they also allow anchoring of nucleic acids for DNA vaccine applications [98]. AuNPs have been modified with different adjuvants as well, such as chitosan [99] and CpG oligodeoxynucleotides [100, 101], and many alternative surface functionalization strategies have been explored to further enhance immune potentiation. AuNPs surface modified with cetyltrimethylammonium bromide (CTAB), poly(diallyldimethylammonium chloride) (PDDAC), and polyethyleneimine (PEI), have been used as a DNA vaccine adjuvant for human immunodeficiency virus (HIV) [102]. These modifications were found to further boost the adjuvanticity of the AuNP carrier.

Gold's unique malleability makes AuNPs an attractive platform for vaccinology studies [103]. AuNPs can be fabricated into myriad of shapes (i.e. sphere, rod, cube, etc.) [104] with tunable yet sharply distributed size range between 2–250 nm [105]. This morphological tunability adds an additional dimension of virus mimicry with the introduction of non-spherical particles. Gold nanorods, for instance, have been used as a vaccine vector for the delivery respiratory syncytial virus (RSV) F protein [106]. These non-spherical particles bear resemblance to rod-like viruses that can also be frequently observed among different virus genera [107]. The size modularity of AuNPs also allows for examining the impact of particle size on vaccine delivery, which will be discussed in details in later sections of this review. Also worth noting is the more recent discovery that AuNP size and shape can modulate the inflammatory responses at the cellular level [104]. In a study that compares gold nanospheres, gold nanorods, and gold nanocubes, Niikura *et al.* showed that whereas the nanospheres and the nanocubes induced tumor necrosis factor- α (TNF- α), IL-6, IL-12, and granulocyte macrophage colony-stimulating factor (GM-CSF), the nanorods induced interleukin-1 β and interleukin-18 via an inflammasome-dependent mechanism [104]. This shape-dependent immunological property may be due to the differing surface energies associated with different nanoscale features, which may promote varying levels of stress upon cellular uptake [108].

Other nanoparticles

Silica nanoparticles offer a range of particle sizes and shapes via controlled synthesis using sol-gel chemistry. An abundance of silanol groups on silica nanoparticle surface allow for functional modifications for increasing specific cellular recognition, facilitating attachment of specific biomolecules, and modulating cellular uptake

[109-111]. Nanoscale pores can be integrated into silica nanoparticles, yielding mesoporous silica nanoparticles (MSNs) with more versatile cargo-carrying capacity for vaccination purposes [112, 113]. The pore size and surface functionalization of MSNs can be modified to control the encapsulation and release of antigens or adjuvants of choice [114-117]. Use of silica nanoparticles in vaccine applications include formulations against snake venom, *E. coli* [118], porcine circovirus [119], HIV [120], and other model antigens [121].

Carbon-based nanoparticles, such as carbon nanotubes and graphene, have also been studied as vaccine carriers [122, 123]. Owing to their high aspect ratio and large surface area, these carbon-based nanoparticles may carry a high proportion of antigens for immune activation. In a study on anticancer vaccination, Villa *et al.* conjugated single-wall carbon nanotubes (SWCNTs) with Wilm's tumour protein, an antigen upregulated in many cancers. Antigen conjugated SWCNTs showed good uptake by dendritic cells and macrophages *in vitro*, and subcutaneous immunization with these SWCNTs promoted induction of antigen-specific IgG. In contrast, the free peptide formulation failed to induce an antibody response against the tumor antigen [124]. In another study, graphene nanosheets were used to deliver antigens to facilitate antigen cross-presentation to CD8+ T cells [123]. Xu *et al.* also demonstrated the use of a dual polymer-modified graphene formulation as an effective adjuvant to enhance the immunogenicity of *H. pylori* derived antigen (Alum-Ure B) [125].

Semiconductor quantum dots have been applied for vaccine applications, offering a versatile platform for nanoparticulates antigen delivery with the added benefit of particle tracking. In a study by Cambi *et al.*, antigen-conjugated quantum dots of virus-like dimension were shown to combine antigen delivery and bioimaging functionalities, enabling immune cell tracking following antigen uptake. It presented the possibility of tracking antigen-presenting cells *in vivo* [126]. Along similar lines, Sen *et al.* showed that quantum dots can induce T cell proliferation and IFN- γ production *in vivo* while being traceable within lymph nodes [127]. Other nanoparticle platforms with imaging functionality, including polymeric upconversion nanoparticles [128], and iron oxide nanoparticles [129], have also been studied as antigen carriers for vaccine applications. These platforms offer the capability for examining the function mechanisms behind the benefits of nanoparticle vaccines.

Nanoscale morphology and lymph node delivery

One of the biggest advantages of nanoparticle

vaccines is their ability to efficiently drain and accumulate to lymph nodes for enhanced immune processing. The lymphatic system is a subset of the circulatory system that consists of a complex network of vessels, tissues and organs. The lymphatic vessels conduct lymph between different parts of the body. As blood exits the blood vasculature to become interstitial fluid, the lymphatic system provides a return route for this interstitial fluid to the blood vessels in the form of lymph. In the process, it regulates fluid balance within tissues [130]. Underlying the lymphatic system are numerous lymph nodes that scatter throughout the body. These lymph nodes are the homing sites of migratory dendritic cells that present engulfed antigens. Resident in these peripheral lymphoid organs are an abundance of specialized macrophages and other lymphocytes which play a major role in antigen capture and processing for adaptive immune responses. As lymph passes through lymph nodes, resident macrophages further capture passing antigens [131]. In other words, the lymphatic system functions as a filter system of bodily fluids, trapping antigens in the lymph nodes for immune processing.

Viruses and virus-like nanoparticulates can accumulate in lymph nodes via both cell-mediated lymphatic delivery and convective lymphatic transport. The cell-mediated transport is mediated primarily by migratory dendritic cells, which take up antigens outside of the lymphatic system (i.e. skin and lung) and enter lymph nodes via either high endothelial venules or lymph vessels [132, 133]. In comparison to small protein antigens, viruses and nanoparticle vaccines are more favorable to this hitchhiking mechanism. Their particulate nature promotes receptor-mediated, complement-mediated, or other intracellular uptake mechanisms [134, 135]. Antigens associated with nanocarriers are routinely observed to be more efficiently uptaken by dendritic cells compared to soluble antigens, thereby enabling more effective lymph node delivery and cross presentation [128, 136-139]. On the other hand, the nanoscale morphology of viruses and virus-like particulates allows them to move freely in lymphatic vessels to draining lymph nodes. Upon lymph node entry, a special subset of macrophages is responsible for the capture of these nanoparticulates. In a study on lymphatic tracking of viruses by Jun *et al.*, macrophages in the subcapsular sinus and in the medulla of lymph nodes were shown to be responsible for the lymph node accumulation of subcutaneously administered inactivated vesicular stomatitis virus, adenovirus, and vaccinia virus [140]. Depletion of these macrophages resulted in an enhanced virus level that circulated back to the blood,

highlighting both viruses' convective transport in the lymphatic system and the macrophages' role in virus filtration. This gatekeeper function by the lymph node-resident macrophages serves to limit blood-borne infection and promote immune processing. Exploiting the same transport mechanisms aimed at detaining viruses, nanoparticle vaccines can effectively target immune cells in lymph nodes, delivering antigens or adjuvants following administration in peripheral tissues [13, 141, 142] (**Fig. 3A**). The benefit and focus on lymph node targeting also explain why particulate vaccines are most commonly administered subcutaneously as opposed to the conventional intramuscular route for subunit vaccines; free lymphatic drainage and access to immune cells in lymph nodes following injection into the interstitium likely outweigh the "depot effect" afforded by the intramuscular route [143, 144]. Lymphatic targeting by nanoparticles have also been observed following administration via different delivery routes, including pulmonary [145], oral [146], intra-peritoneal [147] routes. Such favorable distribution profile allows tailoring of nanoparticle vaccines towards targeting specific immunological compartments against different infectious threats.

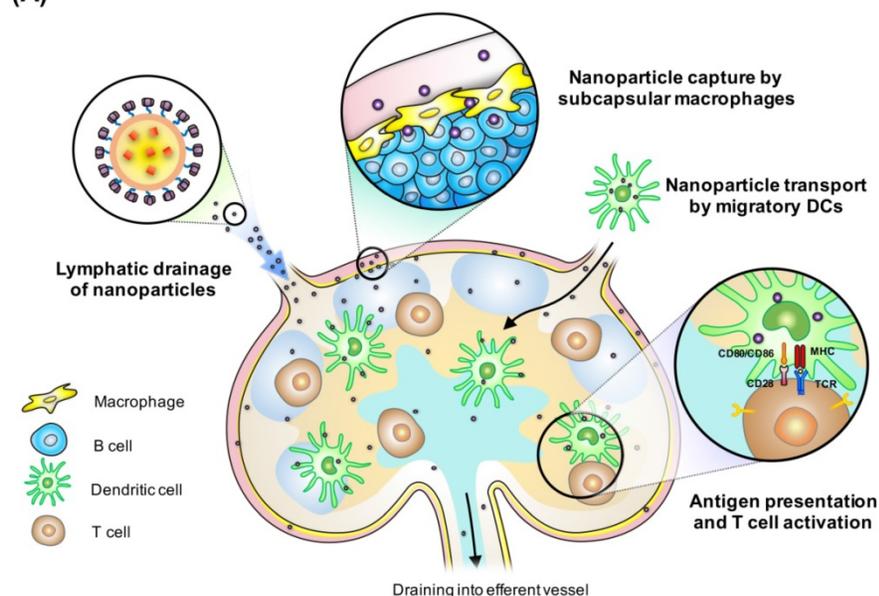
Nanoparticle size and lymph node targeting

Studies on the influence of nanoparticle size on lymph node targeting began in the 1980s as scientists aimed to maximize lymphatic delivery of drugs for treating metastatic cancer. It was generally observed that following subcutaneous injections liposomes smaller than 150 nm were able to enter the lymphatic capillaries whereas larger liposomes remained at the injection sites [148-150]. In a study by Oussoren *et al.* using isotope labelled liposomes between 40 to 400 nm in diameter in lymphatic tracking, lymphatic uptake was found to be inversely proportional to the liposome size. 40 nm, 70 nm, 170 nm and 400 nm liposomes had approximately 76%, 61%, 30%, and 18% of the injected dose entering the lymphatic system, respectively. Curiously, despite higher lymphatic entry by smaller liposomes in the study, liposome accumulation in the draining lymph node was similar across the differently sized formulations. It was found that the majority of the small liposomes, which consisted of egg-phosphatidylcholine and egg-phosphatidylglycerol, passed through the lymph node and were ultimately captured by the liver and the spleen [151]. The result highlighted the dynamic relationship between particle size and lymph node accumulation; as smaller particles are more likely to enter the lymphatic system, they are also more likely to evade the filtering mechanism of lymph nodes. The authors showed that incorporating

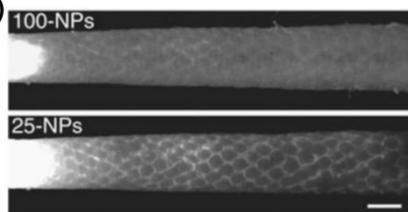
phosphatidylserine, a lipid more susceptible to macrophage recognition and capture, increased the lymph node accumulation by 3-fold. Other lipid modification strategies, such as steric stabilization and ligand functionalization, have also been reported to influence the lymph node accumulation of liposomes following lymphatic uptake [152].

Later studies using solid nanoparticles on examining the effect of particle size on lymph node delivery echo earlier findings based on liposomes. Using polystyrene beads, Manolova *et al.* confirmed the size-dependent particle transport in the lymphatic system. Upon subcutaneous delivery, large particles between 500 to 2000 nm were found to be associated with dendritic cells from the site of injection, and small nanoparticles between 20 to 200 nm could drain freely in the lymphatic system, effectively targeting lymph node-resident dendritic cells and macrophages [139]. In a study by Reddy *et al.* on examining nanocarriers as a vaccine delivery platform, Pluronic-stabilized polypropylene sulfide nanoparticles of well-defined sizes were investigated.

(A)



(B)



(C)

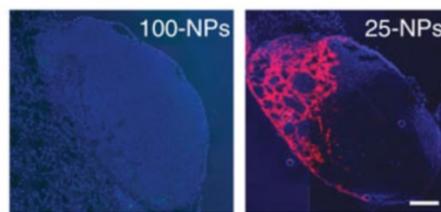


Figure 3. (A) Schematics illustrating the mechanisms behind and advantages of lymph node delivery by nanoparticle vaccines. Nanoparticles can exploit both cell-mediated and convective transport for lymph node localization. Particles that enter lymph nodes via interstitial-lymphatic drainage are captured by lymph node-resident macrophages. Enhanced antigen delivery by nanoparticles facilitates antigen presentation and T cell activation. (B) Fluorescence microlymphangiography imaging of 100 nm and 25 nm nanoparticles following tail based injection. 25 nm particles more effectively traverse through the lymphatic network. (C) 25 nm nanoparticles can more efficiently accumulate in lymph nodes as compared to 100 nm nanoparticles as evidenced by fluorescence microscopy. Images in (B)(C) are reproduced with permission from ref. 14, 2007 NPG.

Using fluorescence microlymphangiography, the investigators showed a clear distinction between 25 nm and 100 nm particles regarding their lymphatic uptake. Following injection into mouse tails, 25 nm particles were efficiently drained to the lymphatic vessels, whereas the interstitial transport of 100 nm particles was less efficient. In contrast to prior studies with liposomes, the 25 nm particles also showed higher lymph node accumulation, resulting in a 10-fold enhancement in lymph node delivery as compared to the 100 nm particles (Fig. 3B, C) [13]. Such enhanced lymph node delivery, which was absent in earlier liposomal studies, could be attributed to both increased colloidal stability of the polymeric nanoparticles and the particles' ability to elicit complement activation. As the polypropylene sulfide particles were functionalized with surface hydroxyl groups to trigger the proteolytic cleavage of C3 complement protein, the danger signal associated with the complement activation could facilitate macrophage uptake upon lymph node entry [153], thereby reducing particle escape from the lymph node's filtering mechanism.

The size-dependent lymph node targeting was also observed in other solid particle platforms. In a study by Gao *et al.* that compared 30 nm and 90 nm gold nanoparticles as antigen carriers, 30 nm gold nanoparticles yielded 2.5-fold higher lymph node accumulation in terms of total gold delivery. Upon conversion to particle number and total particle surface area, 30 nm gold nanoparticles had an enhancement of 67-fold and 7.5-fold as compared to 90 nm particles, respectively [154]. In general, nanoparticles between 20 and 200 nm, a length scale that coincides with viral particles, can exploit interstitial flow for lymphatic delivery. Within this length scale, smaller particle size tends to favor lymph node accumulation.

Antigen delivery by nanoparticle vaccines

Given the privilege of nanocarriers in lymphatic transport, nanoparticles have been shown to enhance the delivery of target antigens to lymph nodes and resident immune cells for processing and immune activation. In the aforementioned study on polypropylene sulfide particles, for instance, Reddy *et al.* demonstrated increased resident dendritic cell activation in the lymph node by ovalbumin-conjugated nanoparticles. In their animal study, strong anti-ovalbumin humoral response was observed [13], highlighting the benefit of nanoparticle-mediated lymph node delivery on enhancing antigen processing. Moon *et al.* also showed nanoparticle vaccines can promote preferential accumulation of antigens in the draining lymph nodes and enhance expansion of antigen-specific T cells. Using interbilayer-crosslinked multilamellar vesicles (ICMVs), a lipid-based nanoformulation consisting of multiple layers of lipid vesicles interconnected via thiol chemistry, the investigators demonstrated enhanced antigen delivery to total DCs, macrophages and plasmacytoid DCs in the lymph nodes [50]. Interestingly, liposomes of comparable sizes were much less effective in shuttling antigens to draining lymph nodes in the same study. This observation highlighted the importance of nanoparticle stability in vaccine design as the ICMVs were more colloiddally stable than liposomes. ICMV-mediated antigen delivery resulted in significantly higher humoral and cellular responses as compared to the free antigens and the liposomal formulations. The effect of nanoparticle carrier on antigen transport was also shown in a study by Chen *et al.*, who demonstrated effective vaccination against coronaviruses using gold nanoparticle-adsorbed viral antigens. These antigen-coated nanoparticles were structurally analogous to coronaviruses in terms of size and antigen display. Immunofluorescence quantification showed that viral spike proteins delivered with 100 nm gold nanoparticles increased lymph node delivery by approximately 6-fold compared to free spike proteins. These virus-like particles showed high immunogenicity in both murine and avian models and enhanced anti-viral IgA and IgG titers and cellular immune responses in comparison to free protein antigens and a commercial WIV vaccine [155].

Adjuvant delivery by nanoparticle vaccines

In addition to delivering antigens for more effective immune processing, nanoparticles have been extensively applied to localize immunological adjuvants to lymph nodes for improved safety and potency. While conventional adjuvants such as alum

have been widely employed clinically to promote humoral responses [156], more recent development in adjuvant research has identified many pathogen-associated molecular patterns (PAMPs) as promising adjuvant candidates towards promoting both humoral and cellular responses [157]. These molecular danger signals are often similar to viral pathogens regarding their immune potentiating mechanisms, triggering innate immunity and in turn facilitating adaptive immune responses. Many PAMPs (i.e. CpG-ODN, Poly(I:C), and cyclic dinucleotides) as well as other molecular agonists of toll-like receptors (TLRs) (i.e. imiquimod and resiquimod) are known to induce strong immune responses. However, their potency poses safety concerns over the likely induction of systemic inflammation. Nanoparticle-based delivery thus offers a desirable strategy in guiding these immunological modulators to lymph nodes, increasing their effective concentration and reducing their systemic reactogenicity. In one example, Nunh *et al.*, constructed a pH-degradable nanogel platform ligated with imidazoquinoline (IMDQ), a TLR7/8 agonist, and showed retention of the adjuvant at the injection site and the draining lymph node. The adjuvant in its free form elicited systemic inflammatory responses, but this side effect was largely obviated with the nanogel formulation. The targeting effect of the nanoformulations also resulted in recruitment of monocytes to the draining lymph node. A large number of immune cells, including B cells, DCs, and macrophages were shown to readily take up these adjuvant-loaded nanogels [158]. In another study by Ilyinskii *et al.*, synthetic vaccine particles encapsulating resiquimod (R848, a TLR7/8 and TLR9 ligand) augmented humoral and cellular immune responses to both soluble and nanoparticle-delivered proteins compared to that observed with free adjuvants. The adjuvant-loaded nanoparticles promoted local cytokine induction in the lymph nodes and reduced systemic cytokine production observed with free R848. Moreover, while injection of the nanoformulation led to sustained expressions of IFN- γ , IL-12, and IL-1 β in lymph nodes after 48 hours, free R848 induced only modest levels of IL-12 and IFN- β [159]. CpG-ODN, an agonist of TLR-9, is another adjuvant that's frequently coupled with nanocarriers for vaccination studies [11, 67, 100, 101, 160, 161]. Some of the primary advantages of nanoparticle-based CpG formulations include strong T cell responses, dosage sparing, and reduced systemic side effects. Such formulations have been commonly applied in anticancer vaccination efforts owing to the need for high cell-based immune responses for effective tumor containment.

Co-delivery of antigens and adjuvants by nanoparticle vaccines

Transport of viral antigen and immune-potentiating adjuvants by viruses to immune cells is a highly coordinated event as viral particles shuttle both antigen targets and adjuvanting nucleic acids simultaneously. Such antigen/adjuvant coordination, or its absence, has been shown to strongly influence immune cell activation [162]. In addition, the immune system can respond to viruses through multiple PAMPs, including glycoprotein, DNA, dsRNA, and ssRNA, activating a broad spectrum of signalling pathways for heightened antiviral immunity [163]. The synthetic flexibility of nanoparticles has thus been exploited to emulate the co-delivery capacity of viral pathogens to boost immune responses. In some cases, antigen and adjuvant are localized on the same nanoparticles for synchronized delivery. Ma *et al.* incorporated a hepatitis B surface antigen (HBsAg) and CpG adjuvant onto PLGA nanoparticles via conjugation with dopamine, allowing the particles to display both the viral antigen and the immune activator. The study showed that the pathogen-mimicking particle enhanced the recruitment of immune cells to the injected site, activated bone marrow derived dendritic cells, and induced strong humoral and cellular immune responses [164]. In another study conducted by Kuai *et al.*, a disc-like synthetic high-density lipoprotein (sHDL) was applied to integrate both target antigens of CD8⁺ T cells and CpG-ODN for anticancer vaccination. The nanodisc drastically improved the co-delivery of antigens and adjuvants to lymph nodes compared to soluble vaccine and induced a 31-fold enhancement in antigen-specific CD8⁺ cytotoxic T-lymphocyte response as compared to the free peptide and adjuvant control.

Besides incorporating antigens and adjuvants on the same particle for co-delivery, a number of studies demonstrated that delivering antigen and adjuvant in separate but similar nanocarriers can also elevate antigen-specific immune responses. In one example, an HIV antigen (HIVgp41) was anchored on the surface of liposomes and co-delivered with a liposomal formulation of cd-GMP, a potent agonist of the STING pathway. The study showed a substantial accumulation of the STING agonist in draining lymph nodes [165], and it is expected that the liposome-bound peptide antigen was delivered in a similar fashion. As a result, enhanced activation of antigen presenting cells and increased levels of antibodies against the HIV antigen were observed. Immune stimulation by formulations containing separate antigen- and adjuvant-loaded nanoparticles was also reported in a study exploring the benefit of

multi-adjuvant loaded particles. By incorporating multiple distinctive activators of TLRs, including monophosphoryl lipid A (a TLR4 agonist) and R837 (a TLR7 agonist), Kasturi *et al.* demonstrated adjuvant synergism that triggered elevated antigen-specific humoral responses [70]. Using ovalbumin and hemagglutinin of influenza viruses, the investigators demonstrated long-lasting humoral responses and evidence of memory B cell formation following immunization with the nanoparticle vaccine. These examples highlight the functional versatility of synthetic nanoparticles, which can facilitate different modes of virus mimicry for immune activation.

Repetitive antigen display for immune activation

In addition to the role of lymph nodes in trapping nanoscale particles for immune processing, the immune system has also adapted to the repetitive antigen display on viruses for effective potentiation. Virus surfaces display antigenic epitopes in an ordered and highly repetitive fashion, and the presentation of repetitively arranged and appropriately spaced antigens on the surface of virus or virus-like particles has been linked to enhanced immune responses [166]. Many reports have shown that numerous components of the mammalian immune system have evolved to respond strongly to the repetitive antigen patterns frequently found on pathogens [167-169]. In comparison, non-repetitive antigens are usually less effective in inducing immune responses [170-173]. Repetitive motifs on viral surfaces are also found to activate the complement system [174] engaging the CD19-CD21 complex, which further facilitates B cell activation and amplify other immune processing pathways [175].

Understanding the link between structural features of antigen display and immunological induction is vital in designing nanoparticulate vaccines. Multivalent interactions promote B-cell receptor (BCR) clustering and signaling and facilitate receptor-mediated internalization of antigen. Antigen features, such as epitope affinity, valency, or co-receptor recruitment can impact B and T cell signaling. In a study that used antigen-conjugated polymer to assay the impact of antigen valency on B cell activation, Puffer *et al.* showed that clustering of BCRs by multivalent antigens is crucial for antigen-dependent signaling. The antigen-conjugated polymers clustered unbound BCRs and contributed to enhanced intracellular signalling [176]. Whereas the multivalent antigen-polymer conjugates elicited antibody production, free antigens failed to trigger humoral responses (**Fig. 4A**). Highly repetitive surfaces are also known to bind strongly to natural

IgM antibodies through multivalent, high-avidity interactions [177]. Such antibody binding also facilitates cellular uptake of particles by macrophages and dendritic cells, which can in turn enhance immune processing through increased antigen presentation. It is also worth noting that many important components in the humoral arm of innate immunity, such as complement C1q, pentraxins, ficolins and collectins, are multimeric structures that favor high-avidity interactions with repetitive pathogen surfaces [178] (**Fig. 4B**). These observations highlight how the immune system has been primed to respond to repetitive motifs frequently found on viral particles.

Unlike free subunit antigens, nanoparticle vaccines present a high concentration of antigens on their surfaces. A nanoparticle can be surface functionalized with up to hundreds of antigens, effectively emulating the antigen display on viral surfaces [179, 180]. The multivalent antigen display on nanoparticles enhances antibody responses by efficiently cross-linking BCRs, activating complement, and facilitating antibody binding. These factors play synergistic roles in promoting B cell differentiation and stimulating DC-mediated T cell priming.

Although synthetically prepared nanoparticles have yet to show the level of ordered antigen arrangement found on viruses and virus-like particles derived from cell culture systems [181, 182], ongoing studies continue to demonstrate emerging techniques to couple antigens of interest to nanocarriers. In the last section of the review, we review commonly used methods for coupling antigens with nanoparticles. These strategies may spur novel approaches for preparing nanoparticle vaccines.

Strategies for antigen functionalization in nanoparticle vaccines

Association of protein antigens with nanoparticles can be divided into particle encapsulation and surface association. While many studies show excellent nanoparticle vaccine potency with encapsulated antigens, surface associated antigen may benefit from the innate immune factors and immune processing mechanisms described in the previous section. Techniques to associate protein or peptide antigens can be categorized into multiple categories. The different modes of antigen association are highlighted as follows.

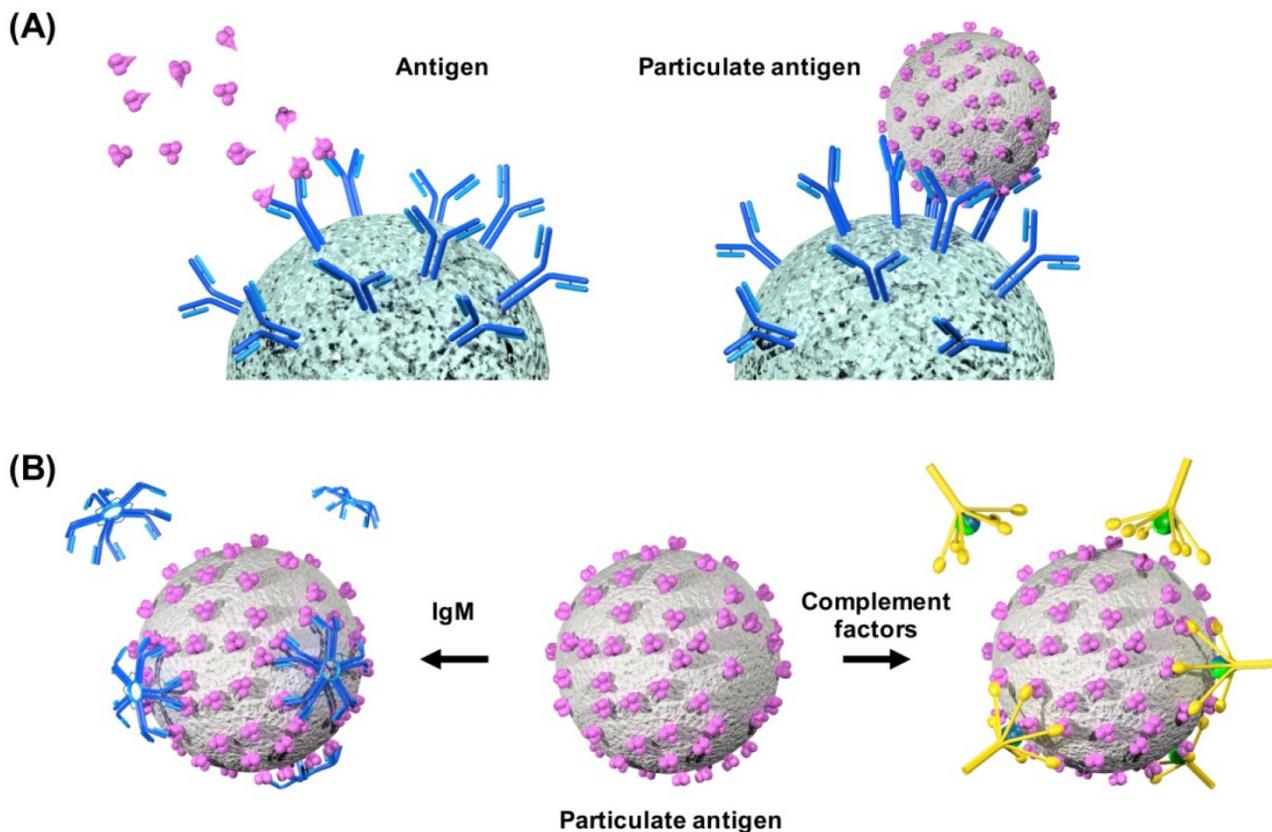


Figure 4. (A) Schematics showing multivalent interactions by nanoparticle vaccines promote B cell receptor clustering and facilitate receptor-mediated internalization. (B) Schematics illustrating enhanced binding of multimeric immune factors, such as IgM and complement factors, to nanoparticle vaccines.

Chemical conjugation

Thiol and amine groups on protein or peptide antigens are frequently exploited for bioconjugation with nanoparticles. The thiol group on a cysteine amino acid is a powerful nucleophile with the capacity to form covalent linkage. Several linker groups, such as maleimide and succinimidyl 3-(2-pyridyldithio)propionate (SPDP), facilitate the conjugation between thiol-containing antigens with nanoparticles [183]. In a maleimide-thiol reaction, the nucleophilic thiolate anion attacks the π -bond of maleimide, forming the enolate intermediate and yielding the desired conjugate. This technique has been widely used to assemble nanoparticle vaccines [184-186]. In a study on refining a liposomal formulation of HIV vaccine, thiol chemistry was exploited to control the physiological conformation and density of the target antigen to modulate immune responses [186]. Thiols groups also readily associate with gold surfaces. The strong interaction between sulfur and gold drives the sulfur atom to fill the free orbitals of a gold atom, creating a coordinate covalent bond [187, 188]. Such approach has been extensively applied to associate nucleic acids [188-192] and antigens [193, 194] with gold nanoparticles.

Amine groups, on the other hand, are present on all protein and peptide antigens, which can be linked to nanocarriers via amide bond formation, typically through carbodiimide crosslinker chemistry. Bioconjugations with amine-containing antigens are commonly performed using 1-ethyl-3-(3-(dimethylamino)propyl)carbodi-imide (EDC) and N-hydroxysuccinimide (NHS). NHS or its sulfonated form (sulfo-NHS) is efficiently coupled to carboxyl groups with the aid of EDC to form NHS esters. The NHS esters then covalently conjugate to primary amines to form an intermediate compound that is subsequently hydrolysed to the desired conjugate [195]. Many nanoparticle vaccines have been prepared with the EDC/NHS conjugation method with a high coupling yield [196, 197]. Polydopamine functionalization is another conjugation approach that is increasingly applied to associate proteins and peptides with nanoparticles. In alkaline pH, dopamine undergoes oxidative self-polymerization to form a layer of polydopamine that can coat almost any type of material [198-200]. This mussel-like adhesive layer enables a secondary reaction with biomolecules containing thiol or amine groups [201, 202]. The technique has been demonstrated on both organic and inorganic nanoparticles [203, 204]. Dopamine-incorporated polymers have also been employed to adsorb proteins for macrophage-targeted delivery [205]. The versatile

technique can be applied to conjugate multiple cargoes for vaccine applications [164].

Electrostatic interaction

Electrostatic attraction between oppositely charged antigens and nanoparticles have been exploited to prepare nanoparticle vaccines. In general, cationic nanocarriers are prepared for the association of anionic protein antigens. In the case of liposomes, cationic lipid DOTAP is frequently applied to render the nanocarrier positively charged. DOTAP-based liposomes have been shown to absorb HPV E7 peptides [206], enhancing antigen-specific CD8 T cell response and increasing antitumor responses. Strategies have also been employed to prepare antigens with added anionic moieties via recombinant protein engineering. By expressing HPV E7 protein and ovalbumin with an anionic lipoprotein, Shen *et al.* demonstrated enhanced antigen association efficiency and retention to DOTAP liposomes. The vaccine formulation elevated antigen-specific cellular responses and inhibited tumor growth in a mouse model [207, 208].

In another work based on PRINT (Particle Replication in Non-wetting Templates) technology, electrostatic interaction was exploited to generate nanoparticle vaccines of cylindrical shape. Cationic particles were prepared by blending positively charged polymers with PLGA prior to the imprint lithography process. Upon mixing with anionic hemagglutinin proteins of influenza viruses, a high level of antigen binding to the particle surface was achieved [209]. Immunization with the nanoparticle vaccine elicited a more potent anti-influenza antibody response compared to a commercial vaccine based on inactivated subunit influenza viruses. Unlike other spherical nanoparticle vaccines, the platform offers the ability to mimic the filamentous shape that can be found among many virus species.

Physical adsorption

Synthetic nanoparticles have high surface energies owing to their large radii of curvature. As a result, adsorption of protein can occur spontaneously owing to a combination of weak interaction forces, leading to the formation of protein corona formation [108]. This phenomenon was demonstrated to facilitate the assembly of synthetic virus-like nanoparticles [155]. Upon mixing gold nanoparticles with viral antigens, rapid protein adsorption took place on the nanoparticle surface. This adsorption led to colloidal stabilization of gold nanoparticles, preventing their aggregation in biological buffers. Biochemical analysis showed the facile assembly method surrounded each nanoparticle with

approximately 900 coronavirus spike proteins, inducing enhanced anti-viral immune responses. The adsorption approach has been used extensively to associated antigens with nanocarriers, including nanoparticles made of gold [210], calcium phosphate [211, 212], aluminium hydroxide [213], carbon [214, 215], silica [117, 216], and organic polymers [217, 218]. The range of adsorbed biomolecules vary from DNA [217, 219, 220], adjuvant [221], small peptides, and protein antigens [155, 218, 222, 223]. It is important to note that formation of protein corona is a dynamic process that can be strongly influenced by both nanomaterials and antigen of interest [224]. Studies have also shown that antigens can undergo conformational changes upon nanoparticle adsorption [225], which could have both positive and negative effects on the resulting vaccine formulations.

Biomembrane coating

Coating nanoparticles with cellular membranes is an emerging functionalization approach that can pave ways to novel vaccine formulations with virus-mimetic features. It has been shown that following dispersion of cell membrane vesicles with nanoparticles, highly controlled membrane coating can be achieved, yielding unilamellar membrane cloaked nanoparticles retaining membrane proteins in their right-side-out orientation [226-228]. These nanoparticles are structurally analogous to enveloped viruses consisting of cell-derived lipid membranes stabilized via viral capsids or matrix proteins [229]. The membrane coating approach has been adapted for vaccine preparations against cancer and bacteria [154, 230-232]. Enhanced dendritic cell activation and presentation of a melanoma-associated tumor antigen (gp100) was demonstrated by Fang *et al.* using PLGA nanoparticles cloaked in B16-F10 membranes [232]. The enhanced immune response to the membrane antigen was attributed to increased cellular delivery and colocalization with immunological adjuvant facilitated by the nanoparticles. Gao *et al.* also showed that bacterial outer membrane vesicles (OMVs) can be rendered more immunogenic following coating on gold nanoparticles. The OMV-coated nanoparticles significantly increased antigen delivery to lymph nodes and elevated production of cytokines associated with bacterial containment [233]. The membrane cloaking approach offers a titillating strategy towards future vaccine designs as the method enables coupling of membrane-anchored antigens in their native conformation with immunogenic nanocarriers.

Concluding remarks

Advances in nanotechnology and its adoption in

vaccinology have helped push the boundaries of non-live, subunit vaccines, resulting in many exciting demonstrations of effective immune potentiation by nanoformulations. Not only can nanoparticle vaccines enhance humoral responses against target antigens, they have been shown to promote cell-based immunity as well as immunological memory. These are hallmarks of good vaccine formulations that are often inherent to live attenuated viruses. An increasing number of studies have shed light on the mechanisms behind the benefits of nanoparticle vaccines, including lymph node targeting, multivalent antigen display, and coordinated delivery of antigens and adjuvants. These features can find their mechanistic analogues in the immunological processing of viruses. Given the frequent semblance between nanoparticle vaccines and viruses regarding their size, morphology, antigen display, and adjuvanticity effect, nanoparticles present a compelling platform in bridging the gap between live and non-live vaccines. Emerging techniques in nanoparticle functionalization also pave ways to novel formulation designs that promise controlled immune modulation. Going forward, understanding of virology may assist scientists and engineering in preparing emerging nanoformulations with advanced virus-like features. Such vaccine nanotechnology is envisioned to improve vaccine safety, potency, and availability, offering compelling platforms towards addressing the many public health threats yet to have effective prophylaxis and treatment options.

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Competing Interests

The authors have declared that no competing interest exists.

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