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Vaccine Adjuvant Delivery Systems Constructed Using Biocompatible Nanoparticles Formed through Self-Assembly of Small Molecules

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Additional information is available at the end of the chapter

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Abstract

Subunit vaccines are playing a critical role in controlling numerous diseases and attracting more and more research interests due to their numerous advantages over conventional whole microbe-based vaccines. However, subunit vaccines are weak immunogens and thus have limited capacity in eliciting the humoral and cellular immunity against pathogens. Recently, nanoparticles (NPs) formed with certain small molecules through self-assembly have been employed as an effective carrier for subunit vaccines to play roles of adjuvant, delivery and stabilization of antigens, thus engendering a vaccine adjuvant-delivery system (VADS), which shows promises to overcome the hurdles in developing subunit vaccines. In particular, the small molecule-self-assembled NPs as a VADS can not only deliver vaccine ingredients to immune cells but also influence the immunoresponse toward a Th1 (type 1 T helper cell) and Th2 balanced pathway to establish both humoral and cellular immunity. This chapter describes the innovative VADSs based on the small molecule-self-assembled NPs, such as metal NPs (mNPs), emulsions, liposomes, and ISCOMs, which are elaborately designed for the development of subunit vaccines.

Keywords: nanoparticle, self-assembly, immune response, mucosal vaccination, cellular immunity, nanocarrier, inorganic particle, danger-associated molecular pattern, targeted delivery

1. Introduction

Modern vaccine development began from the use of vaccinia against smallpox by British physician Edward Jenner in the late eighteenth century and ever since has brought many products that have saved countless human lives from being claimed by numerous infectious pathogens, such as smallpox, measles, and rabies [1]. However, many pathogens such as human immunodeficiency virus (HIV), herpes simplex virus (HSV), and Ebola virus (EBV), still lack vaccines and are still posing a great threat to human health and life [2].

Vaccines are developed based on the antigenic components, which can stimulate the host immune system to set up immunity able to clear the abnormalities and usually include three types: the live attenuated whole microbe vaccines, the killed whole microbe vaccines, and the antigenic component-based subunit vaccines [3]. The former two types are regarded as the conventional vaccines with a high capacity of defending against deleterious organisms but, unfortunately, are also linked to a relatively poor safety profile due to their possible reversion of virulence and induction of deviated immunoresponses leading to unprotective and even harmful immunity and unacceptable inflammation. In contrast, the subunit vaccine is elaborately formulated with defined components including antigen (Ags) to induce immunoresponses which is accurately targeting the matched objects, thus causing few safety concerns and can be employed to fight both infectious pathogens and detrimental neoplasms carrying the identical Ags. Subunit vaccines since the notion emergence have attracted great research interests with numerous visible and all imaginable advantages [4], including high safety without reversion to virulent state; their production needing no dangerous microorganisms; providing an alternative solution to the problematic culture for attenuating some pathogens; low risk of allergic or autoimmune reactions; customization to recognize certain pathogen-associated targets; feasible developing anticancer vaccine; carrying several peptide epitopes targeting different stages in the life cycle or subtypes of a pathogen; production in large scale in a pure state, in an economically and highly reproducible manner; high solubility allowing lyophilization to form stable dry products [4].

However, subunit vaccines often show a weak immune induction potency, due to lack of a large fraction of components associated with pathogen structural characteristics, which are broadly shared by pathogens but distinguishable from host molecules, collectively referred to as pathogen-associated molecular patterns (PAMPs) and able to activate the pattern recognition receptors (PRRs), such as the toll-like receptors (TLRs), the nucleotide-binding oligomerization domain-like receptors (NOD-like receptors), retinoic-acid-inducible gene-I-like receptors (RIG-I-like receptors), and C-type lectin receptors, to trigger as immunostimulators or an adjuvant mammalian innate immunoresponse sponsoring a series of adaptive reactions required for establishing the Ag-specific immunity [5]. Thus, while subunit vaccines are usually safer and less reactogenic than the whole organism vaccines, they often need an adjuvant or a vaccine adjuvant delivery system (VADS) to synergistically stimulate professional Ag-presenting cells (APCs) such as dendritic cells (DCs) and macrophages (MPs) for enhancing their immunization efficacy [6–10]. An adjuvant is a non-specific immune-potentiating substance, which is capable of enhancing the body's immune

response to the Ag or changes the type of immune response with some mechanisms that are not exactly known but are argued relevant to two fundamental aspects: (1) giving off dangerous signals by imposing damages on cells/tissues to activate the innate immune cells; (2) exciting PRRs of the innate immune cells, such as DCs, MPs, histiocytes and mast cells, to sponsor the subsequent adaptive responses [11, 12]. The available adjuvants are mainly micron- or nanometer-sized particles or aggregates which may be classified into two types: (1) the natural or synthetic substances with intrinsic adjuvanticity, such as alum (insoluble aluminum salt), squalene/squalane, saponin, chitosan, hyaluronic acid (HA), and various pattern recognition receptor agonists (PRRs); (2) functional carriers capable of playing roles of both adjuvant and delivery, thus regarded as vaccine adjuvant delivery system (VADSs), which are often formulated with nanoparticles (NPs) fabricated with various biocompatible materials, such as liposomes made of phospholipids and cholesterol, immune stimulating complexes (ISCOMs) of saponin and lipids, polymeric NPs made of PLGA or polystyrene, virus-like particles (VLPs) made of viral proteins, emulsions made of squalene and surfactants, and the metal NPs (mNPs) made of aluminum or gold metal compounds, which prove an efficient VADSs able to enormously enhance vaccination efficacy [3, 13].

In this chapter, we describe design principles, main formulations and the state-of-the-art advances in developing novel VADSs constructed with different types of NPs, which are formed through small molecule self-assembly and herein, include liposomes, nondegradable inorganic metal NPs (mNPs), emulsions, ISCOMs. These small molecule-based NPs have been devised as a VADS with the potential to stimulate the Ag-specific humoral and cellular immune responses and are promising in preparation of next generation vaccines against a range of infectious pathogens.

2. VADS constructed with different types of NPs formed by self-assembly of small molecules

2.1. VADS constructed with metal nanoparticles (mNPs) formed by self-aggregation

Alum is the micron-sized aggregates of water-insoluble aluminum salts and is the first substance that was discovered able to boost the efficacy of vaccines and coined the term “vaccine adjuvant,” a concept which was put forward when scientists came to realize that certain materials irrelevant to pathogens but able to enhance immunoresponse induced by vaccine [14]. Since 1926, when first being used as an adjuvant, alum, such as aluminum phosphate, aluminum potassium sulfate, and aluminum hydroxide had been the only clinically used adjuvant in many subunit vaccines as well as the inactivated pathogen-based vaccines, until the approval of adjuvant calcium phosphate in diphtheria/pertussis (DT) vaccines [15]. Subsequently, an O/W nanoemulsions formed of squalene/Span 85/Tween 80, called MF59[®] was marketed as a VADS for delivering an influenza vaccine (Fluad[®]) in 1997, followed by AS04 (MPL/alum mixture) for delivering human papillomavirus (HPV, Cervarix[®]) and hepatitis B virus (HBV, Fendrix[®]) vaccines [16]. Alum forms a micron-sized VADS by just mixing the insoluble salt with other vaccine components or Ags and tends to function eliciting humoral over cellular immunity when

intramuscularly administered to humans. Though having successfully been used for nearly a century in human vaccines against numerous infectious diseases, such as hepatitis A and B, diphtheria-tetanus-pertussis (DTaP, Tdap), *Haemophilus influenzae* type b (Hib), HPV, and pneumococcus infection, alum is still argued to be associated with a potential risk for causing autoimmunity, long-term brain inflammation and neurological complications, as evidenced by the observation of severe disorders in recipients of alum-adjuvanted vaccines [17].

Thus, frustrated by the reactogenicity and the injury adverse effects associated alum while expected to enhance its capability to induce humoral and even cellular immunoresponses, researchers have for years endeavored to reshape the micron-sized salt adjuvant in mainly two ways: forging the micron-sized salt into NPs and coating surfaces with biocompatible materials. Recently, to develop an effective HIV vaccine, which is known a huge challenge almost since this virus discovery, Neutra's group conjugated peptide epitopes derived from HIV-1 gp120 glycoprotein to the Al_2O_3 NPs with a size of about 350 nm, which showed able to stimulate the moderate antibody responses after intraperitoneal injection but failed to stimulate mucosal immunity [18]. Also, Cui's group engineered 112 nm-sized aluminum hydroxide NPs and aluminum oxyhydroxide nanosticks with a length of 80 nm, long aspect ratio of 10 and low degree of crystallinity and showed that both aluminum NPs were able to facilitate in vitro APC uptake of the loaded protein Ags and induced in mice a stronger Ag-specific antibody response but milder local inflammation in the injection sites, compared with traditional aluminum microparticles [19, 20]. Furthermore, aluminum NPs proved able to stimulate in vitro APCs to produce uric acid, and, when injected into peritoneal cavity of mice, induced production of increased levels of uric acid, to contrast micron alum which did not in either case. The results suggest that aluminum has a stronger adjuvant activity in the form of NPs, as opposed to microparticles, may be partially attributed to their higher ability to induce endogenous danger signals such as uric acid [21].

Based on the phosphophilicity of aluminum, Wang and coworkers engineered the phospholipid bilayer-coated aluminum nanoparticles (PLANs) formed via chemisorption between phospholipid and aluminum using a procedure of reverse ethanol injection-lyophilization (REIL) [7]. The researchers demonstrated that the anhydrous Ag-PLANs had a high stability satisfying the prerequisite requirements for distribution with the controlled temperature chain instead of the integrated cold chain [22] and that upon rehydration the Ag-carried PLANs could be instantly reconstituted to form an aqueous dispersion maintaining vaccine activity. Further exploration confirmed that the PLANs remarkably enhanced APC uptake of the delivered vaccines and when given subcutaneously to mice, induced more robust Ag-specific humoral as well as cellular immunoresponses, while stimulated less local inflammations, in comparison to microparticle alum, proving that the PLANs are an efficient VADS and possess numerous advantages over alum, which has been the widely used for clinical immunization for nearly a century [7].

In recent years, other types of NPs made of metal substances, such as calcium and gold, have also become a popular VADS, owing to their certain unique physicochemical properties including inertness with good biocompatibility, facile surface modification with functional molecules, and easy size and shape control. Chiu and coworkers coated the 25 nm-sized amorphous cores of calcium phosphate nanoparticles (CaP-NPs) with peptide Ags, thus

producing a particulate vaccine with a hydrodynamic size of 60 nm and found that the small core-shell assemblies induced in mice a 3-fold increase of anti-Ag titers 3 weeks post-injection, compared to a commercial aluminum phosphate adjuvant, suggesting that CaP-NPs may be an effective VADS delivery of vaccines [23]. Morcol et al. demonstrated that CaP-NPs were also a good VADS for the inactivated influenza A/CA/04/2009 (H1N1pdm) vaccine and could enormously boost production in the intramuscularly vaccinated mice of hemagglutination inhibition (HAI), virus neutralization (VN), and IgG antibody titers, at all dose levels, relative to the nonadjuvanted vaccine. In particular, the CaP-NP vaccine equally protected mice against influenza virus at 1/3 of the Ag dose of the nonadjuvanted or alum-adjuvanted vaccines, indicating that CaP-NPs are a promising VADS which may play a crucial role in production of a dose-sparing vaccine which is of a great importance during, in particular, an influenza pandemic [24]. Also, Powell and coworkers constructed calcium carbonate NPs which had an average diameter of 200 nm and based on opposite charge attraction, coated with polylysine and polyglutamic acid and showed that this type of the CaCO_3 NP-based VADS could efficiently facilitate maturation of DCs, which were simultaneously induced capable of cross-presentation of Ags. Notably, after a single injection in mice, CaCO_3 NPs induced strong humoral and cellular immunity without triggering secretion of inflammatory cytokines, proving CaCO_3 NPs are an efficient and safe VADS [25].

Gold nanoparticles (AuNPs) have unique physicochemical properties, such as an ultra-small size, large surface area to mass ratio, and high surface reactivity, presence of surface plasmon resonance (SPR) bands, biocompatibility and ease of surface functionalization, allowing this type of mNPs able to act as a versatile VADS bearing numerous beneficial features including, particularly, targeted delivery and stimulus-sensitive release. Chen et al. engineered gold NPs (AuNPs) with sizes ranging from 2 to 50 nm conjugated with foot-and-mouth disease virus associated peptide Ags and proved that gold NPs with a size of ranging in 2–17 nm induced strong humoral response, which was correlated to spleen uptake of gold NPs [26]. Gill's group prepared gold NPs conjugated with M2e peptide, an extracellular domain of influenza A virus ion channel membrane matrix protein 2 (M2e) and demonstrated that intranasal administration to mice of AuNP-M2e plus soluble CpG induced lung B cell activation and robust serum anti-M2e antibody response, resulting in high levels of both IgG1 and IgG2a subtypes [27]. Also, the group revealed that the antibodies generated by AuNP-M2e/CpG stimulation could bind to the homotetrameric form of M2 expressed on Madin-Darby canine kidney (MDCK) cells, which as an immunosorbent had been infected with H1N1, H3N2 or H5N1 strain of influenza viruses. Moreover, mice intranasally immunized with AuNP-M2e/CpG obtained 100, 92, and 100% protection against lethal challenges with A/California/04/2009 (H1N1pdm) pandemic strain, A/Victoria/3/75 (H3N2), and the highly pathogenic avian influenza virus A/Vietnam/1203/2004 (H5N1), respectively, proving AuNP-M2e/CpG a promising VADS for developing a universal influenza vaccine, a desired Holy Grail for controlling the most prevalent infections [27].

2.2. VADS constructed with emulsions formed by self-assembly of surfactants

Emulsions are formed of two immiscible liquid phases, generally oil phase and water phase, with one phase organized into small droplets (inner phase), which, depending on composition and manufacturing process, have a size in a range of from tens of nanometers to several

microns, and are dispersed in a distinct continuous phase (outer phase) under stabilization by an interfacial surfactant layer. Emulsions, based on structural characteristics, are made of three classical types of single emulsions, double emulsions and Pickering emulsions: single emulsions include oil-in-water (O/W) type denoting oil droplets being emulsified in a bulk aqueous phase, and vice versa, the water-in-oil (W/O) type; double emulsions include O/W/O and W/O/W emulsions; and Pickering emulsions are a special type with an emulsifier of solid NPs replacing surfactants [28].

Notably, the emulsions formed of special oils, such as lanolin oil, cottonseed oil, and paraffin oil, were found, like alum, in some serendipitous way, of adjuvanticity in the early twentieth century and have ever since been widely used as a VADS to produce vaccines against pathogens. For example, lipovaccines used in the 1920s were in fact the formulations consisting of killed bacterial vaccines suspended in lanolin or cottonseed oils and proved able to induce immunoresponses with additional functions of dose spare and stability enhancement [29]. Freund adjuvants are the mostly known potent emulsion-based VADS including two types: incomplete Freund adjuvant (IFA), which is essentially a viscous crude W/O emulsion containing Ags in water phase using mineral paraffin as an oil phase and mannide monooleate as a surfactant; complete Freund adjuvant (CFA), which forms by addition to IFA of heat-killed mycobacteria (*Mycobacterium tuberculosis*) and has thus a high immunostimulating potency but also a high reactogenic toxicity, rendering the adjuvant to be used only in veterinary vaccines [30]. Though IFA is rather safe compared to CFA and was actually administered to hundreds of thousands of humans as an adjuvant in polio and influenza vaccines in the mid-twentieth century, the severe local reactogenicity excluded the adjuvant from continuing clinical use [31].

Discarding the flaws of unacceptable toxicity and uncertain component associated with early emulsion adjuvants, modern emulsions as a VADS are usually formulated with well-defined factors, such as particle size, component and concentrations, and compatibility with antigens as well as human bodies, which are related to efficacy, safety, and stability [32]. Important lessons highlighted by early emulsion vaccines and deep insights into problems arising in use of the adjuvant inspired researchers to commit to developing an emulsion VADS with clear thoughts in several issues: (1) using biodegradable oil and the surfactants with an established safety profile in humans; (2) using the O/W instead of W/O emulsions to lower oil content for enhancing tolerability as well as the ease of use due to reduced viscosity; (3) enhancing potency with emulsions having a size <500 nm to promote APC uptake. As a result of the efforts directed toward these aspects, a breakthrough was made in the development of emulsion VADS in the 1980s when the squalene was explored as the oil phase of emulsions, which were thus rendered with an acceptable reactogenicity profile and potent adjuvant effects and were subsequently licensed as several proprietary products, including MF59 by Novartis, AS03[®] by GSK and AF03 by Sanofi Pasteur. MF59 is an O/W emulsion which is produced with a microfluidizer (MF) and contains squalene oil droplets stabilized by surfactants Tween 80 and Span85 guaranteeing the size of 160 nm for sterilization by filtration and as a VADS has proven of potent immunogenicity and low reactogenicity for a range of Ags [16]. MF59 can induce robust immunoresponses through triggering vaccinated tissue-resident immune cells to secrete a number of chemokines, which recruit other immunocytes to amplify the chemokine gradient, resulting in a significant signal magnification and immune cell influx to establish

anti-Ag immunity. MF59 became the first emulsion-based VADS approved for delivering the seasonal influenza vaccine of Fluad® for human immunization in 1997 and followed by AS03, which is a 200 nm-sized O/W emulsion consisting of squalene/DL- α -tocopherol/Tween 80 and was approved for human use in GSK's A/H1N1 pandemic flu vaccine Pandemrix® [33]; and then AF03, which is a 80 nm-sized O/W emulsion consisting of squalene/polyoxyethylene cetyl-stearyl ether/sorbitan oleate/mannitol and was approved for clinical immunization in Sanofi Pasteur's pandemic influenza vaccine, Humenza® [34].

Now, novel types of emulsions are still actively formulated using various functional materials to constitute a VADS possessing desired properties, including high potent immunogenicity, targeting delivery of vaccines toward draining lymph nodes (dLNs) and APCs, enhanced cellular uptake, controlled release of Ags, rendering vaccine lysosome escape, and directing immunoresponses toward the Th1/Th2 type biased or balanced pathway [32]. Meanwhile, attempts in pushing into clinical trials of emulsion VADSs for delivery of cancer vaccines and other applications have also increasingly continued and are accompanied by endeavors in shedding light on the mechanisms involved in the action of emulsion adjuvants. Recently, Schmidt et al. using squalane as an O and distearoylphosphoethanolamine (DSPE) as an emulsifier engineered the TLR3a poly(I:C)-entrapping cationic nanoemulsions with a size of 200 nm and demonstrated that when given to mice the cationic nanoemulsions drained rapidly to the LNs and activated cross-presenting DCs, MPs as well as B cells, resulting in strong Ag-specific CD8⁺ T-cell responses [35]. The results suggest the squalane-based cationic nanoemulsions may be a promising VADS with the ability to induce strong CTL responses, offering an alternative way to make vaccines against pathogens that can hardly be protected without activated CTLs. Interestingly, using squalene as O but the 100 nm-sized poly(D, L-lactic-co-glycolide) (PLGA) NPs as a stabilizer, Ma and coworkers formulated 2 μ m-sized Pickering emulsions as a VADS, which retained the force-dependent deformability and lateral mobility of loaded Ags [36]. Mouse experiments proved that the Pickering emulsions enhanced the recruitment, Ag uptake, and activation of APCs which initiated robust humoral as well as cellular immunoresponses, which effectively supported mice to survive a lethal challenge of influenza virus. The outcomes hint that the pliability of vaccine carriers and lateral mobility of Ags may well count in triggering immune reactions and, as such, may well be taken into account when developing certain types of VADS.

In summary, as one of a few types of VADSs that have been approved for human use, certain types of emulsions prove by numerous clinical and preclinical evaluations capable of eliciting strong humoral and/or cellular immunity against heterologous pathogens meanwhile maintain an excellent safety profile, depending on the components as well as the structural characteristics of this fluid carrier. Further development of emulsion VADSs may focus on elucidating the mechanisms underlying the immunopotentiating functions in regard of particularly the relationship between emulsion efficacy, systematic characteristics, and molecular structure of squalene, squalane or other unidentified active materials [32]. Further efforts may well be committed to improving the stability of emulsions to construct a VADS allowing the products to be distributed, at least for some time, out of the cold chain [37], thus facilitating global vaccination against various infections in, especially, some low-income countries or districts, where integral cold chain may not be available.

2.3. VADS constructed with liposomes formed by self-assembly of phospholipids

Liposomes are the phospholipid bilayer-enclosed vesicles and have attracted many research interests in the development of drug delivery system (DDS) as well as VADS ever since its discovery by Bangham et al. in the early 1960s [38]. Liposomes usually consist of one or more concentric lipid bilayers alternating with aqueous spaces [39, 40], with the components of one, or more type of amphiphilic phospholipids such as phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylglycerol (PG), and sphingomyelin (SM), which though form the frame structure of liposomes and are often supplemented with ingredients, such as cholesterol (CHO) and other charged lipids such as stearylamine (SA), N[1-(2,3-dioleoyloxy) propyl]-N,N,N-triethylammonium (DOTMA), 1,2-dioleoyloxy-3-(trimethylammonium propane) (DOTAP), and 3 (N,N-dimethylaminoethane)-carbamyl cholesterol (DMACHO), which are purposely used for tailoring the property of liposomes. Depending on ambient temperature and the nature of the lipids, the liposome bilayers may exist in either a “fluid” state when the ambient temperature is above the T_c (a gel to liquid crystalline transition temperature—the temperature at which the acyl chains melt) of liposomes, or a “rigid” state when the ambient temperature falls below the T_c of liposomes [40]. However, when CHO is homogeneously incorporated into phospholipid bilayers, for example, at the mole ratio of CHO/PC between 1/3 and 2/3, the membrane rigidity may be significantly strengthened, and as a consequence, liposomes are blurred of T_c lowering content leakage. Bearing a common weakness of instability associated with a colloidal system, liposomes are often superficially PEGylated (modification with polyethylene glycol, PEG) to engender a steric stabilization effect, and/or are charged with an appropriate zeta potential value by the incorporated ionic lipids to generate an electrostatic repulsion for preventing aggregation, or for flocculating particles according to DLVO theory. Also, lyophilization is often employed to engender liposomes into a dry entity, which has a high stability completely satisfying the shelf-life requirements for clinical application, as it can be rehydrated to reconstitute the initial vesicles with little cargo leakage just in the presence of disaccharide as an effective lyoprotectant [41].

Liposomes possess numerous distinct properties enabling them to fulfill the functions of an excellent VADS, which can be summarized as aspects including good biocompatibility, high loading capacity for various ingredients, and the ease for preparation and surface decoration to engender specific functions such as targeting delivery, lysosome escape and controlled release [42]. With ability to entrap hydrophilic, lipophilic as well as amphiphilic molecules in the inner aqueous phase or lipid bilayers, liposomes have been formulated for delivering a large range of therapeutic agents, including small molecules, DNA/RNA fragments, peptides, and even proteins with a large molecular weight (MW), which exhibit respective therapeutic activities [39]. Also, liposomes are frequently employed as a VADS fitting diverse immunization routes, including intravenous, intramuscular, subcutaneous, intranasal, oral uptake, pulmonary inhalation, and topical skin or mucosal administration, for delivering vaccines to resolve problems associated with free Ags, for instances, averting premature inactivation caused by environmental chemicals, and ensuring Ags to approach APCs and even the intracellular organelles without off-targets [43]. In particular, beneficial for acting as a VADS, liposomes possess the intrinsic adjuvant properties as established by Gregoriadis and coworkers in as early as 1974 when strong humoral immune responses to liposome-entrapped

diphtheria toxoid were observed after injection of the liposome vaccines into mice [40, 44, 45]. It is generally accepted that liposomes as a VADS function with the adjuvanticity regardless of the carrying mode of Ags, including entrapment within vesicles, attachment on surfaces, or simply mixing together [46, 47], which allows diverse modifications to be carried out on liposomes without concerning the Ag damage through measures, including PEGylation, decoration with PAMP molecules or the pattern recognition receptor agonists (PRRs), such as lipid A for TLR4, CpG-ODN for TLR9, and synthetic mannose derivatives for C-type receptors on APCs [8, 9, 37, 48, 49].

Notably, multifunctional liposomes have also been developed in combination with novel administration devices to form a VADS which can be employed to enhance immunization efficacy via convenient administration [47]. In particular, Wang's group developed the multifunctional liposome-based VADSs through fabrication of liposomes adorned with TLR4a lipid A and loaded with Ags into biodegradable microneedle arrays, which can efficiently exert penetration of mucosa enhancing topical delivery efficiency [37, 48, 50]. Going further, Wang and coworkers engineered two types of multifunctional liposomes, the 200 nm-sized mannosylated lipid A-liposomes (MLLs) and the 50 nm stealth lipid A-liposomes (SLLs), both of which were loaded with Ags and NH_4HCO_3 and then packed together into microneedles, forming the proSLL/MLL-constituted microneedle array (proSMMA), which proved able to rapidly recover the initial MLLs and SLLs upon rehydration by tissue fluids [47]. Mice vaccinated with proSMMA by vaginal mucosa patching established robust Ag-specific humoral and cellular immunity at both systemic and mucosal systems, especially, in the reproductive and intestinal ducts, owing to the action processes involving the facts that the MLLs reconstituted from the administered proSMMA were mostly taken up by vaccination site-resident DCs for mucosal responses, whereas the smaller SLLs traveled to the dLDs wherein picked up by macrophages for efficient use of Ags. Furthermore, the delivered Ags were displayed by APCs via cross-presented with MHC-I thanks to lysosome escape and ROS (reactive oxygen species) stimulation, which were caused, respectively, by expansion of CO_2 gas and induction of excessive $\text{NH}_4^+/\text{NH}_3$ both sourcing from the liposome-released NH_4HCO_3 , leading to a mixed Th1/Th2 type response promoted further by liposomal lipid A and activation of TLR4. Thus, though the large-scale production of the proSMMA seems still a problem owing to the complex procedure for products and the instable entrapment of volatile NH_4HCO_3 in vesicles, the multifunctional VADS constructed with liposomal microneedles for vaginal immunization provides an alternative strategy to elicit immunity against various pathogens, especially, the sexually transmitted ones. Moon et al. fabricated a novel VADS based on a special type of liposomes, which were called interbilayer-crosslinked multilamellar vesicles (ICMV) and formed by crosslinking headgroups of adjacent phospholipid bilayers within multilamellar vesicles [51]. Further investigation showed that the stable Ag/adjuvant-carried ICMVs rapidly released the loaded cargos in response to catalysis by endolysosomal lipases, and when given to mice elicited robust endogenous T-cell and antibody responses, suggesting ICMVs a stimulus-sensitive VADS which may open up new possibilities for vaccination against infectious diseases and cancer.

Summarily, liposomes are the most diverse carrier for delivering various agents and can be employed through diverse modifications with various functional molecules to constitute

different types of multifunctional VADS satisfying different vaccination requirements. As a proved by numerous experiments, at least in animal models, these multifunctional liposome VADSs are highly effective in both targeting delivery of vaccine to APCs and enhancing Ag presentation by APCs to related T-cells to set up the Ag-specific immunity against pathogens, fulfilling a dual function of adjuvancy and delivery for vaccines [7, 37, 47–49].

2.4. VADS constructed with ISCOMs formed by self-assembly of saponin and lipids

The immune stimulating complexes, named ISCOMs, are a type cage-like NPs with a size of 40 nm constructed of linked nanoring subunits with a size of 12 nm, and usually formed through self-assembly of the main components of phospholipids, cholesterol and, importantly, saponin which, as a crude mixture of numerous triterpene derivatives extracted from the cortex of the South-American Tree *Quillaja saponaria Molina*, has potent adjuvant activities [52]. ISCOM was first coined the name in 1984 by Morein et al. [53], who demonstrated that ISCOMs contained saponin Quil A, a heterogeneous mixture containing up to 23 different saponin compounds [54], and virus membrane proteins were at least 10 times more potent than micelles formed by aggregation of the protein Ags alone, but caused no severe side effects, such as hemolysis, associated with saponin. The strong immunostimulatory effects were argued to be resulted from large exposure of protein Ags in ISCOMs and the intrinsic adjuvant activity of saponin Quil A, while no severe adverse effects of hemolysis associated with saponin were noticed thanks to its tight association with cholesterol.

Being explored for high potency and low toxicity, Quil A was purified using reversed phase high performance liquid chromatography (RP-HPLC), by which Kensil et al. identified adjuvant activity in 10 of the fractions including the four most abundant compounds, termed QS-7, 17, 18, and 21, with the numbers corresponding to their relative elution time, which is dependent on their degree of hydrophobicity using C4 resin column with RP-HPLC [55]. Similarly, Rönnberg et al. isolated three different RP-HPLC fractions of Quil A: QH-A sequences eluted early, further two sequences of the more hydrophobic fractions QH-B and QH-C, which were examined by pre-clinical toxicology and animal testing, resulting in an optimized combination of 7 parts QH-A, 0 parts QH-B and 3 parts QH-C, known as QH-703 or ISCOPREP™703 (Isotec AB, Sweden) [56], which was further developed into proprietary product ISCOPREP™ saponin by omitting QH-A fraction [57].

The identification of purified adjuvants from crude saponin allows ISCOMs to be formulated with more defined ingredients, such as monomer of QS21, ISCOPREP™ 703, and ISCOPREP™ [54], to constitute a VADS which can induce robust immunoresponses with Ags whether incorporated in the carrier or just physically mixed with the carrier [58, 59]. Formulation requiring no Ag incorporation not only simplifies the process of preparing the ISCOM vaccines but also expands the delivered Ags to include the hydrophilic ones; and the findings further supports the hypothesis that encapsulation of Ags in a carrier is not necessarily the prerequisite requirements for stimulating immunoresponses [60]. Duewell et al. developed the palmitified OVA-incorporated ISCOMs consisting of ISCOPREP, PC and cholesterol and showed that subcutaneous injection of OVA-ISCOMs to mice resulted in a substantial influx and activation of immune effector cells in dLNs in control of the vaccinated site and promoted natural killer (NK) and NK T cells to produce IFN- γ . Also, facilitated by the efficient Ag cross-presentation CD8 α^+ DCs in dLNs, a high frequency of different tumor cell killing Ag-specific CTLs was

differentiated and proliferated from relevant precursors [61] through MyD88 (the myeloid differentiation primary response gene 88) adapter protein-expression pathway, as revealed by Wilson et al. [62]. Notably, ISCOMs were upgraded by Schiött and coworkers to the next generation VADS, denoted Posintro™, which were cationic NPs formulated with cholesterol, DC-cholesterol (3 β -(N-(N',N'-dimethylaminoethane)-carbamoyl) cholesterol hydrochloride), POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine), Quil A and HBsAg in the weight ratio of 3:1:4:20:5, engendering a new HBV vaccine of Posintro™-HBsAg [63]. In the intradermally (i.d.) immunized animal models of mice and guinea pigs, Posintro™-HBsAg induced the strong response with high titers of HBsAg-specific antibody and high levels of cytotoxic T lymphocyte (CTL), demonstrating that Posintro™-HBsAg is promising both for the protection against HBV infection and as a potential therapeutic vaccine.

Notably, to develop effective vaccines against the deadly Ebola virus (EBV), which causes a fatal hemorrhagic fever in humans with a mortality of around 50%, as evidenced by the 2014–2016 West Africa Ebola epidemic which claimed 11,310 lives in 28,600 infection cases [64], Bengtsson et al. engineered the 2014 EBV Makona strain glycoprotein (EBV/Mak GP) trimer VLPs (virus-like particles) with a size of 30–40 nm using the Sf9 (engineered *Spodoptera frugiperda*) insect cell-recombinant baculovirus expression system [65]. In mice, adjuvanted with the Matrix-M which consists of two populations of 40 nm ISCOMs: 85% Matrix-A of saponin QH-A fraction +15% Matrix-C of saponin QH-C, EBV/Mak GP VLPs induced a rapid onset of specific IgG and neutralizing antibodies, increased frequency of multifunctional CD4+ and CD8+ T cells as well as effector B cells. Noteworthy, the immunity established in the vaccinated mice conferred a 100% protection against a lethal viral challenge, suggesting the Matrix-M adjuvanted EBV/Mak GP VLP NPs an effective VADS for developing subunit vaccines against the deadly Ebola infections. Similarly, the group using Sf9 insect cell platform engineered a recombinant trivalent NP influenza vaccine (tNIV), which when intramuscularly administered with Matrix-M to ferrets induced high levels of broadly neutralizing antibodies against A (H1N1) strain, B strain and, especially, a panel of all historic (2000–2017) A/H3N2 strains [66]. In particular, in a clinical trial involving 330 adults, the 60- μ g dose of tNIV/50 μ g Matrix-M induced significantly greater HA inhibition antibody responses against a panel of wild-type A (H3N2) strains than did the inactivated trivalent vaccine Fluzone [67], showing that Matrix-M/tNIV may be an efficient strategy for developing the effective universal influenza vaccines with additional advantage in avoidance of the mismatching Ags as occurred in conventional procedures.

Summarily, the nanosized cage-like ISCOMs constituted through self-assembly of a combination of saponin, phospholipid and cholesterol are a multifunctional VADS, which can deliver or adjuvant Ags and, in both cases, can enormously boost the efficacy of subunit vaccines. In particular, ISCOMs can be combined with other adjuvants such as TLRs to further improve the immunostimulatory effects for enhancing function of adjuvanted Ags, thus providing a diverse platform for making therapeutic as well as prophylactic vaccines against pathogens or malicious neoplasms.

3. Conclusions

The NP-based VADSs provide an efficient strategy for delivering and enhancing efficacy of subunit vaccines, which are weak immunogens but represent the current trends in the

development of vaccines against various pathogens including cancer. The NPs formed through self-assembly of small molecules, especially, those possessing intrinsic adjuvanticity, are an attractive and promising VADS due to their numerous advantages, such as acceptable safety profile, ease for preparation and modification with functional materials as well as control of size, and fitting different vaccination routes, which may confer the carried vaccines multiple functions capable of eliciting the Ag-specific humoral as well as cellular immunity at both systemic and mucosal levels providing a strong protection against pathogens. Encouragingly, some subunit vaccines developed with the VADSs that are based on small molecule-assembled NPs have already been approved for clinical vaccination, and typical products include the virosome-based hepatitis A vaccine (Epaxal[®]) and influenza vaccine (Inflexal V[®]), MF59-based influenza vaccine (Fluad[®]), AS04-based HPV (Cervarix[®]) and HBV (Fendrix[®]) vaccines, and AS01-based malaria vaccine (Mosquirix[®]). Hopefully, as many problems associated with NP VADSs, such as high cost for products, and undefined mechanisms underlying immune reactions and associated adverse effects, are finally settled, more NP VADS-based subunit vaccines will be pushed into markets for conquering human life-threatening diseases, such as HIV infection, MERS infection, and even intractable cancers.

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Conflict of interest

All the authors declared no conflict of interest.

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