Chapter 3

Polymeric Nanoparticles Engineered as a Vaccine Adjuvant-Delivery System

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Additional information is available at the end of the chapter

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Abstract

Global immunization saves millions of human lives each year through using vaccines, which include whole microbe-based products and the subunit ones formulated with just the components of antigens able to stimulate immune system to establish specific immunity against diseases. Subunit vaccines show numerous advantages, such as defined components, high safety profile, and production without the use of dangerous pathogens, but also limited capacity in eliciting immunity due to the lack of other components than antigens, including the immunostimulatory elements of pathogen-associated molecular patterns which are able to activate the innate immunoreponses. Recently, nanoparticles (NPs) formulated with polymeric materials, such as poly(lactic-co-glycolic acid), viral proteins, chitosan, hyaluronic acid, and polystyrene, with some bearing intrinsic adjuvanticity, are widely employed as vaccine adjuvant-delivery systems (VADSs) and show great potential in developing subunit vaccines. Particularly, the polymeric NPs engineered with functional materials possess many features, such as targeting delivery, lysosome escape, anti-damaging protection, and ability to guide immune reactions toward a Th1 (T helper type 1) and Th2 pathway, which are crucial for establishing humoral and cellular immunity. This chapter describes polymeric NP-based VADSs designed for developing subunit vaccines able to elicit Ag-specific immunity at both systemic and mucosal levels via different vaccination routes.

Keywords: polymer, nanocarrier, immune response, mucosal vaccination, cellular immunity, pathogen/danger-associated molecular pattern (PAMP/DAMP), targeting delivery

1. Introduction

Vaccines have saved countless human lives from lethal infections since the modern use of vaccinia against smallpox by British physician Edward Jenner in the late eighteenth century and are today playing more and more crucial roles in fighting life-threatening diseases, of which, due to great advances made in branches of the field and related fields such as immunology and biotechnology, the scope has enormously expanded, ranging from the earlier aim of microorganism infections to the novel targets of autoimmune disorders, allergic reactions, and even malicious cancers [1]. However, the list of infectious diseases for which vaccines are urgently needed but do not yet exist is till long and in particular many pathogens such as HIV (human immunodeficiency virus), HSV (herpes simplex virus), and HCV (hepatitis C virus), showing on their surface elusive or ever changing immunogenic features to continuously dismantle a variety of potential arsenals; let alone numerous malignant tumors, autoimmune disorders such as multiple sclerosis, diabetes, and rheumatoid arthritis, which are all aggressively threatening human health and life, posing a big challenge to developing effective vaccines or immunotherapy [2].

Vaccines function against diseases through stimulating the host immune system with the antigenic components featured by pathogens or neoplasms to establish the antigen-specific immunity which is able to clear the abnormalities bearing the identical antigens. Vaccines developed for handling infectious diseases are mostly manufactured using the live attenuated or killed whole microbes, which have a high potency in triggering immune system but are argued to be associated with possible reversion to virulence due to mutation of administered strains, as evidenced by gene sequencing in vaccinated sufferers, thus causing safety concern. As many mechanisms underlying immunoresponses are revealed and great achievements are made in relevant fields, subunit vaccines, which are formulated with defined components including Ags to induce immunoresponses accurately targeting the matched objects thus causing few safety concerns, are now more and more employed to fight not only infectious pathogens but also other illnesses, providing, in particular, outstanding ways to overcome the previously intractable diseases, such as cancer [3]. Compared to conventional whole microbe vaccines, subunit vaccines possess many distinct properties which are beneficial for clinical applications, summarily including high safety profile, production needing no dangerous microorganisms, no redundant components to cause allergic or autoimmune responses, diverse usage including anticancer, and high capacity for several peptide epitopes targeting different stages in the life cycle or subtypes of a pathogen [4].

However, subunit vaccines are often weak immunostimulatory products, due to lack of such components as the pathogen-associated molecular patterns (PAMPs), which are expressed on a microbe surfaces and are able to activate the pattern recognition receptors (PRRs), such as the TLRs (toll-like receptors), NOD-like receptors (the nucleotide-binding oligomerization domain-like receptors), RIG-I-like receptors (retinoic-acid-inducible gene-I-like receptor), and C-type lectin receptors, thus facilitating host immunoresponses [5]. As such, subunit vaccines are often formulated with an adjuvant, which is a nonspecific immunopotentiating substance able to elevate, either in advance or simultaneously with the vaccine Ags, the immune

response of recipients to the Ags, or change the type of immune responses; otherwise, subunit vaccines are engineered with functional carriers to form a vaccine adjuvant-delivery system (VADS), which is frequently made of a range of NPs (nanoparticles) using various materials capable of targeting the professional Ag-presenting cells (APCs) such as dendritic cells (DCs) and macrophages (MPs) to boost enormously the immunostimulatory activity of a vaccine and is thus making full use of Ags [6–10].

This chapter elaborates the material basis, formulation, rationale, and the state-of-the-art advances in the development of a VADS constructed with polymeric NPs which are made of certain crucial types of polymers such as PLGA, HA, polystyrene, or VLPs, which possess many beneficial features for eliciting immunity against a range of diseases. Thus, this comprehensive introduction will provide a useful reference to interested readers who may thus be attracted to denote their innovative talents to the development of vaccines based on VADS constructed, probably, with polymers.

2. Different polymeric NPs designed as a VADS

Polymeric NPs, namely NPs made of highly biocompatible polymers, such as polystyrene, PLGA, proteins, chitosan, and hyaluronic acid (HA), have recently been widely explored as a DDS (drug delivery system) as well as VADS and show many excellent properties beneficial for therapeutic delivery of agents, for example, high stability can shelter the loaded Ags from environmental detriment and in vivo unwanted degradation; biocompatibility can reduce toxicity to recipients and their compliance; ease in modulation of particle size, surface charge, and specific binding characteristics allow developing multifunctional VADS [3, 4]. Thus, through fulfilling multiple functions, polymeric NPs are able to improve the efficacy of vaccines, for example, they can form a depot to enhance vaccine efficacy via elongated release and exposure of Ag at the site of injection; they can targetedly deliver vaccines to APCs promoting cellular uptake of Ags and thus enhancing Ag stimulation efficiency; they can alter intracellular process of Ags adapting immune responses toward the beneficial humoral and/or cellular pathways; and also they can provide diverse administration routes for vaccination to elicit the desired immunity at circulation system as well as mucosal sites [6, 11].

2.1. Polystyrene NPs as a VADS

Although polymers suitable for constructing VADSs are usually thought to be biodegradable one since they cause no size-limited excretion and associated toxicity concern, certain nonbiodegradable materials possessing certain specific properties, such as chemical inertness and ease for fabricating stable NPs, are also the preferred candidates by researchers for engineering the kind of NPs with an accurate size and special shape, so as to be employed reliably to investigate these physical properties on the immune system and immune responses. For example, Plebanski and coworkers using nonbiodegradable polystyrene NPs performed studies on VADS and showed that polystyrene NPs loaded with OVA epitopes induced different immune responses in a size-dependent manner needing no additional adjuvant, and

that among different particles with a size ranging from 20 to 2000 nm, 40 nm NPs induced the strongest cellular and humoral immunity [12]. Further investigations demonstrated that covalent linkage of peptide to NPs is a requirement for eliciting immunization efficacy and also proved that 40-nm-sized NPs serve as a VADS owing to their preferential uptake by APCs and their ability to traffic to lymph nodes to induce strong immune responses compared to their larger counterparts [4, 13].

Notably, Schöttler et al. recently reported the counterintuitive research results on cellular uptake of NPs with PEGylation (modification with polyethylene glycol), which is gold standard in removal of immune clearance of in vivo NPs through the mechanism of reducing nonspecific cellular uptake of nanocarriers [14]. The researchers documented that polystyrene NPs, which had been modified with PEG or poly(ethyl ethylene phosphate) (PEEP), only had been exposed to plasma proteins, could exhibited a lowered cellular uptake by macrophages (RAW264.7 cells), whereas those not exposed to plasma proteins showed high nonspecific uptake. Further mass spectrometric analysis revealed that the plasma-exposed nanocarriers formed a protein corona which was identified to contain just an abundance of clusterin proteins (known as apolipoprotein J) and to be the decisive factor controlling lowered nonspecific cellular uptake of the PEGylated or PEEPylated polystyrene NPs, and to contrast, the classic conception that PEGylated NPs free of immune clearance is resulted from avoidance of protein adsorption. These outcomes indicated that PEG as well as PEEP can affect the composition of protein adsorption by polystyrene NPs, and that the presence of certain type proteins may be just a prerequisite in preventing nonspecific cellular uptake of NPs, defying the conventional belief that PEGylation reduces protein adsorption thereby conferring a stealth effect [15].

2.2. Virus-like particles as a VADS

Virus-like particles (VLPs) consist mainly of viral proteins devoid of viral genomes to mimic the natural structure of virions and have been engineered to carry agents for various applications, including, particularly, for constructing a VADS for delivering subunit vaccines based on their viral envelop structures suitable for presenting functional spikes on NP surfaces to maintain the intrinsic immunogenicity apt to trigger immunoresponses [16]. VLPs are usually manufactured using protein expression systems based on bioengineered bacteria, yeast, insect, avian, mammalian or plant cells, or using cell-free protein synthesis system (CFPS), which provide an alternative to construct effective VADSs with beneficial characteristics, such as having defined structure formed through self-assembling, large cargo loading capacity, easy functionalization with ligands, and high stability and low toxicity [17]. As a VADS for producing vaccine candidates, VLPs are often designed with characteristics identical to native virus especially in the aspects of the immunochemical properties, 3-D (3-dimensional) architectures and morphological conformations, through engineering on their particulate structures, which, like the native virus, include the nonenveloped and enveloped types. The nonenveloped VLPs mainly consist of one or more pathogenic components, but do not contain any components of the expression hosts, while the enveloped VLPs generally consist of matrix proteins which are enveloped in a lipid membrane derived from the expression hosts, possibly, with glycoproteins embedded into the bilayered membranes.

VLPs are widely used as a VADS because they possess several clear advantages, including induction of immunity with a broad cross-protection rendering onetime immunization to

protect against different virus genotypes, high potency to trigger immune responses providing an option to conquering intractable pathogens such as HIV and HCV (hepatitis C virus), high thermostability possibly requiring no integral cold chain to keep viability favoring global vaccination, and high manufacturing efficiency in large scale while at low cost offering a strategy to handle the emergency arising from infectious diseases, such as Ebola outbreak and epidemic. For example, HCV infection is still a significant public health problem, though it has been partially addressed with the advent of directly acting antiviral agents (DAAs), which represent a major advance toward controlling HCV but confer little protection against reinfection [18]. Presently, around 71 million people in the world are living with chronic HCV infection, and each year nearly half a million of them will die of HCV infection or its complications, rendering it urgent to develop an effective vaccine capable of eradicating HCV, which may well be produced using the VLP-based VADS, in reference to HBV vaccines. Recently, a quadrivalent genotype 1a/1b/2a/3a HCV VLP vaccine was successfully engineered by researchers using scale-up production methods of Huh7 cell factories containing a recombinant adenoviral expression system representing each HCV genotype, followed by cell lysing and purification with iodixanol ultracentrifugation and stirred cell ultrafiltration [19]. When given subcutaneously to mice, whether in the presence of an adjuvant (system) or not, the quadrivalent vaccine consistently induced production of Ab and nAb (neutralizing Ab) together with robust T and B cell responses for eliciting broad humoral and cellular immunity, indicating the VLP-based VADS a useful tool which may be employed for the production of an effective HCV vaccine [20].

Globally, HIV continues to be a major public health issue and also claims approximately 1 million lives each year, although total people living with HIV have the opportunity to receive antiretroviral drugs (ARVs), which may effectively control the virus from transmission and causing illness but are also found to be undermined in efficacy by pathogens that had evolved with drug resistance [21]. Still, a highly effective vaccine is believed to be the ultimate weapon able to erase HIV and associated disease AIDS (acquired immune deficiency syndrome), though at present, there are no such a product in markets and, moreover, many products developed in previous years bearing such expectations failed to show clinical efficacy in fighting this rapidly mutating pathogen, including especially the big trial, known as STEP, which was halted in 2007 after the vaccine was found to increase the risk, instead of prophylaxis, of HIV infection [22]. Nevertheless, scientists are getting closer than ever to developing such an effective product, as evidenced by a large-scale clinical study conducted in 2009 in Thailand (called RV144) showing that immunization with a combination of two HIV vaccines prevented about 31% of new infections through a prime-boost combination regime [23], which comprises four priming intramuscular injections of ALVAC-HIV, which is a recombinant canarypox vaccine express HIV-1 Gag, Pro and gp120-gp41, plus two boosting intramuscular injections of AIDSVAX® B/E, which is an alum-adjuvanted bivalent HIV-1 gp120 vaccine of subtypes E and B [24]. Though the low prevention rate of vaccination with ALVAC/AIDSVAX combination excludes the products from approval for clinical prophylaxis of HIV, the moderate effects displayed in human trial not only provoked scientists to make deep explorations on the causes for the failures, but also presented researchers a great encouragement to commit further efforts to developing efficacious HIV vaccines. Subsequently, based on the virion features which are more and more clearly elaborated in structure and function, scientists set out to handle the obstacles identified to the development of a preventive HIV vaccine from several aspects, such as accurately targeting the conserved antigenic proteins, seeking Ags able

to induce the broadly neutralizing Abs (bnAbs), and formulating a highly efficient VADS [25]. As mentioned above, a VLP-based VADS proves a highly potent inducer for Abs and helper T cell responses and also able to elicit robust cytotoxic T cell responses necessary for preventing primary infections and erasing infected cells, thus offering researchers an alternative tool to engineer effective vaccines against HIV, which is regarded as the most challenging foe owing to its poor immunogenicity, fragile surface glycoprotein, and the ability to overpower the cell immune system [26].

Recently, Chapman et al. constructed an MVA (modified vaccinia Ankara)-mGag (an HIV-1 subtype C mosaic Gag immunogen) and a DNA-mGag vaccine, which were designed to address the tremendous diversity of HIV, and showed that mGag budded from cells infected and transfected with MVA-mGag and DNA-mGag, respectively, formed VLPs [27]. In mice, the DNA-mGag homologous prime boost vaccination elicited predominantly CD8+ T cells, and the homologous MVA-mGag vaccination induced predominantly CD4+ T cells; in contrast, a heterologous DNA-mGag prime MVA-mGag boost induced strong, more balanced Gag CD8+ and CD4+ T cell responses that were predominantly of an effector memory phenotype. Also, it was found that DNA-mGag homologous vaccination induced much higher cumulative Ag-specific IFN-γ secretion responses and generated significant higher levels of cytokine-positive CD8+ T cells than DNA-nGag (natural Gag), indicating a heterologous prime-boost regimen with DNA and MVA vaccines expressing HIV-1 subtype C mosaic Gag as an Ag is highly immunogenic and may be an effective VLP-based VADS for eliciting strong immunity to HIV. Yao's group formulated a VLP-based HIV vaccine, which was composed of HIV_{IIIB} Gag and HIV_{BaL} gp120/gp41 envelope as a pseudovirion vaccine capable of presenting Ags in their native conformations and was engineered through using HEK (human embryonic kidney cell)-derived cell line expression system [28]. The researchers demonstrated that mice vaccinated by intranasal prime followed by two sub-cheek boosts with VLPs adjuvanted with liposomes entrapping TLR3 ligand dsRNA were stimulated to secrete high titers of Abs against the Ags, with predominant IgG2c over IgG and produce a significantly increased germinal center B cells and T follicular cells, suggesting that the VLP-based VADS is superior for induction of a Th1-biased immune response, while prolonging lymph node germinal centers, T follicular cells, and generating neutralizing antibodies, and thus is rather suitable for making HIV vaccines [26].

Notably, certain types of pathogens that are once known to cause only a mild and self-healing illness and therefore never listed in dangerous items and may abruptly cause the unexpected problems associated with human and population health, hinting the existence of undisclosed infection mechanisms and pathophysiological processes or the emergence of mutations relevant to severe toxicity. For example, during the 2015–2016 South American Zika epidemic, the mosquito-borne virus which used to cause mild symptoms, such as fever, skin rash, and joint or muscle pain, was eventually identified able to cause severe damage to fetal brain through infecting pregnant women and thus finally recognized as the culprit responsible for thousands of microcephaly affected new borns, raising a great social problem and concern [29]. Unfortunately, up to now, still there are no licensed vaccines for prophylaxis of Zika, though several conventional approaches have been tried on developing such as an urgently needed products, including inactivated, recombinant live-attenuated viruses, protein subunit vaccines, RNA and DNA vaccines, as well as the VLP-based VADS [30]. Recently, using

HEK293 cell (Human embryonic kidney 293 cell) expression system, Salvo et al. engineered a VLP-based VADS composed of Zika prM/E (pre-membrane and envelope) glycoproteins for making vaccines to defend against Zika and demonstrated that mice injected with Zika VLP combined with adjuvant alum secreted high levels of the Ag-neutralizing Abs [31]. In particular, the vaccinated mice all survived without morbidity or weight loss after receiving the lethal challenge with the dose of 200 PFU of Zika strain H/PF/2013, proving the protective efficacy of the VLP-based Zika vaccine which may be tested in humans as a prophylactic candidate with minimal safety concerns to protect unborn babies whose mothers become infected with Zika during pregnancy.

Similarly, Espinosa and colleagues formulated a ZIKV vaccine based on virus-like particles (VLPs) which were generated in HEK293 cells transiently transfected with the prM/E genes of Zika placed downstream from a heterologous signal sequence and observed efficient induction of neutralizing antibody and a dose-sparing effect of alum in VLP-immunized mice (C57Bl/6 x Balb/c) [32]. In addition, passive transfer experiments showed that AG129 mice received the sera from immunized mice prior to Zika infection manifested significantly reduced viral replication as indicated by viral RNA levels in the blood and successfully conquered the infection to contrast control mice which succumbed to infection, underscoring the protective effect of the humoral immunity elicited by this VLP-based Zika vaccine candidate.

In summary, the VLP-based VADSs are a potent inducer of Ab and cellular responses and also possesses the prerequisite features required to prepare the vaccines that are able not only to prevent the primary infections but also to clear infected cells, thus representing an alternative tool promising to engineer efficacious vaccines against the intractable pathogens, such as HCV, HIV, and even parasites [33].

2.3. Chitosan NPs

Chitosan, a linear polysaccharide composed of randomly distributed β-(1,4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit), is usually made through hydrolysis of the chitin shells of shrimp and other crustaceans with an alkaline substance, such as sodium hydroxide [34]. Chitosan has a positive charge under neutral conditions due to protonation of basic amine groups, which contributes to the enhancement of solubility of the compound; however, the dissolution behavior of chitosan in aqueous media is also negatively influenced by the number and structural form of acetylated groups, allowing chitosan able to be used for agent delivery in several distinct forms, including solution, hydrogel, and especially nano/microparticle, which can be obtained via crosslinking, ionotropic gelation or precipitation-coacervation [35]. Interestingly, novel chitosan derivatives with customized biochemical properties are still continuously synthesized through facile conjugation of side chain moieties of functional molecules to solvent-accessible amine and hydroxyl groups, rapidly expanding chitosan in application range and dosage form [36].

In retrospect, in 1980s, researchers observed that chitosan of 70% deacetylated chitin could activate peritoneal macrophages [37] and induce production of various cytokines in mice [38], which was subsequently further explored by Illum et al. to demonstrate that in mouse model chitosan, via nasal immunization, could act as an efficient VADS able to remarkably enhance

local as well as systemic Ab responses toward the vaccines containing filamentous hemagglutinin from *Bordetella pertussis* [39]. The researchers also proved that chitosan could significantly elevate immunogenicity of the nasal vaccine of diphtheria toxoid (DT) and enhanced vaccines in induction of high levels of Ag-specific IgG, secretory IgA, toxin-neutralizing Abs, and T cell responses, predominately of Th2 subtype [40].

Chitosan, as a cationic polysaccharide bearing lots of reactive groups, possesses beneficial properties for vaccine formulation, including biocompatibility, flexibility in terms of formulation and degree of deacetylation, and efficacy when administered via mucosal route, and ability to promote immune responses [41, 42], and thus is thought a superior alternative to alum which only favors promoting humoral responses [43]. Also, chitosan is thought suitable for constituting a mucosal VADS thanks to its bio-adhesive character and intrinsic adjuvanticity, which may arise from chitosan-mediated inflammasome activation [44], or from triggering certain type of PRRs such as TLR4 on immune cells [45], in consistence with its parent molecule chitin which is confirmed able to activate immunocytes via binding to mannose receptors and TLR2 to initiate innate immune responses [46]. However, in spite of numerous research outcomes confirming its strong immunostimulatory potency, the exact mechanism underlying the intrinsic adjuvanticity of soluble chitosan remains yet elusive and needs to be fully discovered through deep exploration. More recently, it was demonstrated that after APC uptake, the intracellular chitosan induced mitochondrial damage, characterized by the generation of mitochondrial ROS and release of endogenous DNA into the cytosol, both of which culminated in the activation of the cytosol DNA sensor cGAS (cyclic-di-GMP-AMP synthase) and subsequent STING (stimulator of IFN gene) pathway, leading to translation of type I IFN and the type I IFN-dependent APC maturation to sponsor cellular immunoresponses [47].

In comparison to free chitosan, chitosan NPs that are design as a VADS capable of enhancing both humoral and cellular immune responses to delivered vaccines are proposed to function relevant to not only chitosan properties but also several aspects associated with NPs, including antigen protection, depot formation, enhanced antigen uptake, and presentation, triggering APCs via different pathways to regulate immune reaction pathways [41]. Recently, Dhakal et al. using the ionic gelation method engineered the chitosan NPs that were loaded with killed swine influenza A H1N2 virus (KIV-CNPs) and demonstrated that the nursery pigs intranasally vaccinated with KIV-CNPs produced high levels of systemic IgG and secretory IgA in nasal mucosa, bronchoalveolar lavage fluids, and lung lysates, which, more importantly, were cross-reactive against homologous (H1N2), heterologous (H1N1), and heterosubtypic (H3N2) influenza A virus strains [48]. Also, the vaccinated pigs demonstrated high frequency of Ag-specific CTLs and lymphocyte proliferation, and stimulation-recalled IFN-γ secretion, leading these pigs to experience reduced severity of macroscopic and microscopic influenza-associated pulmonary lesions after challenge with heterologous viruses, firmly confirming that the NPs composed of chitosan may function as an effective mucosal VADS favoring noninvasive immunization. In another report, Lebre et al. prepared chitosan-aluminum nanoparticles (CH-Al NPs) with a size of 280 nm and a positive surface charge and proved that CH-Al NPs loaded with hepatitis B surface antigen (HBsAg) were more stable in physiological environment and more efficient in inducing cellular immunity than common chitosan NPs, suggesting the two combination immunostimulants chitosan and aluminum salts may

be a promising VADS system for antigen delivery [49]. Interestingly, to develop a vaccine able induce robust mucosal immunity against *Chlamydia trachomatis* (Ct), which is the most common sexually transmitted infection in humans, Rose and cowokers fabricated the PLGA NPs covered with the mucoadhesive chitosan and used as a VADS for delivering recombinant Ct fusion Ag CTH522 [50]. Mice intranasally immunized with the optimized chitosan-coated PLGA NPs containing Ct Ags established potent Ag-specific systemic as well as mucosal immunity, characterized by high levels of anti-CTH522 IgG/IgA Abs in the lungs and the genital tract and high frequency of IFN-γ producing Th1 cells, suggesting that chitosan-coated PLGA NPs may be a promising mucosal VADS for delivering vaccines against sexually transmitted *Chlamydia trachomatis*.

Notably, in a randomized two center phase I clinical trial, an HIV vaccine consisting of HIV-I Clade C-CN54GP140 envelope glycoprotein was administered to HIV negative female volunteers through intramuscular (i.m.) immunization with glucopyranosyl lipid adjuvant (GLA), intranasal (i.n.) immunization with 0.5% chitosan, and intravaginal (i.va.) immunization with an aqueous gel vehicle [51]. The results indicated that, compared to subjects with i.n. or i.va. immunizations, recipients with three i.m. immunizations at the dose of either 20 or 100 μg CN54 gp140 secreted greater systemic and mucosal antibodies, but even in the i.m. immunized subjects, only modest neutralizing responses against closely matched tier 1 clade C virus were triggered; and the i.n. primed subjects were induced the strongest CD4+ T cell response, and, following additional i.m. boosting, were also induced an anamnestic antibody response, suggesting i.n. immunization of HIV vaccines formulated with chitosan may be an effective prime for i.m. boost.

Summarily, due to good tolerability, safety, and, particularly, mucosa-adhesive properties, chitosan and derivatives represent a promising polymer suitable for constructing mucosal VADS to provide great opportunity for developing mucosal vaccines against numerous pathogens which invade hosts through mainly mucosa. However, the available clinical results indicate clearly that to construct an effective vaccine with chitosan to handle the intractable pathogens, such as HIV, further efforts are needed to commit to optimizing formulation, seeking optimal immunization routes, as well as exploring combination with appropriate adjuvants.

2.4. PLGA NPs

PLGA represents one of the most popular polymers for constructing a VADS due to its excellent safety profile, biodegradable properties, ease for processing NPs through double emulsion method, diverse modification to bear functional groups, and also the established use in several marketed products for controlled or targeted delivery of drugs [52]. It is now clear that in vivo PLGA hydrolyzes into metabolite monomers of lactic acid and glycolic acid, both of which are endogenous and easily metabolized by the body via the Krebs cycle, leaving behind little systemic toxicity, allowing wide use of PLGA as a VADS or DDS (drug delivery system). Notably, one of the appealing issues associated with the use of PLGA NPs as a VADS is attributed to the confirmation that, after cellular internalization via fluid phase pinocytosis or clathrin-mediated endocytosis, PLGA NPs may rapidly escape the endolysosomes and carry the loaded cargoes to cytoplasm, avoiding lysosomal degradation into null fragments and thus enhancing vaccine delivery efficiency [53].

To develop an effective VADS, Noormehr et al. fabricated 500-nm-sized PLGA NPs which were covalently conjugated with recombinant Ags Leishmanial CPA (cysteine peptidase A) and CPB, and proved that mice intra-peritoneally immunized with the inhomogeneous Ag-NPs secreted high levels of NO (nitric oxide) by peritoneal MPs and high levels of IFN-γ by splenocytes, which significantly lowered *Leishmania major* burden, suggesting the Ag-conjugated PLGA NPs can be used as a VADS able to deliver vaccines to protect against the tough pathogen of parasites [54]. To investigate the function of multiple adjuvant-combined VADS, Ebrahimian and colleagues formulated the TLR 7/8a resiquimod- or TLR4a MPLA-loaded PLGA NPs which were physically covered with polyethylenimine (PEI) forming PLGA/PEI NPs and then mixed with CpG ODN (cytosine-phosphorothioate-guanine oligodeoxynucleotide) to engender a complexed entity of resiquimod- or MPLA-PLGA NPs/PEI-CpG ODN [55]. Given to BALB/c mice, the multiple adjuvant-constituted PLGA NPs loaded with Ags induced robust and efficient immune responses, as confirmed by evaluation of vivo cytokine (IFN-γ, IL-4, and IL-1β) secretion and antibody (IgG1 and IgG2a) production, demonstrating using a combination of adjuvants in a context-dependent manner may a feasible strategy for engineering a potent PLGA-based VADS. To make subunit vaccines suitable for immunization via skin, which is an attractive but also very challenging immunization site due to the presence of affluent APCs while difficulty of administration, recently, Bouwstra's group fabricated the hyaluronan (HA)-based dissolving microneedles (MNs) entrapped with PLGA NPs which co-encapsulated ovalbumin (OVA) as an Ag and poly(I:C) as an adjuvant for intradermal immunization [56]. Further investigation indicated that the immunogenicity of the PLGA NPs after administration of dissolving MNs was compared with that of hollow MN-delivered PLGA NPs in mice, while immunization with free Ag in dissolving MNs resulted in equally strong immune responses compared to delivery by hollow MNs. However, humoral and cellular immune responses evoked by PLGA NP-loaded dissolving MNs were inferior to those elicited by NPs delivered through a hollow MN, suggesting several critical parameters should be fully evaluated in engineering the PLGA NP-loaded dissolving MNs as an intradermal VADS to avoid unnecessary efforts on the complexed formulations.

At present, still a large fraction of vaccines require a multiple dosing schedule with a 1- to 2-month gap between administrations to guarantee establishing the Ag-specific immunity strong enough to protect recipients, as such, however, engendering a big challenge to worldwide vaccination, especially, in the developing countries, where healthcare workers are not only in shortage but also confronting difficulty in reaching the subjects multiple times to administer booster shots [57]. Conceptually, this challenge may be conquered using a VADS that are constructed with a functional carrier which release vaccine ingredients in pulses with an appropriate time gap between vaccinations, thus simplifying the vaccination schedule to consist of only once injection to exclude additional visits by a healthcare worker. For this, Tzeng et al. engineered a controlled release VADS consisting of bPEI (branched PEI)-modified PLGA microparticles which contained in inner core Ags of IPV (inactivated polio vaccine with three antigens) and an Ag stabilizer poly(L-lysine) [58]. Further investigation indicated that the bPEI-PLGA microparticles stabilized IPV in its active conformation inside the particles for months but in an aqueous medium released two bursts of IPV with an interval of just 1 month, vividly mimicking a typical twice vaccination schedule. Moreover, one injection of the controlled-release formulations elicited a similar or better Ag neutralizing response in rats compared to

multiple injections of liquid vaccine, suggesting the VADS constructed with the bPEI-PLGA microparticles has big potential to elevate vaccine coverage in the developing world.

Conclusively, PLGA is a biodegradable, safe, and clinically used polymer, which, using the double-emulsion method, can be conveniently engineered into NPs to constitute a VADS with appropriate features and abilities to render vaccine lysosome escape, thus enhancing vaccination efficiency.

2.5. Hyaluronic acid (HA)-modified liposomes

Hyaluronic acid (HA) as a polysaccharide consists of alternating units of D-glucuronic acid (GlcA) and N-acetyl-D-glucosamine (Glc-NAc), connected to each other with β-1,3- and β-1,4-glycosidic bonds, having nearly perfect chemical repeats except for occasional deacetylated glucosamine residues to form a very hydrophilic linear high molecular weight (HMW) biopolymer [59]. HA ranges in size from 5 kDa to 10 million Da (corresponding to 25,000 disaccharide units), with the most common forms of 1–8 million Da in humans and can absorb water to expand its solid volume by up to 1000 times forming a very viscous and elastic gel [60]. HA GlcA carboxyl group is dissociated at physiological pH values to engender a negatively charged polymer which is readily combining with the most prevalent extracellular cation of Na^+ to form sodium hyaluronate, suggesting that the molecule is not ionized [59]. In fact, while native HA with a high molecular weight (HMW) acts mainly as a constructive stuff and a control on tissue hydrodynamics, low molecular weight (LMW) HA usually participates in cell signaling through interaction with certain types of cell surface receptors, such as CD44 as the primary one, but also TLR2 and TLR4, thus contributing to several physiological and biological activities [61, 62].

As an abundant endogenous polymer, HA has been widely exploited to construct the functional carriers for delivering various bioactives with expectation of improving human health, given many of its desirable merits that can be employed for optimizing delivery effects [63]. Most attractively, HMW HA possesses numerous physicochemical and physiological features, such as biocompatibility, biodegradation, mucoadhesive property, bearing negative charges in a neutral condition, possessing active groups allowing various functional modifications that are all beneficial characters for engineering carriers to deliver agents [64]. Another interesting aspect lies in that LMW HA binds to several receptors, especially CD44, which is ubiquitously expressed on various cells, and especially overexpressed on many types of cancer cells, presenting bases for developing a tumor-targeting drug delivery system (DDS) with attractive advantages, such as the ease of associating drugs with the polysaccharide or its carrier thus solving any solubility problems, improving a drug's blood plasma half-life thus playing a similar role to PEG, and high tumor-targeting efficiency, and as such is currently the main trend in the HA-based delivery research [65]. More recently, LMW HA is focused on its ability to activate CD44 or TLRs on immune cells involving regulation of certain signaling pathways associated with APC maturation, cytokine production and innate immune responses for immunization, and even, in a CD44- and TLR4-independent manner, to enhance CCR7 expression on DCs promoting DC recruitment to tumor regional lymph nodes and restraining DC migration toward tumor tissue [62, 66–68], thus providing not only a comprehensive option for engineering functional nanoparticles fitting a VADS, but also a promising candidate for improving DC maturation in the context of DC-based vaccine development.

Up to now, most of the HA-based carriers used as a VADS have been developed by making use of the "nonbioactive" properties, which has little physiological interference on the body and as such, is used just as constructive stuff. Moon's group formulated the HA-PEG-shelled cationic DOTAP/DOPE liposomes carrying F1-V, a candidate recombinant antigen for *Yersinia pestis*, as a stable and potent nasal VADS, which exhibited markedly decreased cytotoxicity associated with DOTAP liposomes to BMDCs, and when further incorporated with MPLA promoted BMDC maturation and induced a strong Th1/Th2-balanced immunoresponse toward Ags, as evidenced by high titers of F1-V-specific total IgG, IgG1, and IgG2c produced in intranasally immunized mice [69]. Huang's group engineered mLCP (the mannosylated lipid-calcium-phosphate NPs) and LPHa NPs (liposome-protamine-HA-anisamide NPs) for, respectively, targeting delivery of the tumor antigen Trp 2 peptide/CpG ODN to APCs and the TGF-β-silencing siRNA to tumor cells which overexpress sigma receptors with a ligand of anisamide [70]. They demonstrated that the delivery of Trp 2/CpG ODN to DCs by mLCP-based VADS elicited a potent systemic immune response to tumors in mice but generated, to later stage B16F10 melanoma, a marginal efficacy, which however, was remarkably boosted through silencing the immune-suppressive cytokine TGF-β in tumor cells with siRNA-loaded LPHa NPs to engender increased tumor infiltrating CD8+ T cells and decreased regulatory T cells within tumor microenvironment. Wu's group fabricated a microneedle array (MA) with HA with a deep cave formed in the basal portion of each microneedle, into which BCG (Bacille Calmette-Guerin bacillus) powder could be packaged directly, thus producing a painless VADS of MA-BCG, which after vaccination by patching on skin of mice caused no overt skin irritation, but elicited strong humoral and cellular immunity comparable to that of intradermal immunization [71]. Notably, other researchers showed in a clinical trial that HA-constructed MA containing trivalent influenza hemagglutinins (A/California/07/2009 (H1N1), A/Victoria/210/2009 (H3N2), and B/Brisbane/60/2008, 15 μg each) induced immune responses against A/H1N1 and A/H3N2 strains equal to that by subcutaneous injection groups without stirring severe local or systemic adverse reactions and engendered the efficacy against the B strain much stronger than that by the injection group, proving HA-MA a promising practical use as an easy and effective method to replace conventional injection systems [72].

Recently, Hahn's group conjugated an antigenic peptide of myostatin fragment (MstnF) to HA with a LMW (17 kDa) for transdermal vaccination against Duchenne muscular dystrophy (DMD), which is a neuromuscular disorder accompanied with muscle weakness and wasting with myostatin emerging as a key negative regulator [73]. In vivo experiments demonstrated that HA-MstnF conjugates efficiently penetrated into deep skin layers, and HA exerted a boosting effect on the immunization of MstnF in the transdermally vaccinated mice, which not only secreted high levels of antibody titers against myostatin but also showed a significant improvement in the pathological status of skeletal musculature as well as functional behaviors. Gonzalez-Aramundiz et al. prepared protamine/LMW HA (162 kDa) NPs using a mild ionic cross-linking technique and showed that in vitro Ag (rHBsAg)-loaded anionic NPs (protamine/HA of 1:4, w/w) induced the secretion of cytokines including TNFα, IL-1α, and IL-6 by macrophages more efficiently than the cationic NPs (protamine/HA of 4:1), whereas in mice, by either intramuscular or intranasal administration, the cationic NPs induced more robust immune responses than the anionic NPs did, as proved by the higher levels of the IgG

against the hepatitis B antigen in the cationic NP group, indicating that the protamine/HA NPs depending on physical features may be an effective VADS for delivering subunit HBV vaccines [74]. Kim et al. using LMW HA (215 kDa) synthesized HA-OVA conjugates, which proved able to facilitate DC maturation *in vitro* and, after topical application to penetrate into the dermis in murine skins, efficiently induced secretion of the anti-OVA IgG levels in serum as well as IgA levels in bronchioalveolar lavage, which could promptly respond to an OVA challenge after 8 weeks rendering a strong immune-recall humoral response, especially, under the condition of pretreatment of the skin using nonablative fractional laser beams to save Ag dose, strongly supporting of the adjuvant role that LMW HA can play for developing the painless topical VADS [75].

In summary, HA is a biodegradable and safe endogenous polymer, which can be used to engineer either inert NPs with high molecular weight HA or cell-targeting NPs with low molecular weight HA based on the fact its selective binding to several receptors, such as CD44 and TLR4, which may possibly trigger innate immune responses, allowing HA-based NPs to be conveniently employed to construct multifunctional VADS able to efficiently deliver various subunit vaccines.

3. Conclusions

At present, various polymeric NP-based VADSs have been designed for delivering as well as adjuvanting vaccines to elicit robust Ag-specific humoral and cellular immunity at both systemic and mucosal levels to provide extensive protection against infectious pathogens. In particular, many types of polymeric NPs can be tailored as a multiple functional VADS to render Ags lysosome escape after APC uptake, allowing vaccine epitopes not only to avoid being degraded into null pieces but also to selectively bind to MHC-I or -II for presentation to dictate immune responses toward a Th1 and/or Th2 pathway to set up immunity fitting medical aims. Encouragingly, a few of the polymeric NP VADS-based subunit vaccines have been approved, as mile stones, for clinical vaccination, typical products including the virosome-based hepatitis A vaccine (Epaxal®) and influenza vaccine (Inflexal V®), VLP-based HBV vaccine and malaria vaccine. Undoubtedly, as many of the uncertainties and problematic issues associated with polymeric NPs, such as safety of synthetic materials, scale-up production, and cost of products, are ultimately resolved, more and more polymeric NP VADS-based vaccines will be developed and licensed to enter markets.

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Conflict of interest

All the authors declared no conflict of interests.

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