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Applications of nanomaterials as vaccine adjuvants

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Vaccine adjuvants are applied to amplify the recipient's specific immune responses against pathogen infection or malignancy. A new generation of adjuvants is being developed to meet the demands for more potent antigen-specific responses, specific types of immune responses, and a high margin of safety. Nanotechnology provides a multifunctional stage for the integration of desired adjuvant activities performed by the building blocks of tailor-designed nanoparticles. Using nanomaterials for antigen delivery can provide high bioavailability, sustained and controlled release profiles, and targeting and imaging properties resulting from manipulation of the nanomaterials' physicochemical properties. Moreover, the inherent immune-regulating activity of particular nanomaterials can further promote and shape the cellular and humoral immune responses toward desired types. The combination of both the delivery function and immunomodulatory effect of nanomaterials as adjuvants is thought to largely benefit the immune outcomes of vaccination. In this review, we will address the current achievements of nanotechnology in the development of novel adjuvants. The potential mechanisms by which nanomaterials impact the immune responses to a vaccine and how physicochemical properties, including size, surface charge and surface modification, impact their resulting immunological outcomes will be discussed. This review aims to provide concentrated information to promote new insights for the development of novel vaccine adjuvants.

Introduction

Adjuvants have been widely applied to increase the magnitude of an antigen-specific immune response by vaccination. Incorporation of an adjuvant into a vaccine can amplify, guide and/or accelerate the immune response toward the most effective form for each infection or malignancy.[1,2] With the growing understanding of the essential role of adjuvants in vaccines, the development of novel adjuvants is urgently needed due to increasing demands for unmet clinical needs. The expectations for a new generation of vaccine adjuvants are concentrated on the increased immunization efficacy of weak antigens, enhanced T cell responses of desired types and generation of multifaceted broadening immune responses without compromising safety. With the growing advances in material science and nanotechnology, the rational design and manufacture of novel adjuvants with desired activity and safety are becoming possible.

Nanotechnology has been a rapidly developing area since the last decade of the 20th century. To date, significant breakthroughs have been made in the design and manipulation of materials at the nanoscale to impact their performance in biomedical applications. Many types of substances, including chemical drugs, proteins as well as vaccines can be delivered by nanomaterial-based delivery systems to meet the criteria of high bioavailability, sustained and controlled release profiles, targeting, imaging and so forth.[3-6] Antigens delivered by nanomaterials can be protected from degradation and released in a sustainable manner, and their uptake by antigen-presenting cells (APCs) is more efficient.[7-11] Furthermore, a number of studies have shown the inherent regulatory activity of nanomaterials in cellular and humoral immune responses.[9,11-14] Therefore, integration of both delivery and immunomodulatory effects of nanomaterials in adjuvant applications will largely benefit the immune outcomes of vaccination.

The applications of nanomaterials as vaccine adjuvants have been increasingly investigated for immune protection and immunotherapy for infectious diseases and malignant cancers, and these materials have shown implicational advantages.[15-19] In this review, we will address the current achievements of nanotechnology in the development of novel adjuvants. Nanomaterials that perform adjuvant activity by enhancing antigen delivery for antigen presentation and via their inherent immunoactivation effect will be reviewed. We also aim to elucidate the underlying mechanisms for how nanomaterials impact the immune responses to a vaccine. Because physicochemical properties are believed to largely determine the adjuvant activity of nanomaterials,[12-14,20] the major properties of nanomaterials, including size, surface charge and surface modification, that impact their immunological outcomes will be discussed. We hope this review and discussion will provide new insights for the development of novel adjuvant formulations.

Current Understanding of Adjuvants and the Development of Nano-Adjuvants

Adjuvants are essential components in vaccines for enhancing or directing antigen-specific immune responses to immunization. An adjuvant is necessary in a vaccine mainly for the following

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reasons: (1) dose sparing, which means the adjuvant helps to promote sufficient immune responses with less antigen or fewer numbers of immunization; (2) enabling a broad antibody response against pathogens with antigenic drift or variations; (3) the ability to shape the immune response toward a functionally appropriate type to provide qualitative and durable protection against infection; and (4) promotion of a more rapid immune response.[1,2] Although numerous molecules and materials have showed immunomodulatory activities, only a small percentage of candidates have been licensed or applied in clinical tests. Indeed, many adjuvant candidates have failed due to their low efficacy, poor stability, unacceptable tolerability, and difficulty to manufacture or toxicity.

Adjuvants currently licensed in the US and Europe in human vaccines include alum (aluminum salts), squalene-in-water emulsions (MF59, AS03, and AF03), virosomes and AS04 (monophosphoryl lipid A preparation [MPL] plus alum).[1,11] Among the licensed adjuvants, MF59 (Novartis) is a nano-adjuvant which is 165 nm in diameter with the ability to recruit neutrophils, monocytes and dendritic cells (DCs) and enhance antigen uptake. In particular, MF59 has shown more potent adjuvant activity than alum in inducing humoral and T helper type 1 (Th1) immune responses.[21,22] Other nano-adjuvants, including virus-like nanoparticles (VLNs) (15–30 nm), poly(lactide-co-glycolide) (PLGA) nanoparticles (100–200 nm), cationic liposomes, nanoemulsion $W_{805}EC$ (400 nm), and cholesterol-bearing nanogel (30–40 nm), are under investigation in clinical trials nearing completion.[11] Emerging evidence shows that the ability to engineer and integrate desired properties and functions into nanomaterials will significantly contribute to generating novel adjuvants. For example, 15–30 nm VLNs mimic the structure of viruses, and their size and surface structure facilitate tissue penetration and lymph node (LN) targeting and also activate toll-like receptor (TLR) signaling.[23,24]

Based on the function and application, adjuvants can be divided into three major categories: immunomodulatory molecules, non-immunostimulatory delivery systems, and combinations of the former two. A number of immunomodulatory molecules have been applied in widespread experimental use or clinical trials. In particular, ligands of innate immune signaling receptors, including TLRs, Nod-like receptors (NLRs) and retinoic acid-inducible gene 1 (RIG-I)-like receptors, are the major types of immunomodulatory adjuvants.[1,2,25] For other classes of adjuvants, the delivery system mainly works to enhance antigen presentation to immune cells.[8,11,14,15,26-28] The two most well-developed delivery systems for vaccines are liposomes and virosomes. Other unconventional delivery systems are also rapidly being developed, including lipid-based particles, dendrimers, polymers, assembling structures of biomolecules, etc.[8,11,14,26,27]

To develop an ideal adjuvant, both the immunomodulatory activity and delivery function of an adjuvant should be considered. Next-generation adjuvants should be optimized for both activities by virtue of their multifunctional materials, and nanomaterials can represent a promising platform for combining delivery and immunostimulatory functions. As a versatile system for antigen delivery, nanomaterials can enhance antigen presentation through more efficient uptake by APCs. Due to the precise design of the surface of nanoparticles, DC targeting can be achieved via conjugation with the ligands of the mannose receptor, Fc receptor (FcR), CD11c/CD18 receptor, and DC-SIGN onto the nanoparticle surface.[29-37] To further facilitate antigen entry into a cell, cell-penetrating peptides (CPPs) and viral-like nanosurfaces have been applied.[23,24,38-40] More recently, smart nanoparticles have been manufactured using pH-sensitive or redox-sensitive materials;[23,24,40-43] these environment-responsive nanoparticles enable controlled release of antigens at target sites and more adequate release of antigens from endo-lysosomal compartments, thus enhancing antigen presentation. In addition to their delivery function, a great number of nanoparticles have shown immunomodulatory effects, particularly for innate immune signaling. For example, nanoparticles can induce inflammasome activation in macrophages and DCs,[44-46] enhance antigen presentation and APC maturation,[33,34] recruit immune cells,[21,22,47,48] direct T cell differentiation to a particular subtype, and activate the complement system.[12,13,49-51] Therefore, nanomaterials constitute a multifunctional unit to integrate target delivery and immunomodulation effects for clinical use in vaccination.

Recent Progress on Nanomaterials in Adjuvant Development

Nanomaterials for vaccine delivery

Nanomaterials for vaccine delivery are designed to enhance antigen uptake by APCs and/or obtain a controlled release or sustained release for antigen presentation.[52] These nanomaterial delivery systems, which we abbreviate as nanocarriers, offer several advantages for antigen delivery compared with soluble antigen inoculation. First, antigens entrapped in nanocarriers can be protected from proteolytic degradation in vivo, which generally causes the antigen dose required for immunization to be increased. Second, nanocarriers provide a sustainable release profile for antigens prior to their internalization by APCs or within the endosome-lysosomal compartment after internalization. Nanocarriers thus serve to provide a long-lasting depot of antigen to boost the immune system. Third, the particulate form of antigen, either entrapped within nanocarriers or bound to nanoparticles, facilitates APC sensing and uptake compared with the soluble form. In addition, nanocarrier surface modification of ligands or antibodies targeting pattern recognition receptors (PRRs) can enhance antigen delivery to specific APCs through active targeting. Last, nanocarriers enable co-delivery of different immunostimulatory components along with antigens to obtain a synergistic effect. Numerous nanomaterial delivery systems, including solid lipid nanoparticles, polymeric nanoparticles, co-polymer nanogels and liposomes, have been developed for antigen delivery.[7,9-11,13,15,27,53] Recent developments related to nanocarriers for vaccine delivery can be broadly divided into three groups: (1) passively APC-targeting nanocarriers, (2) actively APC-targeting nanocarriers, and (3) cytosolic delivery and smart nanocarriers.

Passively APC-targeting nanocarriers

The antigen sensing and uptake of a particulate form by APCs are superior to those for small soluble proteins.[1,2,14,15] Therefore, antigens bound to or encapsulated in nanoparticles can be more efficiently internalized compared with free antigens. By covalently conjugating bovine serum albumin (BSA) or *Bacillus anthracis* protective antigen (PA) protein to the outer surface of nanoparticles, the antibody responses in mice were increased after immunization. In particular, the anti-BSA antibody response was 6.5-fold stronger than that induced by BSA adjuvanted with alum, and the anti-PA antibody response following immunization of PA-conjugated nanoparticles elicited a quicker, stronger, and more durable protective immune response against a lethal dose of anthrax in mice.[54] Moreover, immunization with human Nogo-66 receptor Fc (hNgR-Fc) fusion protein conjugated to 15-nm gold nanoparticles generated higher titers of anti-NgR antibody, and the antibody responses were stronger than those following immunization adjuvanted with Freund's adjuvant.[55] Other attempts have focused on encapsulating antigens within the nanocarrier, as this strategy can protect the antigen from proteolytic degradation in addition to providing the antigen in a particulate form. PLGA is an FDA-approved biodegradable polymer widely used for controlled drug release for human uses.[28,56] In previous research, peptide Hp91 encapsulated within PLGA nanoparticles induced 5-fold more potent immune responses compared with the free peptide.[57] Moreover, when delivering protective antigen domain 4 (PAD4) of *Bacillus anthracis* via PLGA nanoparticles to Swiss Webster outbred mice, following single-dose immunization with PAD4-PLGA nanoparticles, a robust IgG response of the mixed IgG1 and IgG2a subtypes was induced, whereas the free recombinant PAD4 only elicited a low IgG response of the IgG1 subtype.[58] Furthermore, upon comparing the efficacy of these formulations to induce protective immune responses against a lethal challenge with *Bacillus anthracis* spores, the median survival of mice immunized with PAD4-PLGA nanoparticles was 6 d, whereas the survival time was only 1 d for mice immunized with free PAD4.[58]

Using the co-delivery capability of nanocarriers, double TLR ligands were encapsulated in nanoparticles with antigens to obtain synergistic enhancement and long-lasting antibody responses in mice.[59,60] Kasturi et al. used 300-nm PLGA nanoparticles containing MPL (TLR4 ligand) or R837 (TLR7 ligand) or both, along with an antigen for immunization. Antigens including ovalbumin (OVA), hemagglutinin (HA) from avian influenza H5N1 virus, and protective antigen (PA) from *Bacillus anthracis* were investigated.[59] Immunization of mice with PLGA (MPL+R837) plus PLGA(antigen) induced a synergistic enhancement of the primary and secondary antigen-specific antibody responses compared with PLGA(antigen) alone or together with single PLGA(MPL) or PLGA(R837). Furthermore, PLGA (antigen) plus PLGA(MPL+R848) yielded at least a 5-fold dose-sparing effect compared with antigen alone in the first immunization, and this result was still evident upon secondary immunization. Strikingly, immunization with PLGA(OVA) and PLGA (MPL+R837) induced persistent germinal center formation and stimulated long-lived plasma cell responses in the LN for more than 1.5 y. The triggering of TLRs on both B cells and DCs was believed to contribute to the synergistic enhancement of antibody responses.[59,60]

Another advantage of nanocarriers for delivery is that their surface chemistry can be easily manipulated. For example, antigen uptake can be further enhanced by altering the nanocarrier's surface charge, hydrophobicity and functional groups for APC targeting. To further enhance antigen uptake by adjusting the surface charge, cationic nanocarriers have been applied. Because the cell membrane has a negatively charged hydrophilic outer face, positively charged particles are preferable for cell membrane binding and internalization due to their higher binding affinity than neutral and negatively charged nanoparticles.[61,62] In particular, one study showed that the cellular uptake of cationic polyethyleneimine (PEI)-coated mesoporous silica nanoparticles (MSNP) was considerably enhanced compared with unmodified MSNP (silanol surface) or particles coated with phosphonate or PEG groups (neutral charge).[63] In addition, the rate and amount of cellular uptake were both positively correlated with the positive surface charge.[64] Wegmann et al. showed that the DC uptake of herpes simplex virus type-2 glycoprotein (gp140) alone was much lower compared with gp140-PEI complexes. At 24 h after intranasal administration, gp140 was primarily found associated with DCs in draining LN. The antigen-specific IgG titers in the serum were about 100-fold higher when gp140 was administered with PEI than alone and up to 6-fold higher than those elicited by another licensed mucosal adjuvant cholera toxin B subunit (CTB). Herein branched PEI forms of 750 kD (B750) and 25 kD (B25) gave higher titers of antigen-specific mucosal IgA than linear PEI forms (L40 and L160).[65] Self-assembled cationic poly(ethylene glycol)-b-poly(L-lysine)-b-poly(L-leucine) (PEG-PLL-PLLeu) hybrid polypeptide micelles also showed high efficiency as a simple and potent vaccine delivery system, as OVA encapsulated in this cationic polypeptide micelle stimulated robust specific antibody production that was up to 70–90-fold greater than that generated using free OVA. These cationic polypeptide micelles were also capable of inducing immature DC (iDC) maturation, enhancing antigen presentation and promoting the formation of a germinal center. Furthermore, by simultaneously co-delivering OVA with polyribocytidylic acid (PIC), a TLR3 agonist, this vaccine formulation synergistically augmented the tumor-specific cytotoxic T lymphocyte (CTL) response.[66] Several other examples of passive target delivery of antigens by nanocarriers and the relevant immunological outcomes are listed in **Table 1**.[67-73]

Actively APC-targeting nanocarriers

A number of nanocarriers are functionalized with a specific ligand or antibody to target DCs because DCs are the main APCs in the primary immune response. Antigens are first taken up by iDCs in peripheral tissues and processed and presented as major histocompatibility complex (MHC)/antigen peptide complexes to activate the adaptive immune system. Therefore, DC has become an attractive cellular target for vaccine delivery. Specific ligands or antibodies allow the vaccine material to interact with specific DC membrane

Table 1. Nanomaterials for antigen delivery

Function	Nanocarrier	Antigen	Molecular target	Immunological outcomes	References
Passively target APCs	PLGA nanoparticles	Human gp100 (25–33) TRP2 (180–188)		Stronger antigen-specific T cell responses than peptides mixed with Freund's adjuvant, delayed growth of subcutaneously inoculated B16 melanoma	67
	PLGA nanoparticles	Hepatitis B core antigen (HBcAg)		Altered the Th2-biased peptide-induced immune responses toward the Th1 type	87
	PLGA nanoparticles	Protective antigen domain 4 (PAD4)		Robust IgG response of mixed IgG1 and IgG2a subtypes; the median survival of PAD4-PLGA nanoparticle-immunized mice was 6 d, as opposed to 1 d for free PAD4	58
	Cationic micelles poly(ethylene glycol)-b-poly(L-lysine)-b-poly(L-leucine) (PEG-PLL-PLLeu) hybrid polypeptides	OVA		70–90-fold enhanced antibody production; synergistically augmented tumor-specific CTL responses when encapsulating a TLR3 agonist (PIC) simultaneously	66
	PLGA nanoparticle	Hp91		5–20-fold more potent immune response than free Hp91	57
	Lipid-based nanoparticles	Plasmodium vivax circumsporozoite antigen, VMP001,		Expansion of follicular T helper cells; antibody responses more durable than traditional adjuvants, even when using 10-fold less antigen	68
	Poly(methylmethacrylate) (PMMA) co-polymer	HIV-1 Tat protein		Long-lasting cellular and humoral responses	69
	PLGA coated iron oxide-zinc oxide nanoparticles	Carcinoembryonic antigen (CEA)		Visible on MRI, faster antigen uptake	70
	Amphiphilic poly (γ-glutamic acid) nanoparticles	OVA		25–50-fold higher DC uptake, 10–40-fold stronger cellular immune response	88
	Poly(propylene sulfide) (PPS) nanoparticles	OVA		Efficient accumulation within the draining LN; expansion of memory $CD8^+$ T cells	71
	Poly(lactide) (PLA) polymer	TT		Sustained anti-TT antibody response (more than 5 mo)	72
	Quantum dots	OVA		Dynamic monitoring and greater efficiency in T cell proliferation and IFN-γ production in vivo compared with free antigen	73
	Lecithin-based nanoparticles	BSA or Bacillus anthracis protective antigen (PA) protein		Faster and 6.5-fold stronger antibody responses than BSA adsorbed onto aluminum hydroxide	54
Actively target APC	Mannosylated cationic liposome	Por A (antigen of Neisseria meningitides)	Mannose receptor	Increased localization in draining LNs and increased antibody responses and IL-12 production	32
	Mannosylated liposome	Tetanus toxoid (TT) OVA	Mannose receptor	Enhanced expression of MHC class II, CD80, CD86 and CD83; and 6-fold greater uptake and 2.5-fold greater T cell proliferation compared with non-targeted liposomes	33
	Mannosylated polyamidoamine (PAMAM) dendrimer	OVA	Mannose receptor	Enhanced OVA-specific $CD4^+$/$CD8^+$ T cell responses and antibody responses; cross-presentation of OVA	74
	Mannosylated PLGA nanoparticles	OVA	Mannose receptor	Enhanced antigen-specific T cell responses; upregulation of CD80, CD86, CD40, HLA-DR and CD83 expression on DCs; increased IL-12 production	34
	Lipid calcium-phosphate (LCP) nanoparticles, mannose-modified	Tyrosinase-related protein 2 (TRP-2) peptide	Mannose receptor	Protected against later-stage B16F10 melanoma	35
	Liposome, Fc fragments modified	OVA	FcR	MHC class I-restricted presentation; increased IL-2 secretion	149

(*Continued on next page*)

Table 1. Nanomaterials for antigen delivery *(Continued)*

Function	Nanocarrier	Antigen	Molecular target	Immunological outcomes	References
	Liposome, Fc fragments modified	Luteinizing hormone releasing hormone (LHRH)	FcR	Increased iDC uptake; 2-fold greater lymphocyte proliferation	29
	Gold nanoparticles, Fc fragments modified	LHRH	FcR	Increased iDC uptake; visible; greater lymphocyte proliferation	29
	Quantum dots	Le X ligand, HIV envelop gp 120	DC-SIGN	Visible; more efficient uptake	75
	PLGA nanoparticles modified with antibody hD1	TT/BSA	DC-SIGN	10–100-fold less antigen for induction of antigen-specific T cell responses than non-targeted PLGA	36 37
	Glycan-modified liposome	OVA	DC-SIGN	Enhanced antigen uptake	30,31
	Liposome conjugated with DEC-205 antibody	OVA	DEC 205 receptor	6-fold greater uptake by DCs than non-targeted liposome	76,77
	Monomer cross-linked with DEC-205 antibody	OVA	DEC 205 receptor	Increased IFN-γ production and OVA-specific CTL activation	78

receptors, resulting in receptor-mediated endocytosis. Surface modifications targeting the mannose receptor, FcR, DEC-205 receptor and DC-SIGN have been widely utilized for developing targeted-delivery nanocarriers.[29-36,74-78]

A luteinizing hormone-releasing hormone (LHRH) vaccine was first developed for the treatment of prostate cancer over 20 years ago. In particular, synthetic LHRH-TT (tetanus toxoid fragment) immunogens have been linked to gold nanoparticles or encapsulated in liposomes to enhance antigen presentation.[29] Fc fragments have also been added onto both gold and liposome nanoparticles for targeting the IgG FcR. The uptake of LHRH-TT, either conjugated to gold nanoparticles or encapsulated by liposomes, was enhanced by the Fc fragment-targeting motif, thereby inducing increased antigen presentation in DCs. In addition, gold nanoparticle conjugation has enabled the monitoring of peptide intracellular localization by transmission electron microscopy.[29] Surface modification of OVA-encapsulated particles by anti-DEC-205 anibody can enhance OVA uptake by DCs almost 2-fold compared with non-targeted particles. In addition, mice injected with anti-DEC-205-conjugated particles showed 30% fluorescent DEC-205-containing DCs in the draining inguinal LN, indicating efficient uptake of the particles by DCs. Accordingly, CTL responses against MHC class I/OVA peptide-expressing cells from mice vaccinated with anti-DEC-205-conjugated particles were greater than those from mice vaccinated with free OVA.[78] Because the interaction strength between the nanoparticle surface ligand and the membrane receptors can be controlled by the type of ligand (i.e., affinity) and by changing the surface ligand density (i.e., avidity), nanomaterials with an optimum ligand surface density can actually improve binding and cellular uptake, which may be preferred for therapeutic and diagnostic purposes. Other approaches to enhance targeted delivery of antigen for antigen presentation and the relevant immunological outcomes are listed in **Table 1**.

Cytosolic delivery nanocarriers and smart nanocarriers

APCs process and present antigens through MHC class I molecules and/or MHC class II molecules, depending on the intracellular localization. Endogenous antigens (i.e., those produced by viral pathogens) degraded by the proteasome are released into the cytosol and presented by MHC class I molecules for $CD8^+$ T cell recognition. In contrast, exogenous antigens (those internalized from the extracellular environment) are degraded in the endo-lysosomal system, and the resulting peptides are loaded onto MHC class II molecules and transported to the plasma membrane for $CD4^+$ T cell recognition. Some particular APCs, such as $CD8^+$ DCs, can also cross-present exogenous antigens through MHC class I molecules. Therefore, by regulating the intracellular location of engineered nanocarriers, the choice of antigen-presentation MHC molecules as well as the resulting antigen-specific responses can be modulated.[1,2,9] In addition, PRRs, which sense pathogens and endogenous danger signals and function as co-stimulators of the T cell response, also exist in different locations within the cell. For example, TLR4 and TLR2, members of the TLR family, appear on the plasma membrane for sensing lipopolysaccharide (LPS) and lipoteichoic acid, respectively, whereas TLR7, TLR8, and TLR9 are mainly present on the endosomal compartment for recognizing bacterial RNA or DNA. NLRs, such as NLRP3 (NACHT domain-, leucine-rich repeat-, and PYD-containing protein 3) are present in the cytosol for sensing crystalline particles and endogenous danger signals.[1,2,9] Therefore, utilizing nanocarriers for vaccine delivery can guide antigen presentation by specific MHC molecules as well as pathogen-associated molecular patterns (PAMPs) to activate PRR signaling.

The majority of nanomaterials are taken up via endocytosis or phagocytosis, and nanomaterials present in early endosomes are either targeted to late endosomes or lysosomes.[79] Therefore, nanocarriers used to deliver antigens to lysosomal compartments for MHC class II presentation are more easily accessed. However, cytosolic target delivery is performed if the intracellular target is in the cytoplasm, especially for DNA vaccines that require expression in the cytosol for antigen production. Several strategies have been developed to overcome the endosomal barrier using viral capsids or non-viral delivery systems.[40,80] For non-viral delivery systems, pH-responsive nanoparticles, cationic nanoparticles, and nanoparticles functionalized with CPPs have been explored for this cytosolic delivery purpose.[38,39]

Nanoparticles modified with pH-responsive peptides or linkers undergo structural deformation or degradation under acidic pH, which disrupts their transfer across the endosomal membrane.[81,82] For example, OVA encapsulated within pH-sensitive liposomes was shown to be 3–6 times stronger in inducing CTL responses compared with pH-insensitive liposomes.[83] In addition, OVA conjugated to the pH-sensitive poly(propylacrylic acid-co-pyridyldisulfide acrylate) (PPAA-PDSA) polymeric carrier was tested for the presentation of OVA by the MHC class I pathway, and OVA conjugated to PPAA-PDSA showed pH-sensitive membrane-disruptive properties at pH values between 6–6.5. Furthermore, the polymer-OVA conjugates induced 22-fold increases in MHC class I presentation and OVA-specific CTL activation compared with free OVA. Comparatively, conjugates of OVA to pH-insensitive poly(methylacrylic acid) (PMAA) did not induce CTL activation because they did not display membrane-disruptive activities. Together, this study suggested that the pH-sensitive properties of the polymer that allowed it to destabilize the endosomal membrane were critical in increasing MHC class I presentation and CTL activation.[84] Polycations, such as PEI polymers, can also mediate their endosomal escape to cytoplasm through the "proton-sponge effect," where the proton-absorbing polymer induces osmotic swelling of the endosome and its eventual rupture.[65,85] DCs exposed to OVA-PEI complexes significantly enhanced the response of B3Z T cells, a T-cell hybridoma activated by recognition of H-2K^b in association with OVA(257–264) peptide, indicating the PEI's ability to enhance antigen cross-presentation in vitro.[65] Another approach for cytosolic delivery is to regulate the transport of nanocarriers through CPP functionalization.[38,80] CPPs are short peptides typically composed of positively charged amino acids such as lysine or arginine or have sequences that contain alternating patterns of charged amino acids and non-polar, hydrophobic amino acids. CPPs facilitate the bonding of nanoparticles to negatively charged membranes to facilitate cell penetration.[38,80] Tat-modified gold nanoparticles (~14 nm) can negotiate intracellular membrane barriers; these particles are initially found in the cytosol but later enter the nucleus, mitochondria and vesicles.[39] In another study, octaarginine (R8, also a CPP) was attached to liposomes to investigate the cytosolic delivery of OVA for MHC class I presentation. Comparing OVA-encapsulated R8 liposomes to pH-sensitive and cationic liposomes, R8 liposomes showed superior ability to increase MHC class I presentation, OVA-specific CTL responses and antitumor responses over other formulations.[86]

Immunomodulatory Effects of Nanomaterials

In addition to the delivery uses of nanomaterials for improving antigen presentation and immune responses to vaccines, nanomaterials themselves have intrinsic immunomodulatory activity that makes them potentially applicable as adjuvants.[12,13,21,22,44-46,50,51] Their nanoscale size, with all three dimensions ranging from 0.1 to 100 nm, happens to be the size range of the fundamental building blocks of biology (including DNA, proteins, viruses, ultrastructures and organelles). Thus, the ways in which engineered nanomaterials may interfere with the function of the host immune system and how these immunomodulatory activities may affect vaccines are increasingly important questions. In the following sections, the immunomodulatory activities of nanomaterials, including inflammasome activation, recruitment of immune cells and complement system activation, will be introduced.[12,13,21,22,33,34,44-51,87-90] The potential mechanisms for these effects will also be discussed to facilitate an in-depth understanding of the inherent adjuvant activity of nanomaterials.

Inflammasome activation

The adjuvant effect of particulate matter in the promotion of vaccine-specific immunity has been recognized for over 80 y.[91] Alum, one of the most common adjuvants in vaccines, has been applied in clinical use for many years,[92] although its mechanisms of action are not fully understood. Our current understanding of how alum enhances antigen-specific antibody responses acknowledges its ability to induce NLRP3 inflammasome activation.[93,94] Inflammasomes are caspase-1-activating platforms assembled by self-oligomerized scaffold proteins. Upon exposure to whole pathogens, danger-associated molecular patterns (DAMPs), PAMPs, or environmental irritants, NLRP1, absent in melanoma 2 (Aim2), NLRP3, and other members of the NLR family self-oligomerize via NACHT domain interactions.[95,96] Formation of these high-molecular-weight complexes triggers the autocleavage of caspase-1, which in turn controls the maturation and secretion of interleukin-1β (IL-1β) and IL-18 for downstream signaling. The NLRP3 inflammasome is the most fully characterized inflammasome and consists of the NLRP3 scaffold, the ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) adaptor protein and caspase-1. Alum adjuvant-induced NLRP3 inflammasome activation is considered to be critical for eliciting antigen-specific antibody responses, and this inflammasome has also been shown to link innate immunity to adaptive responses targeting tumor growth. In addition, an in vivo study showed that mice deficient in NLRP3, ASC, or caspase-1 failed to elicit a significant antibody response to OVA adsorbed to Imject alum adjuvants (commercial adjuvant product, a mixture of aluminum hydroxide and magnesium hydroxide), whereas the OVA-specific antibody response to complete Freund's adjuvant remained intact.[97,98]

Numerous particulates have been reported to activate the NLRP3 inflammasome, including alum, Imject® alum, asbestos, silica, gout-associated uric acid crystals, calcium pyrophosphate dihydrate (CPPD) and particulate wear debris.[93,99-102] Several nanoparticles have also shown the ability to activate the inflammasome, including carbon nanotubes (CNTs), carbon black nanoparticles, PLGA and polystyrene nanoparticles, titanium dioxide (TiO_2) nanoparticles, silicon dioxide (SiO_2) nanoparticles, and aluminum oxyhydroxide nanoparticles.[44-46,103,104] Crystalline or particulate matter is thought to trigger the inflammasome complex by providing a danger signal such as lysosomal destabilization and reactive oxygen species (ROS) production upon endocytosis of particulates.[93,94,99-102] Lysosome contents,

including factors such as lysosomal cysteine protease cathepsin B, are released into the cytoplasm and serve as danger signals that are sensed by NLRP3, leading to inflammasome activation.[93,94,101,102] Other danger signals triggering the inflammasome are the ROS generated by particulates.[99,100] The potent ability of nanoparticles to generate ROS and destabilize lysosomes also contributes to their inflammasome activation capability. Regarding the ultrasmall size of nanoparticles, it is thought that the relatively large surface area of nanoparticles is directly related to their ROS-generating capability and proinflammatory effects.[105] Different levels of IL-1β production can be generated by TiO_2 nanoparticles with different sizes, shapes and crystal structures, depending on cathepsin B release and ROS production.[103] In addition, polystyrene nanoparticles functionalized by different surface groups were shown to induce surface-charge-dependent inflammasome activation. Amino-functionalized polystyrene nanoparticles (PS-NH_2), which displayed a positive surface charge, triggered NLRP3 inflammasome activation in human macrophages and the subsequent release of IL-1β. Comparatively, carboxyl-functionalized or nonfunctionalized particles, which respectively displayed negative or neutral charged surface, did not show the ability to activate the inflammasome. The reason for this result was likely that the amino group of PS-NH_2 induced proton accumulation in lysosomes during the endocytosis process; as a result, proton accumulation was associated with lysosomal destabilization, cathepsin B release and damage to the mitochondrial membrane.[44] In addition, thioredoxin (TRX)-interacting protein (TXNIP), a NLRP3 binding partner under oxidative stress, was found to bind NLRP3 after PS-NH_2 stimulation.[106] TXNIP is a negative regulator of TRX (an ROS scavenger), and inflammasome activators such as uric acid crystals induced the dissociation of TXNIP from TRX in a ROS-sensitive manner and allowed it to bind NLRP3.[106] Furthermore, during stimulation with PS-NH_2, TXNIP dissociated from TRX and bound to NLRP3, and PS-NH_2-induced NLRP3 inflammasome activation was abolished by the ROS scavenger N-acetyl-L-cysteine, which thereby protected macrophages from mitochondrial damage, caspase-1 autocleavage and IL-1β release.[106] Another study examining carbon-based nanomaterials of different sizes and shapes indicated that long, needle-like CNTs similar to asbestos were more potent at activating the NLRP3 inflammasome compared with carbon black, short CNTs and long, tangled CNTs. Moreover, all CNT-induced NLRP3 inflammasome activation was shown to depend on ROS production, cathepsin B release, the P2×7 receptor, and the Src and Syk tyrosine kinases.[45] The uptake of particulate adjuvant is also required for activating the inflammasome in most cases. Comparing the impact of size on inflammasome activation induced by biodegradable PLGA and polystyrene (PS) microparticles, 430-nm and 1-μm particles induced a dramatic increase in the secretion of IL-1β by DCs due to their efficient uptake. In contrast, PLGA and PS particles of 10 μm and 32 μm in size were unable to activate the inflammasome because they were not able to be endocytosed.[94]

The link between the NLRP3 inflammasome and the immunostimulatory properties of particulate adjuvant remains controversial. Although one critical study showed that the NLRP3 inflammasome mediates the OVA antigen-specific adjuvant activity of IgG1 elicited by alum, other studies have not found any change in antigen-specific IgG titers after immunization of NLRP3-deficient mice.[107,108] Sharp et al.[94] demonstrated that the enhancement of OVA-specific antibody responses administered by PLGA microparticles was independent of NLRP3, whereas the recruitment and activation of a population of $CD11b^+Gr1^-$ cells and enhanced antigen-specific IL-6 production required NLRP3. These discrepant findings indicate that the NLRP3 inflammasome is not essential for, but is able to impact, the immune response. Moreover, the inflammasome-dependent adjuvant activity of particulates might be restricted to particular types of T cell responses and immunization protocols. Additionally, the enhanced OVA antibody responses induced by alum adjuvant were shown to be independent of TLRs, IL-1R, and IL-18R signaling.[109] Thus, whether additional pathways are required to coordinate with or are affected by the NLRP3 inflammasome for adjuvant activity is a principal challenge warranting further research. It should also be investigated whether other inflammasomes such as Aim2 and NLRP6 also play a role in vaccine adjuvant activity.

Complement activation

The complement system contains over 30 soluble and membrane-bound proteins. Complement system activation is a cascade that occurs via three different pathways: the classical, alternative and lectin pathways. C3 is a major component of the complement system whose activation products have shown molecular adjuvant activity in inducing strong antigen-specific humoral immunity.[110]

Recent findings indicate that opsonins (a type of complements) may be adsorbed onto nanoparticles (by the opsonin fragments of C3) when nanoparticles enter the circulation. Opsonins on nanoparticles may thus provide signals to phagocytic cells to promote the recognition and ingestion of particles, a process called opsonization.[13] This process elicited by the host immune response aims to clear the invading nanoparticles and remove nanoparticles from the bloodstream via phagocytosis by monocytes and macrophages. Opsonins can also form a dynamic protein corona that adsorbs onto nanoparticles, and this process is favored by increased hydrophobicity of the nanoparticles.[20,111,112] As a result of these interactions, nanoparticles can significantly impact complement activation.

Several nanomaterials have been investigated for their C3 activation effects during adaptive immune responses.[12,49-51,89,90] The degree of complement activation induced by nanomaterials is determined by their surface chemistry and size. Nanoparticles functionalized with different lipid-anchored gadolinium chelates were shown to induce rapid complement activation-dependent IgM antibody production and were propagated via the classical pathway. Moreover, the extent of this response depended on the chemical structures of the lipid-anchored chelates and surface charge.[51] Reddy et al. compared the complement activation effects of two different forms of pluronic nanoparticles: polyhydroxylated nanoparticles (OH-NPs) and polymethoxylated nanoparticles (CH_3O-NPs). In this study, the degree of complement

activation, assessed by the C3a level, was much higher with OH-NP compared with CH_3O-NP induction. Regarding the impact of surface chemistry and size, 25-nm CH_3O-NPs (CH_3O-25-NPs), 25-nm OH-NPs (OH-25-NPs), and 100 nm OH-NPs (OH-100-NPs) showed selective complement activation. In particular, OH-25-NPs demonstrated the ability to target LNs to induce DC maturation and strongly activate complement, and $CD8^+$ T cell memory was also induced after treatment with OH-25-OVA-NPs but not CH_3O-25-OVA-NPs. Consistently, a strong anti-OVA antibody response was observed only for OH-25-OVA-NPs but not for larger nanoparticles OH-100-OVA-NPs or low-complement-activating nanoparticles CH_3O-25-OVA-NPs. These results indicate that the humoral and cellular immunity triggered by antigens delivered by nanoparticles are impacted by their complement activation capability in a size- and surface chemistry-dependent manner.[12]

Immune cell recruitment

The effects of nanoparticles on immune cell recruitment are mainly related to their ability to induce phagocytes to produce proinflammatory cytokines, chemokines, and adhesion molecules. The secretion and expression of these molecules result in the recruitment of immune cells, including macrophages, DCs, T cells, neutrophils, basophils, and eosinophils.[47,48]

MF59, a nanoemulsion consisting of squalene oil, Tween 80, and sorbitan trioleate (Span 85), has been licensed in Europe for use in clinical vaccinations for influenza. This adjuvant has multifunctional activities, including enhancing antigen uptake, enhancing cytokine and chemokine release, recruiting monocytes and granulocytes to the site of injection, and augmenting the maturation of DCs and upregulation of C-C chemokine receptor 7 (CCR7).[21,22] In addition, pulmonary instillation of plant virus (papaya mosaic virus; PapMV) protein-conjugated nanoparticles containing an ssRNA elicited strong innate immune stimulation in the lungs, and the rapid recruitment of monocytes, macrophages, neutrophils and lymphocytes was observed.[47] Macrophages incubated with KMP-11 (Leishmania antigen kinetoplastid membrane protein 11)-loaded PLGA nanoparticles generated high levels of nitric oxide, superoxide, tumor necrosis factor α (TNF-α), and IL-6. Increased release of chemokines, including chemokine (C-C motif) ligand 2 (CCL2)/MCP-1 and chemokine (C-X-C motif) ligand 1 (CXCL1)/KC, was also observed, which consequently promoted macrophage and neutrophil recruitment. Moreover, PLGA nanoparticles loaded with KMP-11 also significantly reduced the parasite load in vivo.[48]

Major Physicochemical Properties that Determine Nanomaterial Adjuvant Activity and Safety

The understanding of the impact of physicochemical properties of nanomaterials on vaccine delivery and adjuvant activity is critical for the design of nano-adjuvants with desired functions. The trafficking and targeting behaviors and adjuvant activity of nanomaterials are largely determined by their size and surface modifications as well as the route of administration.[113-115] The safety of nano-adjuvants, which is crucial for their application in humans, is mainly impacted by their size, composition and surface charge. In the following sections, we will discuss the major physicochemical properties that control nanomaterial trafficking, adjuvant activity and safety.

Trafficking and targeting behavior of nano-adjuvants under different routes of administration

The major routes of immunization for human vaccines include the oral, intranasal, intramuscular, intradermal, intraperitoneal and intravenous routes. The size and surface properties of nanoparticles are the predominating factors controlling their behaviors in biological barrier transport, tissue and cellular uptake and the induction of immune responses.

During intranasal or aerosolized immunization to boost mucosal and lung immunity,[116] the deposition and distribution of nanoparticles in the respiratory tract is governed by diffusion due to displacement when they collide with air molecules, rather than inertial impaction, gravitational settling, or interception of bulk particles.[117] In previous studies, the fractional deposition of inhaled particles of different sizes in the nasopharyngeal, tracheobronchial, and alveoli regions of the human respiratory tract were simulated using a predictive mathematical model (International Commission on Radiological Protection 1994).[118,119] Microparticles of 1–10 μm in diameter were mostly deposited in the nasopharyngeal compartment; nanoparticles of 10–100 nm in diameter were mostly deposited in the alveolar region; and nanoparticles of 1–10 nm in diameter were prone to deposition in the tracheobronchial region. For example, 5-nm inhaled particles were approximately equally deposited in the nasopharyngeal compartment, tracheobronchial region and alveolar region. Comparatively, approximately 50% of all 20-nm particles were deposited in the alveolar region, with approximately 15% in each of the tracheobronchial and nasopharyngeal regions. Another difference between nanoparticles and microparticles is that, once deposited, nanoparticles appear to readily transfer across barriers to extrapulmonary sites and target different organs. In contrast, large particles are rarely transferred to extrapulmonary sites and are instead cleared by mucociliary movement or phagocytes. Thus, the selection of optimally sized nano- or micro-adjuvants can be based on the site of interest in different regions of the respiratory system.

Upon intradermal injection, nanoparticles are more efficient at overcoming biological barriers in comparison to microparticles.[14] Intradermal-injected pluronic-stabilized polypropylene sulfide (PPS) nanoparticles with a size of 25–40 nm were shown to penetrate tissue barriers and traffic to the draining LN more rapidly than particles greater than 100 nm and were retained in the LN for at least 120 h after injection. In contrast, injected 100-nm PPS nanoparticles were mainly retained at the site of injection and required internalization and trafficking by DCs for transport to the LN.[12,120] In addition, 25-nm PPS nanoparticles were found within 50% of DCs isolated from the LN, whereas 100-nm PPS nanoparticles were only found within 6% of DCs, and clearance from the draining LN took less than 24 h.[12,120]

Nano-adjuvants injected intravenously or nanoparticles that bypass tissue barriers at the site of administration will enter the circulation. It has been suggested that the mechanism of hepatic uptake is mediated by the surface absorption of proteins, leading to opsonization,[121] thereby inducing alterations in blood circulating time. Surface modifications to nanoparticles using specific antibodies or ligands may also significantly affect their distribution and tissue uptake. Active targeting of nanoparticles involves the conjugation of targeting ligands to the surface of nanoparticles. Generally, the active targeting mechanism takes advantage of highly specific interactions between the targeting ligand and certain tissues or cells within the body to promote the accumulation of nanoparticles at a specific site or cell type for highly efficient immunization.[122,123] There is growing literature in this area, as described in the "Actively APC-targeting nanocarriers" section. These types of ligands mainly include antibodies, engineered antibody fragments, peptides and aptamers. In general, an optimum surface density of ligand coating can improve binding and tissue uptake, which may be preferred for vaccine purposes.[124]

Comparing the vaccine delivery and immune responses generated by different-sized nano-adjuvants or even micro-sized adjuvants, it remains unclear what the optimum size range is. However, it is clear that for all purposes pertaining to the targeted delivery of drugs, nanoparticles are superior to large particles because they are more efficient at crossing biological barriers and circulating in the blood, with a prolonged half-life.[14,125] However, reports on vaccine delivery are conflicting as to what size is optimal for the generation of stronger and long-lasting immune responses. For example, the antibody response to BSA entrapped in PLGA particles was stronger after 1000-nm particles were subcutaneously injected than when 200- or 500-nm PLGA particles were injected.[126] Similarly, hepatitis B surface antigen (HBsAg) entrapped in 2000- to 8000-nm PLA particles induced a stronger anti-HBsAg antibody response than HBsAg entrapped in 200- to 600-nm PLA particles.[127] In contrast, another report showed that when subcutaneously injected into mice, 230-nm OVA-conjugated nanoparticles engineered from lecithin/glyceryl monostearate-in-water emulsions induced stronger OVA-specific antibody and cellular immune responses than 708-nm OVA-nanoparticles.[128] Additionally, experiments using TT adsorbed onto PLGA particles also showed that small particles of 100 and 500 nm in size induced significantly greater antibody responses than particles >1000 nm after oral or intranasal administration.[129] Other studies have indicated that there may be an optimal size for vaccination. For example, experiments using OVA conjugated onto polystyrene beads of different sizes (20, 40, 100, 500, 1000, and 2000 nm) showed that particles with a size of 40 nm were most efficient in inducing both antibody and cellular immune responses after intradermal immunization.[130] When studying the cellular uptake of Herceptin (membrane receptor ErbB2-antibodies)-coated gold nanoparticles ranging from 2 to 100 nm in diameter, the 40- and 50-nm sizes demonstrated the greatest receptor-mediated endocytosis efficiency.[131] We believe that the best size for each nanoparticle in vaccine delivery depends on the nanoparticle's surface hydrophobicity, charge, the type of peptide/ligand (i.e., affinity) and the surface peptide/ligand density (i.e., avidity). Moreover, the extent and duration of different types of immune responses (e.g., Th1, Th2) following vaccinations administered by different routes may also vary, even when using the same nanoparticle. Thus, careful interpretation of nano-bio interactions is required for accelerating the future application of nano-adjuvants toward clinical use.

Safety and potential risks

When applying nanomaterials into vaccine uses, the safety of the materials apart from the safety of the loaded antigens is of great concern. Overall, concerns about the potential toxicity of nanomaterials have mainly focused on their biological fate, off-target effects and unpredictable toxicity to susceptible populations such as pregnant women. Nanosized particles are superior to microparticles in terms of their ability to bypass biological barriers. Herein, we focus on nanoparticle transfer across the placental barrier[132-135] and blood–brain barrier (BBB). The BBB is a physical and physiological barrier that regulates the passage of molecules from the systemic circulation into the brain parenchyma. Both organic nanoparticulate systems and inorganic nanoparticles have shown the ability to enter brain tissue, which was unwanted for most vaccine application.[136-141] Moreover, surface functionalization with certain receptors[137,140] or CPPs[139] was shown to increase the BBB translocation of nanoparticles. Nanoparticles administered by nasal inhalation can also translocate into the brain via the olfactory nerve and olfactory bulb without overcoming the brain vascular endothelium.[142,143] The BBB transfer of nanoparticles may be potentially harmful to the neurological systems. Another important biological barrier, the placental barrier, is of particular interest in protecting the developing fetus during pregnancy. The materno-fetal transfer of nanoparticles is thus of great importance in the safety and medical application of nanomaterials.[133,134,144] TiO_2 nanoparticles administered to mice during early pregnancy were shown to reach the fetal brain and cause developmental abnormalities.[145] Yamashita et al. found that silica and TiO_2 nanoparticles with diameters of 70 and 35 nm, respectively, caused pregnancy complications after intravenous injection into pregnant mice. In particular, these silica and TiO_2 nanoparticles were found in the placenta, fetal liver, and fetal brain and induced fetal toxicity.[134] Wick et al. found that fluorescent polystyrene particles with a diameter of up to 240 nm were able to cross the placenta in an ex vivo human placental perfusion model.[144] The materno-fetal transfer of nanoparticles is also gestational age dependent. For example, 13-nm Au nanoparticles were able to translocate into the fetus before embryonic day 11.5 during murine pregnancy, but rarely after day 11.5. The materno-fetal transfer ability of 13-nm Au nanoparticles depended on their surface modification. Fetal accumulation of ferritin- and PEG-modified Au nanoparticles was considerably greater than citrate-modified nanoparticles.[146] Thus, special attention must be paid to the potential risks from unintentional exposure of susceptible populations to vaccines.

Another concern regarding the safety of nanoparticles is their biological fate and the resulting biological consequences,

particularly for non-easily degradable or non-degradable materials that have a high risk of accumulation. Graphene, Au, and TiO_2 are of particular interest in delivery and labeling purposes for biomedical applications. These materials are stable, and hardly undergo any degree of bioprocessing and can only be excreted from cells or accumulate in specific cells and organs. Thus, the potential toxicity resulting from their accumulation would depend on the dose, the properties of the nanoparticles and the site of accumulation. From the limited literature on nanoparticle clearance, removal of non-degradable nanomaterials from live cells seems to occur mainly through exocytosis.[39,147] Diffusional movement of nanoparticles through cell membranes is unlikely to occur under normal conditions. Transferrin (Tf)-coated spherical-shaped Au nanoparticles (Tf-Au) are exocytosed from cells in a linear relationship with their size,[147] whereas smaller Tf-Au particles appear to exocytose at a faster rate and higher percentage than larger Tf-Au particles. It is assumed that this relationship could be extended to other types of spherical nanoparticles in the sub-100 nm size range that have other protein coatings and that enter cells via endocytosis. Therefore, when using these non-degradable nanomaterials for vaccine delivery or labeling precaution and careful testing of their metabolic behavior are necessary to avoid potential long-term accumulation and risks. Moreover, an understanding of the dominant physicochemical factors that control nanoparticle absorption, distribution, metabolism and excretion (ADME) at the systemic level and their uptake and bioprocessing at the cellular level is important for the rational design and evaluation of the efficacy and safety of nano-adjuvants, and two previous reviews have discussed these issues in greater detail.[113,114] Together, these previous studies indicate that the biological behavior, benefits and safety of nanomaterials must be better understood before they can be developed and used in the clinic as nano-adjuvants.

Concluding Remarks

The use of nanotechnology for immunotherapy and prophylactic immunization is increasing, as the ability to manipulate nanostructures offers the opportunity for the unique design of nanomaterials for vaccines. The encapsulated components, the building blocks of the nanocarriers, the surface functionalization and the key features in their regulation of immune responses are expected to be engineered and integrated in a desired way to achieve a synergistic effect for highly efficient immunization. Nanomaterials with unique immunomodulatory activity and efficient delivery properties controlled by their size, shape, hydrophobicity, surface modification, and functionalization will provide new approaches to researchers to obtain the desired immune response. For example, by controlling the physicochemical properties of nanoparticles, it is possible to modify a vaccine to enhance its uptake efficacy in target sites such as LNs and by cell types such as DCs through targeted delivery. Nano-adjuvants will also enable a reduced dose and number of required immunizations by virtue of the antigen-depot effect of nanocarriers. In addition to these advantages, some promising strategies such as using pH-sensitive smart nanoparticles, polycationic nanoparticles, and CPP-modified nanoparticles to deliver the antigen into cytosol have been shown to guide antigen presentation to the MHC class I pathway. These specialized nanoparticles are superior at targeting specific CTL responses and antibody responses, which may be used to promote greater anti-tumor responses in clinical use.

Although nanotechnology has had numerous brilliant applications and formed the basis of some promising strategies for vaccines, several concerns and issues still need to be addressed to bring nano-adjuvanted vaccines to the clinic. Fundamental challenges for how to optimize the biological behaviors and minimize the potential risks of nanomaterials need to be overcome. For example, when using pH-sensitive smart nanoparticles, polycationic nanoparticles and CPP-modified nanocarriers for vaccine delivery, we should take precautions to avoid overly strong binding to membranes due to the cationic groups present on the nanomaterial. If the cationic density is not controlled, these interactions may compromise the integrity of the cell membrane, potentially leading to pore formation and membrane disruption and thus toxicity.[148] The superior biological barrier translocation ability of nanoparticles such as BBB transfer and fetal transfer also requires full assessment before clinical use. The extent of the immune responses induced by nanomaterials also needs to be carefully controlled to obtain optimal adjuvant effects rather than toxic responses. A comprehensive understanding of nano-bio interactions and the dominant physicochemical factors involved in inducing an immune response remains unclear, and this also represents a major challenge for the future application of nanomaterials in the clinic. Because multiple physicochemical properties dictate nanoparticle absorption, translocation, metabolism and clearance, there is still no common guideline specifying the properties of the nanoparticles or their administration. Till now, few studies have sought to predict the effects of certain physicochemical properties on the fate of nanoparticles. Thus, the establishment of standardized practical strategy will require further experimental data to help build a reference database to guide further studies. The lack of more effective monitoring techniques or methodology, particularly in situ, real-time, rapid, and quantitative methods for characterizing the biological behavior of nanoparticles, is a major challenge for studying the causative relationships between the physicochemical properties of nanoparticles and their elicited immune responses. Breakthroughs are urgently needed in this field, as this knowledge will be essential for developing sustainable nanotechnology for vaccines and other biomedical applications.

Disclosure of Potential Conflicts of Interest

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