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Applications and perspectives of nanomaterials in novel vaccine development

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Vaccines show great potential for both prophylactic and therapeutic use in infections, cancer, and other diseases. With the rapid development of bio-technologies and materials sciences, nanomaterials are playing essential roles in novel vaccine formulations and can boost antigen effectiveness by operating as delivery systems to enhance antigen processing and/or as immune-potentiating adjuvants to induce or potentiate immune responses. The effect of nanoparticles in vaccinology showed enhanced antigen stability and immunogenicity as well as targeted delivery and slow release. However, obstacles remain due to the lack of fundamental knowledge on the detailed molecular working mechanism and *in vivo* bio-effects of nanoparticles. This review provides a broad overview of the current improvements in nanoparticles in vaccinology. Modern nanoparticle vaccines are classified by the nanoparticles' action based on either delivery system or immune potentiator approaches. The mechanisms of interaction of nanoparticles with the antigens and the immune system are discussed. Nanoparticle vaccines approved for use are also listed. A fundamental understanding of the *in vivo* bio-distribution and the fate of nanoparticles will accelerate the rational design of new nanoparticles comprising vaccines in the future.

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1. Introduction

Vaccines stand out as one of the most notable triumphs in the history of modern medicine. Vaccination has become a key factor to increase the life expectancy in the past centuries. 400 years after the first utilization of vaccination, a growing number of tools to facilitate the rational design of vaccines have become a reality.¹ Vaccination remains the most effective strategy against infectious diseases and represents the most relevant contribution of immunology to human health.² However, most candidate vaccines currently available were developed empirically with a lower immunogenicity. The safety of attenuated or inactivated vaccines is kept on being improved, while their ability to elicit long-lasting and potent immune responses was limited.^{3,4} There is an increasing need for delivery systems and adjuvants to have safe and potent immunogenicity.

The use of nanotechnology in vaccinology has transformed many areas of the traditional diagnosis and treatment of diseases, and provided fresh insights into previous difficulties to develop effective vaccines.^{5,6} Thus, nanotech-

nology provides the opportunity to design various nanoparticles in different compositions, sizes, shapes, and surface properties, for application in the field of medicine.

Natural or synthetic materials that can package exogenous therapeutics and self-assemble to form nanoparticles (NPs) with submicron diameters in aqueous media have been extensively studied as drug delivery carriers.^{7–10} Nanoparticles can mimic biophysical and biochemical modes due to their similar size to cellular components.¹¹ Nano-carriers with poly(ethylene glycol) (PEG) cloaking are able to delay the clearance of drugs from the body and, thereby, prolong their systemic circulation when compared with free drugs. This could ultimately be beneficial to drug accumulation in the diseased sites. Additionally, NP-mediated delivery systems can provide several unique properties, including high drug loading, sustained release kinetics, and the alleviation of drug toxicity *in vivo*. Consequently, strategies relying on NPs as delivery systems or adjuvants offer new opportunities to enhance the activation of innate immunity and to induce potent immune response to minimal toxicity.

An antigen to activate immunity, an immune potentiator to stimulate the innate immune system and a delivery system to ensure trafficking and targeting of the antigen and the immune potentiator to the antigen presenting cells (APCs) are the key factors of a successful vaccine. To achieve these factors, the design of nanoparticles should focus on composition, size, surface charge and surface properties which showed impact on the modulation of particle biodistribution,

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antigen delivery, immunostimulatory efficiency and overall immune response.^{12–17}

Virus-like particles (VLPs) are ideal nano-vaccine systems derived from virus proteins.¹⁸ γ -PGA (γ -poly-glutamic acid) is one of natural polymer-based particles with the potential to enhance antigen delivery systems and effective vaccine adjuvants generating potent immune response. Emulsions, liposomes and synthetic polymers are nano-size particles that are made into adjuvants for appropriate delivery of immune potentiators.¹⁹

The utilization of nanoparticles in vaccinology has attracted considerable interest in recent years. As new advances increasingly progress to draw on a rational approach for the design and development of vaccine candidates, we have a deeper understanding of the design criteria for synthetic particles to imitate the biophysical and biochemical characteristics of pathogens and induce potent immune response without their toxicities.

In this review, we provided an overview of the types and applications of nanoparticles in vaccines. Accordingly, we outlined different mechanisms of action of nanoparticles. Furthermore, the candidate vaccines approved for medical use were also listed in our review.

2. Types of nanoparticles

In the past decades, an increasing number of proteins and peptides have been emerging as novel potential therapeutics.^{2,20,21} Despite these biotherapeutics revolutionising the pharmaceutical scenario by prospecting novel therapeutic opportunities, their poor bio-pharmaceutical properties and inconvenient pharmacokinetic profiles often represent the bottleneck for their pharmaceutical development, which dramatically hinders their widespread application.²² Their attractiveness is based on various advantages such as enhancement of drug stability, addition of target principles and controlled drug release. Nanoparticles carrying antigens are able to affect the type of immune response and to markedly enhance a cytotoxic T-cell's response against the particle-associated antigen. This is due to the unique capability of some professional antigen-presenting cells (APCs) which can efficiently internalize foreign particulate materials such as microparticles and bacteria. This process called phagocytosis becomes activated by the recognition of the antigenic material in order to process and present both self and foreign antigens to other cells of the immune system. Nanoscale materials can improve the efficacy of vaccines due to their advantages for carrying biopharmaceutical drugs including: small size which allows their enhanced permeation into tumors and retention in tumors in targeted delivery, and nanoparticles has high adjuvancy for vaccine applications; however, the limitations are the non-specific uptake in reticuloendothelial system (RES) organs and the forming immunotoxicity.¹⁹

2.1. Virus-like particles

Influenza virus infections are a major public health concern and have caused significant morbidity and mortality world-

wide. Current influenza virus vaccines are an effective countermeasure against infection but need to be reformulated almost every year owing to antigenic drift. Moreover, these vaccines do not protect against novel pandemic strains, and the timely production of pandemic vaccines remains problematic due to the limitations of the current technology. Improvements in vaccine formulations have been made with the goal of eliciting better protection against seasonal influenza virus strains, in order to induce a stronger, broader and more sustained immune response.

Virus-like particles (VLPs) are self-assembled nanoparticles which contain biocompatible recombinant proteins (*i.e.* capsid of the hepatitis B virus), sometimes in combination with nucleic acids (*i.e.* bacteriophage) or a viral envelop with viral coat proteins (*i.e.* virosomes). VLPs have been used as the most effective nanoparticles in vaccinology due to their naturally optimized characteristics. VLPs can generate robust immune responses even in the absence of adjuvants *via* their optimized particle size and repetitive antigenic structures.²³ They do not possess any viral infectious genetic material, which avoids virus infection and replication. It is possible to produce self-assembled synthetic VLPs (SVLPs) with synthetically derived particles which have a size and composition similar to naturally derived particles. These self-assembled SVLPs can elicit adaptive immunity without the need for adjuvants, and in addition to being optimized, can be produced by various synthetic chemical methods. Recently there have been many VLP vaccines in clinical and preclinical trials, but only a few of them have been approved for use. The first VLP-based vaccine for hepatitis B was commercialized in 1986 and has been administered since then. VLPs work as delivery systems for multiple human papilloma virus vaccines (HPV) including Gardasil and Cervarix. These vaccines provide protection against a vast majority of HPV serotypes making VLPs robustly effective by eliciting highly cross-protective antibody responses.

There are two major problems related to pandemic influenza vaccines that need to be addressed. The first is the lag between pandemic virus identification and vaccine development and distribution. When a novel pandemic virus is identified, it takes months to develop, test, distribute and administer a new vaccine.¹⁹ After vaccination of an individual, it takes an additional 2–3 weeks until a protective immune response is mounted. The second issue is their low immunogenicity. For example, current pandemic candidate vaccines against H5N1 and H7N9 induce relatively weak immune responses. The cause of this low immunogenicity is currently debated, and vaccine formulations and regimens to overcome this problem are being developed.

2.2. Self-assembled protein

To recognize the power of the VLP approaches, various supra-molecular structures are formed from self-assembled proteins.²⁴ However, the self-assembled proteins may not be derived from virus components. Examples include structures

such as viruses, some bacterial micro-compartments and eukaryotic vaults. Self-assembly of proteins can be induced by changes in the lipid length and position in the lipoprotein molecules with significant modification to their morphology and secondary structures. Self-assembled peptide nanoparticles (SAPNs) can be used as drug delivery systems without the risk of anaphylactic shock.^{25,26} These nanoparticles which consist of two helical coiled-coil domains are connected by a short linker region into a single peptide chain. One helix forms a trimeric coiled coil while the other forms a pentameric coiled coil.²⁷ Multivalent synthetic vaccines are made by conjugation of OVA CD4 LPs and the OVA CD8 peptide using azide-alkyne Huisgen cycloaddition.^{8,28} These vaccines have a self-assembling tendency in aqueous media due to their physicochemical characteristics. Chung²⁹ reported that peptide amphiphiles show no evidence of toxicity found within the liver, spleen, kidney, bladder, intestines, lung, and heart. Compared with naked antigens, APNPs (antigens as nanoparticles) can more effectively enter APCs and present antigen epitopes.³⁰ An *in vivo* experiment indicated that APNPs displaying 4E10 and 2F5 epitopes of HIV on their surface (two crucial targets for inducing neutralization of antibodies) induced potent antibody responses against important neutralizing antibody epitopes, which are crucial to prevent HIV infection.³¹ Another kind of self-assembling protein vault proteins (MVPs), which contains ninety-six units with a size of approximately 40 nm wide and 70 nm long, can self-assemble into a barrel-shaped vault nanoparticle.³² Therefore, antigens can be packaged inside the vault nanoparticles by the self-assembling process when mixed with MVPs.

2.3. Inorganic nanoparticles

The use of inorganic nanoparticles in vaccinology has been widely studied as evidenced by various recent publications. Inorganic nanoparticles consisting of an inorganic solid nanoparticle core can be conjugated with antigens, which have been used in vaccinology to make adjuvants of and delivery carriers of antigens to enhance their immune responses. The important attribute of the inorganic nanoparticles lies in their rigid structures and controllable synthesis, although these nanoparticles are mostly non-biodegradable.^{33,34}

2.3.1. Carbon nanoparticles. There have been a lot of references on the use of carbon nanomaterials as adjuvants or carriers for different kinds of vaccines. Their immunity effects are significantly affected by their physicochemical properties, especially surface modifications.

Carbon nanoparticles are widely used as carriers and adjuvants in various vaccines; these nanoparticles have been used for the delivery of antigenic epitopes to APCs because they have the trend towards internalization into a wide variety of cell types and several mechanisms.^{35–40} The effect of carbon nanoparticles was affected by the biocompatibilities and physicochemical properties including their surface modifica-

tions, which were the most influential factors of the adjuvant effects.⁴¹

Carbon nanotubes (CNTs) have a unique geometry with a length of 100–1000 nm and a diameter of 0.8–2.0 nm, which can alter the immunogenicity of appended antigens. Single-walled carbon nanoparticles (SWNTs) as antigen carriers for the delivery of antigens into APCs stimulate humoral immune response *via* binding and presentation of the peptide antigens by the MHC molecules of APCs.⁴² Several forms of carbon nanoparticles including fullerene, CNTs and graphene have also been investigated as promising adjuvant candidates due to their good biocompatibilities.^{43,44} A previous study showed that modified water-soluble CNTs had excellent biocompatibility. They could be internalized quickly by B and T lymphocytes and macrophages, and had a negligible effect on cell viability and immune function (Table 1).⁴⁵ The conjugation of synthesized tuberculin purified protein derivatives (PPD) and carboxylated SWCNTs generated a preferential cellular response in the absence of potential cytotoxic effects in mice.²²

2.3.2. Silica nanoparticles. Silica nanoparticles (SiNPs) are excellent nanocarriers due to their many applications including vaccine delivery, selective tumor targeting and real-time multimodal imaging;^{46–48} they have been used to enable *in vivo* antigen release.⁴⁶ SiNPs interact with cells in different patterns by controlling the sol-gel chemistry to adjust tunable structural parameters including the particle size and shape.⁴⁹ Further modification can be achieved *via* abundant surface silanol groups, which can lead to additional functionalities including cell recognition, improvement of interaction with cells, enhancement of cellular uptake and absorption of specific biomolecules.^{50–52} The antigenic conjugation of these particles was not required to impart their immunostimulatory effect, which made SiNPs good delivery systems.^{53,54} However, the most critical problem concerning the use of NPs in biomedical and biotechnological applications is the toxicity derived from the reducing agents and stabilizers used for the NP synthesis (Table 1).

It is possible to make controlled-release and high-efficiency nano-carriers with a size of 50–200 nm termed mesoporous silica nanoparticles (MSNs), which can be used as both nano-carriers and adjuvants for the delivery of effective antigens.⁵⁵ Novel silica vesicles (SV) as new generation antigen carriers and adjuvants are prepared for the bovine viral diarrhoea virus (BVDV), which is widely distributed in the cattle industry. These nanoparticles with a particle size of 50 nm showed a high loading capacity and controlled release of the codon-optimized E2 (oE2) protein, which is the major immunogenic determinant of the BVDV.

2.3.3. Gold nanomaterials. Gold nanomaterials with sizes ranging from 1 nm to 150 nm can be easily modified into different shapes (*i.e.* rod, spherical, cubic, *etc.*) and surface-fabricated with carbohydrates.^{56,57} Both size and shape are the affecting factors of the immunity effect of gold nanomaterials, which lead to an enhancement in immune response *via* different cytokine pathways. Immune responses of

Table 1 Characteristics of different types of nanoparticles used in vaccines

Types	Size	Shape	Bio-toxicity	Biocompatibility	Disadvantages	Ref.
VLPs	20–800 nm	Particle	Some unpredictable consequences	Highly cross-protective antibody responses	Low immunogenicity	19, 23
SAP	10 nm length (70 nm) and wide (40 nm)	Spherical or barrel	—	Reduce the risk of immunogenicity; improve half-life	Creates a new molecular entity; poor solution stability and aggregation	25, 32
CNPs	30–60 nm length (100–1000 nm) and diameter (0.8–2.0 nm)	Fullerene particle, nanotube or mesoporous spheres	Negligible effect on cell viability	Good biocompatibility	Low immune function	39, 43
GNS	2–150 nm	Particle, rod, spherical, and cubic	Immuno-toxicity	More potent immune response	Immuno-toxicity	56, 58, 59
CNs	50–100 nm	Calcium phosphate particle	Biocompatible and safe	Biocompatibility and easily biodegradable.	—	63, 64, 70
SNs	50–200 nm	The tunable hollow and mesoporous structure	Toxicity degraded	Biocompatibility and selective tumor targeting, real-time multimodal imaging, vaccine delivery	Toxicity derived from the reducing agents	46, 48, 50
LS	25–1000 nm	Spherical	Safely degraded	Stabilize the antigen, biocompatible and stable	—	78, 80
Polymer	10–2000 nm	Particle	Non-toxicity	Antigen loading into polymeric particles under aqueous conditions <i>via</i> a self-healing process	Loss of antigenicity and immunogenicity during particle synthesis	85–87
ISCOM	40 nm	Cage-like particles	Cytotoxicity-mediated immune responses	—	Cytotoxic-mediated immune responses	109, 111
EN	50–600 nm	Oil-in-water or water-in-oil forms	Safe	Safe and potent vaccine adjuvant	—	117, 118

Virus-like particles, VLPs; self-assembled protein, SAP; carbon nanoparticles, CNPs; gold nanomaterials, GNS; calcium nanoparticles, CNs; silica nanoparticles, SNs; immunostimulating complex, ISCOM; liposomes, LS; emulsion, EN.

delivered antigens can be modulated by the rational modification of the surface of Au nanostructures.^{58,59} Furthermore, gold nanoparticles can also be used as carriers of antigens originating from viruses such as influenza, or as a DNA vaccine adjuvant for HIV.⁵⁹ AuNPs can undergo simple oxidation by the sulfur groups found in protein epitopes which makes antigen conjugation and the development of novel nanoparticle vaccines very straightforward.

The use of nanomaterials as vaccine adjuvants has been shown in various kinds of vaccines, including those used in cancer therapy and infectious diseases. Colloidal gold complexed with partial or complete antigens could induce the binding of highly specific antibodies.⁶⁰ Gold nanoparticles (AuNPs) and gold nano-rods (AuNRs) attached to other molecules can be used as adjuvants to induce a more potent immune response compared to naked vaccines.^{59,61} A previous study showed that F1-antigens coated on the surface of 15 nm AuNPs generated a greater IgG antibody response in mice compared to F1-antigens alone.⁶² Immunity effects of gold nanostructures are mainly influenced by their size and shape. Niikura *et al.* investigated the influence of the shape and size of AuNPs on immune responses *in vivo*.⁵⁶

2.3.4. Calcium nanoparticles. Calcium phosphate nanoparticles (CaP-NPs) are created by mixing sodium citrate, calcium chloride and dibasic sodium phosphate following specific criteria. CaP-NPs have been found to act as central binding sites for antigenic proteins.⁶³ Endocytosed CaP-NPs

which will release their encapsulated materials into the cytoplasm will be dissolved when the pH environment changes from neutral to acidic during cellular uptake processes. Therefore, CaP-NPs can naturally release their incorporated contents without any external trigger, which makes them perfect carriers of antigens. Also, complex multi-layered nanoparticles coupled to antigenic adjuvants and epitopes have been synthesized following the simple, versatile chemistry of these nanoparticles, which enhanced the potent immune response. Furthermore, calcium phosphate nanoparticles (CaP-NPs) are stable under physiological conditions and have been widely used as non-viral DNA vectors. They show excellent biocompatibility and are easily biodegraded, non-toxic and useful adjuvants for DNA vaccines and mucosal immunity. Previous studies reported that surface-modified CaP-NPs of 80 nm size could target genes in the liver, protect the inner DNA from external DNase both *in vitro* and *in vivo*, and exhibit a more effective transfection to mammalian cells.⁶⁴

CaP-NPs can decompose *in vivo* into non-toxic and bio-resorbable components, so they are safe under physiological conditions and as a result, have gained wide clinical applications.^{65–69} CaP-NPs also allow for significant surface modification with a size range of 50–100 nm by displaying facile surface chemistry to conjugate antigens of different kinds.⁷⁰ Previous studies have proved that CaP-NPs are useful adjuvants for DNA vaccines and mucosal immunity.⁷¹ Calcium nanomaterials are also used as an alternative to

aluminium adjuvants for some vaccines.^{72,73} Calcium nano-materials are also investigated as an alternative to aluminium as an adjuvant for many vaccines. For instance, CaP-NP injection elicited little inflammation at the site of administration and a little IgE response in animal models, which shows its obvious advantage over injection of commercial alum compounds.⁷⁴ Furthermore, previously *in vivo* experiments have indicated that micrometer-sized CaP aggregates could generate high titers of neutralizing antibody, and showed high protection against viral infection, which is more potent than the aluminium adjuvant.^{41,75}

2.4. Liposomes

Liposomes are spherical nanoparticles composed of one or many lipid bilayers which mimic vesicles in cells.⁷⁶ Lipids possess a hydrophilic head and a hydrophobic tail or tails which under the correct conditions self-assemble in water to form liposomes. The liposome core is hydrophilic while the bilayer interior is hydrophobic. Liposomes can actively fuse with the target cells by displaying the viral proteins (*e.g.* influenza-derived hemagglutinin and neuraminidase) on their surface.⁷⁷ Liposomes can be used as delivery vehicle adjuvants,⁷⁸ whose main functions are to stabilize the antigen by protecting it from the surrounding biological conditions, to slow down the clearance of antigens and to enhance the delivery to antigen presenting cells (APCs). Liposomes have great potential for clinical use since they are biocompatible and stable within the body. They are also easy to manufacture and scale-up.⁷⁹ The ability of liposomal systems to co-deliver antigens and immune potentiators for enhancing immune responses is considered crucial for inducing potent immune response.⁸⁰

Faster clearance from the site of injection and retention in draining lymph nodes (dLNs) can be achieved by pegylation of liposomes consisting of 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), leading to a 10- to 300-fold increase in IgG2a and IgG2b responses compared with soluble antigen vaccination.⁸¹ Incorporation of R8-liposomes with polyinosinic:polycytidylic acid (polyI:C), which is a synthetic dsRNA, has been shown to promote significantly higher CTL responses and to reduce tumor growth, as compared with antigens that were formulated with soluble polyI:C or complete Freund's adjuvant (CFA).⁸²

Additionally, immunization with liposomes greater than 400 nm in diameter led to a 2-times increase in the IgG2a/IgG1 ratio indicative of a Th1-skewed response, compared with 100 nm liposomes.⁸³ Furthermore, previous studies indicated that the size does not play a role, at least for cationic liposome CAF01, composed of dimethyl-dioctadecylammonium (DDA) and immunostimulatory trehalose dibehenate (TDB). Regardless of liposomal size (ranging from 300 nm to 4 μ m), CAF01 liposomes did not show any liposome-size dependent uptake or IgG1/2 responses *in vivo*, whereas a size-dependent increase in cell proliferation was noted with larger particles possibly because of their increased retention in lymph nodes.⁸⁴

2.5. Polymer

A great variety of polymers is used to prepare nanoparticles due to their biocompatibilities, diverse chemistry and slow biodegradation rate, for which these polymeric nanoparticles entrap antigens for delivery to certain cells or sustain antigen release. In this review, four types of polymeric nanoparticles were outlined and we put them into two categories including naturally derived polymers (*i.e.* chitosan and γ -PGA) and synthetic polymers (*i.e.* PLGA and PLA).⁸⁵

Chitosan (CS) is a naturally occurring poly-aminosaccharide derived from the de-acetylation of chitin, which consists of copolymers of D-glucosamine and N-acetyl-D-glucosamine monomers linked *via* β -(1,4) bonds. This polysaccharide is mainly obtained from the exoskeletons of crustaceans and squids and other sources such as fungi. Thus, the cost of extracting chitosan will be low. Chitosan nanoparticles have the specific properties of non-toxicity and the ability to be easily modified into desired shapes and sizes, making them attractive for application in various vaccines and delivery systems including HBV vaccines, Newcastle disease vaccines and DNA vaccines. Chitosan nanoparticles coupled to rHBsAg exhibited an adjuvant potency higher than that of alum, after intramuscular administration to mice.⁸⁶ The up-regulation of co-stimulatory markers in APCs and the release of pro-inflammatory cytokines (*e.g.* IL-1 β , IL-6, TNF- α) enhanced by antigen-loaded chitosan nanoparticles are more potent than those of an equivalent dose of the soluble antigen.⁸⁷

Gamma poly-glutamic acid (γ -PGA) nanoparticles are water-soluble and have been used as both antigen delivery systems and vaccine adjuvants to induce potent humoral and cellular immune responses. Antigens can be encapsulated and delivered *via* γ -PGA nanoparticles including HIV-1 p24, HIV-1 gp120, and the Japanese encephalitis envelope protein.^{88–90} γ -PGA nanoparticles exhibit intrinsic immunostimulatory properties because they were found to induce innate and adaptive immune responses *via* the TLR4 and MyD88 signaling pathways without any additional adjuvant molecules.⁵⁹ Additionally, the size of γ -PGA nanoparticles has shown an influence on antigen uptake in APCs and their maturation and migration to lymph nodes.⁹¹ γ -PGA nanoparticles with 40 nm diameters promoted higher activation of DCs and their migration to lymph nodes than 100 or 200 nm nanoparticles. γ -PGA nanoparticles also have been utilized as a vaccine platform for cancer immunotherapy.⁹²

Poly(lactic-co-glycolic acid) (PLGA) co-polymers can also entrap antigens for delivery to certain cells or sustain the release of antigen and adjuvant molecules for several weeks to months, which has been found to prolong and enhance immune stimulation.⁸¹ PLGA particles have been used to deliver antigens derived from various pathogens, including hepatitis B virus (HBV), VMP001 malaria protein, *Bacillus anthracis*, HIV antigens and tumor-associated antigens.^{93–99} Fine-tuning their molecular composition and weight can easily control the kinetics of antigen and adjuvant release into several

phases after a single vaccination.¹⁰⁰ When they are loaded with ovalbumin (OVA) antigens and monophosphoryl lipid A (MPLA) adjuvants, orally administered PLGA nanoparticles stimulate significantly the production of more potent IgG and IgA antibody titers compared to an OVA antigen solution or PLGA nanoparticles loaded with the OVA antigen alone.¹⁰¹ PLGA as well as other polymers including poly(L-lactic acid) (PLA), poly(ethylene glycol) (PEG), and natural polymers such as polysaccharides can be synthesized as hydrogel nanoparticles, which are a type of nano-sized hydrophilic three-dimensional polymer network.^{102–105}

Poly(L-lactic acid) (PLA) is another synthetic polymer to prepare nanoparticles weighing 85 000–160 000 Da,¹⁰⁶ which has the advantages of being well characterized and commercially available for drug delivery systems.¹⁰⁷ Graphene oxide/PLA-PEG composites exhibited the capacity for a satisfactory paclitaxel load and sustained delivery of *in vitro* drugs.¹⁰⁸

In summary, natural polymers are attractive materials for vaccine delivery applications due to their high abundance in nature, hydrophilicity, biodegradability, and tissue-specific interactions. Recent studies focusing on improving the physical stability of natural polymer-based nanoparticles have led to new vaccine formulations with enhanced efficiency of antigen delivery, especially for mucosal routes of vaccination. Future studies should focus on utilization of the tissue-targeting properties of these biopolymers and elaboration of the mechanism of action, including the inherent immunogenic properties of these biopolymers.

2.6. Immunostimulating complex

Immunostimulating complexes (ISCOMs) are cage-like particles with self-assembled structures composed of bio-molecular components, including saponin adjuvant Quil A, cholesterol, phospholipids, and protein antigens. ISCOMs are about 40 nm large in size.^{109–111} The immunostimulatory function of ISCOMs is due to their ability to trap antigens by apolar interactions. Various antigens have been incorporated into ISCOMs, including antigens originating from influenza,^{112,113} the herpes simplex virus,¹¹⁴ HIV and Newcastle disease,¹¹⁰ which suggested that these spherical particles are quite potent as synthetic vaccines.¹¹⁵ ISCOMs could be synthesized as adjuvants and not as drug delivery systems in vaccine technology. In the rational design of modern vaccines, most antigens (*i.e.* soluble recombinant antigens) are evaluated with ISCOMs since the antigen is no longer incorporated into the particulate. Electrostatic attractive forces predominantly affect antigen association, which partially determines the performance of ISCOMs. ISCOMATRIX is a new iteration of ISCOMs, which is composed of ISCOMs without antigens.^{109,116} However, ISCOMATRIX can be mixed with an antigen, inducing antibody-mediated and cytotoxic-mediated immune responses.

2.7. Emulsion

Nano-size emulsions can be used as adjuvants in vaccines, the droplet size varying from 50 nm to 600 nm.^{117,118} These

nanoparticles can exist as oil-in-water or water-in-oil forms consisting of oil nano-drops stabilized by adequate surfactants.¹¹⁷ Emulsions dissolve the polymers in an organic phase and then emulsify them with an aqueous phase. Depending on its hydrophobicity, the vaccine can be incorporated into either an organic or aqueous phase of the emulsion. The organic phase of the emulsified particles can be removed by diffusion or evaporation, making the drug containing cores become exposed.¹¹⁹ Polymer-coated emulsions act as nano-capsules by forming a core-shell nanostructure. Emulsions can entrap antigens for efficient vaccine delivery either by simply mixing with the antigen or carrying antigens inside their cores.¹²⁰

Emulsions, which have great potential in nanovaccinology and squalene, are currently the oil component of several o/w emulsion adjuvant influenza vaccines, including Prepandrix® (AS03 adjuvant prepandemic influenza), Focetria® (MF59 adjuvant pandemic influenza), Pandremix® (AS03 adjuvant pandemic influenza), Fluad® (MF59 adjuvant seasonal influenza) and Aflunov® (MF59 adjuvant pandemic influenza). In addition to influenza vaccines, the MF59 adjuvants have also been evaluated in clinical trials for use in vaccines, including HSV, HIV, HBV, HCV and CMV.

The biological fate of nanoparticles is one major factor for their potential use as vectors and/or immunoregulators for vaccines. An ideal vector/immunoregulator should be biocompatible, biodegradable, cheap to produce, not antigenic, and be able to promote an appropriate immune response. Among the several kinds of nanomaterials introduced in this review, except gold, all the other materials could be degraded and cleared from the body.

Generally, nanomaterials can cause oxidative stress and inflammation in cells and organisms. In particular, they can induce the production of reactive oxygen species (ROS), which may cause toxic effects to the cells.¹²¹ These toxic effects are generally affected by the intrinsic physicochemical properties of the nanomaterial, including size, shape, surface charge, surface modification, chemical composition, degradation, and the formation of a “protein corona”. However, appropriate modulation of the physicochemical properties such as size and surface functionalization can effectively reduce toxicity and improve the clearance efficiency of nanomaterials from the body. For instance, the cytotoxicity of CTAB-coated AuNRs could be avoided by polyelectrolyte functionalization.¹⁹ This result indicates that reasonable surface chemical modifications can significantly reduce the toxicity of metal nanoparticles. This provides us with a guide as to how to avoid serious side effects caused by nanomaterials in the process of their practical application.

After summarizing some different chemical compositions of nanomaterials, our review shows the diverse biological effects of these nanomaterials for regulating immune responses of vaccines according to their different physical characteristics (shape, size, chemical or biological surface modifications) (Table 1). Therefore, on the nanoscale, the chemical compositions, physical characteristics, and

biochemical modifications of particles determine their biological effects *in vivo*.

3. Nanoparticle interactions with antigen

The necessary components of an effective vaccine include an antigen to induce the adaptive immune response, a delivery system that can ensure targeting of antigens and immunopotentiators to APCs, and an immune potentiator stimulating the innate immune system. The delivery systems and immune potentiators are the classifications of different nanoparticle-based vaccine formulations (Fig. 1).

For nanoparticles which function as a delivery system, encapsulation of antigens and nanoparticles is typically necessary (Fig. 2). The incorporation of antigens with nanoparticles can prevent antigen degradation and increase their stability, (*i.e.* the antigen and nanoparticle are co-ingested by the immune cell, or act as a transient delivery system). Nanoparticle based vaccine delivery systems that can meet these criteria exhibit multi-fold advantages over traditional vaccines; 1) co-encapsulation of antigen and immunostimulatory agents in nanoparticles enhances immunogenicity and potency of vaccines; 2) particles designed for the cytosolic delivery of antigens enhance cross-presentation and MHC-I presentation of antigens, thereby promoting cytotoxic T-cell lymphocyte (CTL) responses; 3) APCs can easily phagocytize and process particles; 4) multivalent presentation of antigens on the surfaces of particles allows crosslinking to B cell receptors for enhanced humoral immune responses; and 5) surface modifications of particles with functional moieties and targeting ligands permit organ- and cell-specific targeting to lymphoid organs and APCs.

An effective delivery system should concentrate the antigen, protect it from degradation, increase its uptake and processing in dendritic cells (DCs), and induce the production of cytokines that create a robust immune response (Fig. 3).^{122,123} Silica-, gold-, and calcium phosphate-based hard material nanoparticles have predominantly been examined for their application as delivery systems and have been engineered to promote antigen attachment. Attachment of antigens has been achieved through simple physical adsorption or more complex methods,

Fig. 1 Nanoparticle-based synthetic vaccines (blue balls: antigen; red balls: nanoparticles) (a) inorganic nanoparticles, (b) polymeric nanoparticles, (c) bio-molecular nanoparticles including liposomes, (d) virus-like particles (e) micelles (f) immunostimulatory complexes.

Fig. 2 Tree functional classifications of nanometer vaccines: (a) cause the proliferation, (b) strengthen swallowing ability of cells, and (c) optimize of the immune.

such as chemical conjugation and encapsulation. Adsorption of antigens onto a nanoparticle is generally based simply on charge or hydrophobic interactions. Therefore, the interaction between nanoparticles and antigens is relatively weak, which may lead to the rapid disassociation of antigens and nanoparticles *in vivo*. Encapsulation and chemical conjugation provide stronger interactions between nanoparticles and antigens. For encapsulation, antigens are mixed with nanoparticle precursors during synthesis, resulting in encapsulation of the antigens when the precursors form into nanoparticles. The antigens are released only when the nanoparticle has decomposed *in vivo* or inside the cell. On the other hand, for chemical conjugation, antigens are chemically cross-linked to the surface of the nanoparticle. The antigens are taken up by the cell together with the nanoparticle and is then released inside the cell. In soft matter nanoparticle delivery systems such as VLPs, ISCOM, ISCOMATRIX™, or liposomes, attachment of BN-antigens is achieved through chemical conjugation, adsorption, encapsulation, and fusion at the DNA level.

For immune potentiator approaches, nanoparticles may enhance antigen processing and improve immunogenicity by activating certain immune pathways. Vaccine adjuvants can be used to elicit potent immune response by enhancing the magnitude and affect the antigen's specificity. For nanoparticles to act as immune potentiators, attachment or

Fig. 3 Several forms of antigen presentation. (a) Receptor, (b) TLRs, (c) receptor-mediated endocytosis, (d) endocytosis and (e) robust release of antigens under the effect of pH.

interaction between the nanoparticle and antigen is not necessary, and may be undesirable in the cases where modification of the antigenic structure occurs at the nanoparticle interface. Soft matter nanoparticles, such as emulsion-based adjuvants MF59TM and AS03TM, have been shown to be adjuvants of a target antigen even when they are injected independently of, and before, the antigen. The molecules that have been used as immunostimulants in vaccine formulations include bacterial toxins (cholera toxin, heat labile *E. coli* toxin and others), saponins (Quil-A or QS-21) and cytokines. In particular, bacterial toxins are known to target their antigen to M cells in the intestinal tract which can enhance the immune response, thereby boosting a strong humoral response at the mucosal level. The cholera toxin (recombinant B subunit) is an example of these toxins, which is used as an adjuvant for a commercialized oral cholera vaccine.¹²⁴ Small molecules including Toll-like receptor (TLR) antigens can target specific receptors present on immune cells and subsequently trigger stronger immune responses.¹²⁵ Agonists for TLRs are the most extensively investigated molecules, including CpG oligonucleotides (TLR 9), poly(I:C) (TLR 3) or imiquimod (TLR 7/8), which have already been explored due to their adjuvant properties in vaccines against malaria, hepatitis B, influenza, as well as in different therapeutic anti-cancer vaccines.¹²⁶ Most immunostimulant adjuvants are ligands for pattern recognition receptors (PRRs), although some can be utilized to provide a key component for the innate responses (cytokines) or stimulate an activation pathway directly, bypassing the innate receptor (toxins). Antigens conjugated with more than one immunostimulant adjuvant, the so called adjuvant system approach, can allow for the ability to induce effective immune responses adapted for both the pathogen and the target population. For successful development of nanoparticles as immune-potentiating adjuvants, more studies are warranted to better understand the mechanism of the interaction between nanoparticles and the immune system (Fig. 4).

4. Nanoparticle interactions with antigen presenting cells

Incorporating antigenic components into nanoparticles has attracted extensive interest with focus on how to deliver antigens more efficiently to antigen presenting cells (APCs) and subsequently induce their maturation and the cross presentation of antigens for activation of potent immune responses.^{127–130} Dendritic cells (DCs) and macrophages are specialized APCs which are often targeted in vaccine design. DCs are the most effective APCs, by which the generation of immune response generally requires peptide presentation.^{131,132} We expect that future studies should focus on exploring DC and macrophage uptake mechanisms and interactions of nanoparticles with APCs for developing efficacious nanoparticle vaccines.^{133,134} The cross-presentation of exogenous antigens by antigenic APCs is required for vaccines to stimulate cytotoxic T cells (Fig. 5). A recently studied synthetic nanoparticle vaccine platform can be used to mount an immune response to a conjugated antigen by targeting

Fig. 4 The combinative styles of nanoparticles and antigens. (a) Connection, (b) containment, (c) adsorption and (d) simple mixture.

lymph node-resident dendritic cells (DCs). A close relationship has been found between the antigen conjugation system to activate both helper and cytotoxic T cell responses and the propylene sulfide nanoparticle vaccine platform.¹³⁵ Previous studies reported that the size, charge and shape of nanoparticles play significant roles in antigen uptake.¹³⁶

Generally, nanoparticles can be easily recognized and taken up efficiently by APCs to induce immune responses due to their size similarities to cellular components.^{137–143} They can enter living cells *via* an endocytosis mechanism, in particular, pinocytosis. The effect of particle size on immunogenicity lies in the enhancement of cellular uptake of the nano-molecules as compared with bigger particles.

The surface charge is an equally important factor in the interaction between nanoparticles and APCs. Particles with a positive surface charge and a 1 μm size showed enhanced uptake.¹⁴⁴ The electrostatic interaction of cationic nanoparticles with anionic cell membranes led to the higher APC uptake of cationic nanoparticles than neutral and anionic particles and hemolysis and platelet aggregation.^{144,145} However, the particle surface charge has a different influence on particles with sizes below 0.5 μm upon interaction with DCs.¹⁴⁴

In addition to size and charge, particle shape also plays a significant role in the activation of immune responses. For

Fig. 5 Two mechanisms of triggering T cells when the nanoparticles enter lymphatic vessels. (a) Small molecular nanoparticles can be directed to the lymph vessels, transported into the lymph nodes, swallowed by DC cells (immature colorless DC), and then activate T cells after DC maturation (color purple). (b) Macromolecular nanoparticles are swallowed by DC cells in the skin, and then the DC cell enters the lymph vessels, moves to the lymph nodes, and activates T cells after its maturation (purple).

big particles ($>1\ \mu\text{m}$), the uptake by APCs is strongly dependent on the local shape of particles-APC interface.¹⁴⁶ Needle-shaped polymer particles were also found to promote transient (non-toxic) permeabilization into cell membranes.¹⁴⁷

Surface modification, vaccine cargoes and the effect of hydrophobicity are some other factors affecting the interaction of nanoparticles with APCs as well.¹⁴⁸ A high immune response has been induced by hydrophobic particles as compared with hydrophilic ones.^{149,150}

5. Conclusion and future outlook

Vaccines benefit greatly from nanoparticle formulations, as they have attracted tremendous interest in recent years due to their utilization as delivery vehicles or immune potentiators, allowing not only improvement of antigen uptake and targeting to APCs but also enhancement of immunogenicity and the slow release of antigens. We covered a wide range of nanoparticle formulations varying in size, composition, and surface charge, all of which are key elements in the modulation of bio-distribution, cellular trafficking, and overall immune responses. Most particulate vaccine platforms in consideration can offer safe and effective alternatives to traditional vaccine formulations including live-attenuated or inactivated/killed pathogens due to their biodegradability, biocompatibility and minimal toxicity.

In addition, nanoparticles have been shown to carry not only the antigen of interest but also a co-adjuvant, including poly(I:C), CpG and MPL. Nanoparticle vaccines provide significant improvement compared to traditional vaccines in terms of their ability to enhance cross-presentation of subunit antigens and generate strong cellular immunity. However, challenges remain due to the lack of understanding regarding adjuvants and their molecular mechanisms of bio-distribution and targeting, which can refine the biophysical and biochemical features of nanoparticles to improve immunostimulation. Furthermore, unmet needs existing in the rational design of novel vaccine systems include the single-dose and needle free delivery, which will become practical in the near future with the integration of some other attractive properties, such as slow release, targeting and alternative administration methods and delivery pathways. Therefore, the manufacturability of these systems should be taken into consideration, as their economic and facile scale-up with reproducibility in industrial settings is a crucial constraint condition for their translation into the clinic.

In general, polymeric nanomaterials as vaccine carriers or immunoregulators are probably the closest to clinical application because of their good biological safety and effectiveness *in vivo*. The rational design for the nanomaterials is still useful to develop safe and efficient vaccine vectors and/or immunoregulators. Rational design in combination with the reproducible production of nanoparticles with desirable properties, functionalities and efficacy becomes increasingly critical. The adoption of new technologies such as microfluidics and the controlled synthesis of nanoparticles will ac-

celerate the development of suitable nanoparticles for pharmaceutical applications.

Abbreviations

APCs	Antigen presenting cells
AuNPs	Gold nanoparticles
AuNRs	Gold nanorods
BVDV	Bovine viral diarrhoea virus
CFA	Complete Freund's adjuvant
CNTs	Carbon nanotubes
CaP-NPs	Calcium phosphate nanoparticles
CS	Chitosan
DCs	Dendritic cells
DOTAP	1,2-Dioleoyl-3-trimethylammonium propane
HBV	Hepatitis B virus
ISCOM	Immunostimulating complex
γ -PGA	Gamma polyglutamic acid
MSNs	Mesoporous silica nanoparticles
NPs	Nanoparticles
OVA	Ovalbumin
PLGA	Poly(D,L-lactic-co-glycolic acid)
PEG	Poly(ethylene glycol)
ROS	Reactive oxygen species
SAPNs	Self-assembled peptide nanoparticles
SWNTs	Single-walled carbon nanoparticles
SiNPs	Silica nanoparticles
SV	Silica vesicles
VLPs	Virus-like particles

Conflicts of interest

The authors state that they have no conflicts of interest.

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