Fabrication of subunit nanovaccines by physical interaction

CHEN HaoLin¹, LIU Hong², LIU LiXin¹ & CHEN YongMing¹,3*

¹ School of Materials Science and Engineering, Key Laboratory for Polymeric Composite and Functional Materials of Ministry of Education, Sun Yat-sen University, Guangzhou 510275, China;
² Zhuhai Jinan Selenium Source Nanotechnology Co., Ltd., Jinan University, Zhuhai 519000, China;
³ Laboratory of Biomaterials and Translational Medicine, Center for Nanomedicine, The Third Affiliated Hospital, Sun Yat-sen University, Guangzhou 510630, China

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Vaccines can improve the quality of human life by preventing the burden of infectious diseases. Also, vaccination is becoming a powerful medication for preventing and treating tumors. Various vaccines have been developed based on the origin of the antigens. Herein, we focus on the subunit vaccines whose antigens are proteins or peptides. The advantage of subunit vaccines is safety for recipients; however, the immunogenicity of subunit antigens is relatively low. Nanoparticular delivery systems have been applied to improve the immunocompetence of subunit vaccines by targeting lymph nodes, and effectively present antigens to immune cells. Moreover, adding appropriate molecular adjuvants may strengthen the antigens to elicit immune response. In this perspective article, we first elucidate the characteristics of immunity induced by subunit nanovaccines and then summarize the strategies to fabricate subunit nanovaccines with delivering materials. Herein we highlight non-covalent interaction to fabricate nanoparticular subunit vaccines.

nanovaccine, protein/peptide, subunit, nanoparticle, immunotherapy

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1 Introduction

Vaccination is one of the most successful and cost-effective preventive interventions in human history and can reduce morbidity and mortality. Since the first vaccine came out centuries ago, vaccination has successfully eradicated many epidemic diseases such as polio and smallpox [1,2]. In December 2019, coronavirus disease 2019 (COVID-19) caused by SARS-CoV-2 brought a significant impact on human society. Vaccination is still regarded as the most powerful tool to control the pandemic. Therefore, unprecedented effort has been involved in developing potent vaccines.

Vaccines include inactivated vaccines, live attenuated vaccines, virus-like particle vaccines, nucleic acid vaccines, and protein subunit vaccines. Attenuated and inactivated vaccines are the most commonly used vaccines. Attenuated vaccines, also known as live vaccines, are obtained simply by reducing the virulence of pathogens, or making from natural attenuated strains. The advantages of attenuated vaccines are that they can produce strong humoral and cellular immune responses in a long time and usually require only one immunization. However, these vaccines are environmentally unstable and require cold chain logistics, making the transport of these therapeutic agents complicated and difficult, especially in developing countries. In addition, the attenuated pathogens could be activated under a specific given situation, and this risk is particularly serious in immunocompromised individuals [2]. Relatively, the inactivated vaccines show a good safety profile and are easy to store and transport because of the stability, so there are no
corresponding side effects or adverse reactions after vaccination [3]. However, the effective antigenic determinants of inactivated pathogens may be damaged or changed during the inactivation process and multiple injections are usually required because of the short-lived immune effect [4]. These problems of whole pathogens may be avoided by developing the components of pathogens as antigens. Based on the origin of the antigens, DNA, mRNA, and subunit vaccines have been developed. For DNA and mRNA vaccines, the genetic materials need to be delivered into immune cells and the protein antigens are expressed on sites. The challenge for DNA vaccines is that it needs to cross cell membrane and enter the nucleus to be transcribed into mRNA. While, mRNA, namely messenger RNA, may transiently express antigen proteins in the cytoplasm of cells and avoid across the nuclear membrane. Since mRNA vaccine is highly potent and has a shorter development cycle, it stimulates much attention to expand to health concerns. The first mRNA vaccine has been used in 2020 against COVID-19 and from then on mRNA vaccine becomes a rising star in vaccine community [5]. Its fatal weakness on stability has been overcome to a certain extent by lipid nanoparticles (LNP), a powerful delivery system.

For subunit vaccines, the antigens are natural or recombinant proteins, peptides, and capsular polysaccharides of a pathogen [6,7]. These subunits as an antigen of vaccines are attractive since they would not revert to their virulence form under specific situations and thus can be applicable for most people, even for those immunocompromised individuals. Also, the fabrication of subunits does not involve the alive virus and thus production of vaccines is safe and much easier. However, the immunogenicity of subunit antigens is relatively low due to easy degradation, poor tissue penetration, and difficult cellular uptake, which obstructs the development of subunit vaccines.

Also, with the development of human genome and DNA sequencing, many tumor-specific antigens (TSAs) and tumor-associated antigens (TAAs) are becoming known. However, these antigens usually are difficult for the autoimmune system to produce sufficient specific immune response, thus leading to uncontrolled proliferation of tumor cells and carcinogenesis. Developing tumor vaccines to efficiently elicit cellular immunity is becoming a very promising medication to treat tumors. However, due to the low efficiency of immune response, ambiguous sequence of specific antigen epitopes, high toxicity of adjuvants, and low efficiency of delivery system, the existing tumor therapeutic and preventive vaccines are still difficult to stimulate adequate cellular immune response, hampering the application of vaccines in tumor therapy. With the rapid development of nanotechnology in immunotherapy, researchers have explored various of vaccine carriers to overcome the barriers during delivery and strengthen the efficacy of antigens [2]. Nanodelivery systems can effectively transport the antigens and adjuvants to the target organs and immune cells, and thus may induce potent innate and adaptive immune responses against pathogen infections or tumors. Various types of nanoparticles with different subunit antigens can overcome immune tolerance and reduce the booster dose indispensable for traditional vaccines [8]. Besides, nanovaccines can be designed to induce cellular immunity or humoral immunity preferentially, and can produce lasting immunogenic memory [9]. Therefore, a rationally developed subunit nanovaccine will be the most promising candidate for combating infection and tumors [10].

To fabricate subunit nanovaccines, delivery materials are needed and the interaction between proteins/peptides is critical for performance. Chemical conjugation and physical interaction are commonly applied to fabricate nanovaccines. In this review, we will introduce the advantages of subunit nanovaccines and summarize the non-covalent strategies for fabrication of potent subunit nanovaccines from physical interaction between subunits and delivery materials (Figure 1).

**Figure 1** (Color online) Subunit nanoparticulate vaccines by physical interaction to co-deliver protein/peptide antigens and molecular adjuvants by material vehicles classified by nanonetwork trapping by materials and hollow particle encapsulation.
Immunity eliciting by subunit nanovaccines

Studies have shown that nanovaccines can induce effective humoral and/or cellular immune responses in the body through a variety of mechanisms to fight viruses and tumors [10,11]. Compared with soluble subunit antigens, nanovaccines have obvious advantages for stimulating immunity (Figure 2). First, based on the good stability of the nanocarrier-based delivery system, nanovaccines protect antigens from enzymatic degradation and prevent low molecular weight peptides or molecular adjuvants from entering the blood system, thereby improving bioavailability. Second, nanovaccine delivery systems can co-fabricate subunit proteins with adjuvant molecules and even comprise targeting molecules for antigen presenting cells (APCs), which mimic live pathogens to effectively activate APCs [12,13]. Third, nanovaccines enable antigen uptake by APCs via clathrin-mediated endocytosis and thus facilitate the intracellular delivery of antigens for enhanced antigen presentation [14,15]. Fourth, nanovaccines can escape from the endolysosomal compartment to the cytoplasm for efficient cross-presentation by peptide-major histocompatibility complex (MHC) molecules, thereby inducing CD4+ and CD8+ T cell immune responses to regulate the humoral and cellular immunity which is critical for combating viral infection and tumor immunotherapy [16,17].

Besides above advantages, nanovaccines can change the way to reach lymph nodes (LNs) by controlling their size [18,19]. The kinetics of nanoparticle transport to the lymph nodes largely depends on the size of the nanoparticle (Figure 2). According to reports, nanoparticles smaller than 5 nm are difficult to reach the LNs directly. Instead, they are likely to diffuse into the circulatory system through blood capillaries for clearance [20]. The nanoparticles with a diameter of 20–200 nm may be transported efficiently by drainage lymph to LNs and then are internalized by APCs through endocytosis to induce T cell responses [21]. While, the nanoparticles with a diameter of 0.5–5 µm are mainly internalized by phagocytosis and then less efficiently transported to LNs [22]. Therefore, the particulate vaccines with correct size may reach LNs more effectively, in which sufficient antigens may be released sustainably to abundant immune cells and thus stimulate robust and long-lasting immune responses with minimal injection frequency. Besides the size, the properties such as shape, flexibility and charge can also affect lymphatic drainage [23–25]. Lymph node targeting seems to be a particularly attractive strategy to stimulate a potent immune response, due to the abundant occurrence of APCs in LNs.

When the nanoparticles are transported to the lymphatic

![Figure 2](Color online) Particulate vaccines of different sizes with different transportation and stimulation mechanism.
tissues through the draining lymph, the delivered antigens are recognized by APCs, subsequently subunit antigens are cross-presented by APCs to CD8+ and CD4+ T cells through MHC class I and class II (MHC II), and then the antigen-specific T cell response is promoted through CD80-CD28, CD86-CD28, CD70-CD27 costimulatory signals, which play important roles against intracellular pathogens such as shingles [26], tuberculosis [27] and tumors [28]. Studies have shown that nanovaccines can induce CD4+ T cells and CD8+ T cells produce more antiviral cytokines such as interferon-γ (IFN-γ), tumor necrosis factor α (TNF-α), interleukin-2 (IL-2) [29,30]. In addition to excellent eliciting cellular immunity competence, nanovaccines have a greater advantage in the activation of humoral immunity. In this process, the sustainable released antigens from nanoparticles in APCs are presented to follicular helper T cells, which play a significant role in the germinal center reaction of LNs with antibody affinity maturation [31]. Studies have shown that nanovaccines can co-deliver antigen and adjuvant combined with B cell receptors to activate B cell proliferation and differentiation into plasma cells [32]. A sustained release of antigens from nanoparticles leads to a higher proliferation of B cells, which induce the body to produce more antibodies, hereby promoting the strong antigen-specific protective immunity [33]. However, circulating antibody levels may drop to sub-optimal levels after the initial immunization, which is not enough to resist the pathogens of the second infection. In this case, the importance of immune memory has become prominent. Nanovaccines can trigger a strong long-term memory response. When re-infected, memory B cells will quickly be activated and proliferate into plasma cells, triggering a stronger humoral response to resist foreign pathogens [34,35].

In summary, vaccines based on nanomaterials can enhance immunity in different ways, stimulate various immune cells to secrete immunomodulatory cytokines, and further enhance cellular and humoral immunity, forming a complete activation cascade, which is effective against infectious diseases and tumors.

3 Approaches for preparation of protein/peptide nanovaccines

Since the performance of nanoparticulate vaccines is influenced by transportation and APCs uptake, it is essential to fabricate nanoparticles of subunit and adjuvant regarding particle size, surface property, location and pattern of subunits and adjuvants [19]. Depending upon the delivery materials and properties of subunits and adjuvants, fabrication of nanoparticulate vaccines includes chemical and physical interaction between materials and delivered items.

The subunits include proteins and peptides. Depending on the source, their molecular weights, hierarchical structure, solubility in water, and pl differ significantly. One has to rationally design the strategy and conditions case by case. For molecular adjuvants, they include agonists of toll-like receptors (TLRs) and stimulators of interferon genes (STING), and proinflammatory molecules. Also, their properties vary dramatically. For example, CpG ODN, unmethylated cytosine-guanine oligodeoxynucleotides, is an agonist of TLR9 and it is hydrophilic and negative charged. Imiquimod (IMQ) is an agonist of TLR7/8 and it is a small hydrophobic organic molecule. Monophosphoryl lipid A (MPLA) is an agonist of TLR4 and it has an amphiphilic character. 2’3’-cGAMP for activating STING pathway is a dinucleotide. These molecules are novel adjuvants that have been applied preclinically in the vaccines against various virus infection and tumors. Here, we will introduce several strategies to prepare protein/peptide nanovaccines by regarding delivery materials.

3.1 Formation nanovaccines based on electrostatic interactions

If the pH of solution is above the pl, the proteins or peptides are negatively charged. In this case, a positively charged delivery material, either synthetic or natural macromolecules, at this condition may form multiple electrostatic charge interaction with the proteins/peptides by releasing counterions. The interaction induces phase-separation in nanoscale and as a result, the nanoparticles with proteins/peptides trapped in electrostatic interacted nanonetworks are generated (Figure 1). Such formation of subunits vaccines is simple and has been applied commonly in the laboratory research. The release of subunits may be governed by equilibrium of electrostatic binding, exchange from electrolytes in body fluids, or degradation of materials. In general, such release profiles are sustainable, which is important for antigens to stimulate immune cells.

Polyethyleneimine (PEI) is a cationic synthetic polymer that has been widely studied as a nucleic acid transfection reagent or DNA vaccine delivery vehicle attributing its amine units that may be ionized in water. Thus, PEI has been applied to form nanovaccines with subunits or adjuvants. Guan et al. [36] used PEI as a carrier to deliver the antigen ovalbumin (OVA) and adjuvant (CpG, TLR9 agonist) through electrostatic interaction to prepare a nanovaccine. The positively charged PEI interacted with negative charged OVA and CpG to form the nanovaccine of ca. 200 nm and 22 mV (zeta potential). Moreover, the particulate nanovaccines with positive charged PEI components promoted uptake of OVA and CpG by DCs and facilitated the endosomal escape into the cytoplasm, leading to a significant stimulation on DCs’ maturation. This vaccine further demonstrated high therapeutic efficiency in vivo anti-tumor by combining with hyaluronidase.
Recently, it has been shown that PEI involves strong adjuvant activity, which can enhance the anti-infection and antitumor effects [37]. Thus, PEI may be used as a carrier as well as adjuvants. Xu et al. [38] reported a construction of the personalized tumor vaccine based on electrostatic interaction by using partially fluorinated PEI with OVA for postoperative tumor immunotherapy. The obtained nanovaccines effectively promoted the cytoplasmic transport of antigens in APCs, and significantly enhanced cross-presentation of antigens. Attributing to PEI with agonist function, it is interesting that the vaccine also stimulated the secretion of cytokines by activating TLR4.

Above direct mixing subunits and charge materials may suffer from weaknesses of poor size control during particle formation and also poor stability in body fluid. Introduction of neutral polymer segment to the nanoparticles may fix the problems. Luo et al. [39] applied a cationic polypeptide based on poly(ethylene glycol)-b-poly(L-lysine)-b-poly(L-leucine) (PEG-PLL-PLLeu) as an effective vaccine delivery system. The triblock copolymers may form micelles with PLLLeu core, PLL middle layer, and PEG shell. The negative charged OVA and polyribonucleic acid (polyI:C, TLR3 agonist) could be loaded simply. Attributed to the PEG corona, the nanoparticles by electrostatic charge interaction showed the improved colloidal stability. Zeng et al. [40] developed a polymeric hybrid micelles by co-self-assembly of PEG phosphorethanolamine (PEG-PE) and PEI-stearic acid conjugate via hydrophobic and electrostatic interactions. By changing feed ratio, the composition of hybrid micelles could be tuned simply and may influence in vivo kinetics of vaccines (Figure 3). They encapsulated Trp2 (melanoma antigen peptide) and CpG, yielding nanovaccine of ultra-small size, which significantly induced cytotoxic T lymphocyte (CTL) response and showed a strong anti-tumor effect in the animal model of lung metastatic melanoma.

Charge interaction is very flexible in fabrication of nanovaccines. One may prepare nanovaccines by interaction of polycations and polyanions in presence of subunits. For example, the primary amines of chitosan can be protonated to produce positively charged soluble polymers that can interact with tripolyphosphate (TPP) [41], heparin [42], anti-PDL1 [43], and other negatively charged substances [44] below pH 6.5. Among them, CS forms a crosslinked network with TPP or heparin, which can trap proteins no matter what charges of subunits are. By this way, CS has been applied extensively by crosslinking with TPP to form nanoparticles to load charged proteins like insulin [45]. Similarly, to prepare nanovaccines, Qiao et al. [41] have applied CS and TPP to load subunit VP1 from EV71 and also either CpG or TNF-α to generate two nanovaccines, NVC and NVT. Also, CS and heparin were applied to prepare nanovaccines delivering recombinant hepatitis B virus surface antigen (rHBsAg) or core antigen (rHBcAg) and CpG or IFN-α [42]. In other work, Li et al. [46] applied interaction between PEI and negative charged alginate to form nanogels, which were then further crosslinked by -S-S-. Then the OVA was loaded by electrostatic adsorption. Relatively to the particles without crosslinking, thus obtained one demonstrated more potent production of antigen specific antibody and cellular immunity. It was believed that the reductive -S-S- crosslinks facilitated cytosolic release of antigens, resulting more MHC I type presentation of antigens. This group also used unimolecular nanoparticles with dense cationic branches to deliver CpG by simple charge interaction [47]. Since this vehicle material had a tailored aspect ratio (AR), we have interpreted the AR dependence of LN targeting and immune cell response. It was shown that the AR of ca. 2 led to a better LN retention and colocalization in TLR9. In coadministration with rHBsAg, the vaccination demonstrated obvious AR dependence in virus clearance.

Though it is simple, the electrostatic charge interaction to prepare nanovaccines has fatal weakness. Because of strong interaction, formation of nanoparticles by mixing in a vial is unlikely to be controllable and the size of particles is broadly distributed. Sometimes, macroscopic precipitates are formed. Even one operator for preparation may not reproduce his/her own results. Moreover, it is impossible to make a scalable production. These problems become bottlenecks of technology translation for subunit nanovaccines. Recently, this group and collaborators have applied the flash nanocomplexation (FNC) process to realize a homogeneous mixing of different components in aqueous phase for fabrica-
cating nanovaccines. FNC is a continuous and fast vortex mixing technology, composed of a small mixing chamber with a designed internal structure and several inlets, from which different solutions of delivery materials, subunits, adjuvants and additives are pumped into the mixing chamber.

We used home-built FNC facility to produce high quality and potent subunit vaccines. Four aqueous solutions of chitosan, TPP, VP1 (subunit of EV71) and CpG or TNF-α were injected into the mixing chamber (Figure 4) [41]. As a result, the outlet solution contained the nanoparticles of VP1 and CpG/TNF-α being encapsulated by nanonetworks of CS and TPP via electrostatic charge interaction. The particle size was in range of 90–110 nm and showed much lower polydispersity relatively to the batch mixing of the same components. By this continuous process, the fabrication shows high reproducibility which is very important for production and quality control. Moreover, this process may be scalable for manufacture. Attributed to fast mixing, one device may produce a solution of nanovaccines at least over 100 liters in one day. Thus, FNC fabrication may be used in pharmaceutical farm for production of nanovaccines.

For the performance, the nanovaccines, NVC (loading VP1 and CpG) and NVT (loading VP1 and TNF-α), with narrow size may demonstrate more clearer properties. Attributed to the small size, two nanovaccines demonstrated a better targeting to proximal LNs and even reached distal LNs (Figure 4). They also resided for roughly two days in LNs of mice. The subunits and adjuvants which are easily degraded by enzymes were stabilized by material encapsulation and gradually released in LNs. Moreover, they demonstrated much high cellular internalization by immune cells. Thanks to these characters, the vaccines promoted VP1-specific antibody and induced strong Th1 and Th2 immune responses in mice, corresponding to cellular immunity and humoral immunity. EV71 is the main course of hand, feed, and mouth disease (HFMD) spread in children. To immunize the EV71 infected mice, NVC and NVT demonstrated 100% protection efficacy, as demonstrated by the vaccine pharmaceutics. This study solved the problems of antigen stabilization, size control and biomanufacturing, demonstrating the translational potential of subunit nanovaccines.

Also, this group used FNC to co-load rHBsAg or rHBcAg and CpG through charge complexation between chitosan and heparin [42]. The obtained nanovaccines promoted the maturation and activation of DCs, and produced more pro-inflammatory cytokines IL-1β, IL-6, and IL-12. Furthermore, the nanovaccines of about 70 nm enhance the LNs targeting and retention, which are necessary for inducing strong humoral and cellular immunity. Also the nanovaccines elicited potent antigen-specific antibody in wild BALB/c mice. Excitingly, the nanovaccines broke the immune tolerance in the mouse chronic hepatitis B model and 90% of the mice recovered the hepatitis B virus (HBV) specific immune response. In addition, the vaccine induced long-term immune memory in mice cured from hepatitis B and protected them from virus reinfection.

Apart from the above studies which constructed the nanovaccines by using exterior materials. It is worth noting that nanovaccines can be prepared by direct charge interaction between subunit antigen and charged adjuvants. Shi et al. [48] prepared peptide vaccines by using cell-penetrating peptide conjugated with CD8+ epitope which is positive charged. By mixing with negative charged CpG, the nanovaccines of ca. 200 nm were obtained and here CpG had dual functions, delivery materials and adjuvant to DCs activation. Such vaccines demonstrated LN targeting and robust cellular

Figure 4  (Color online) Fabrication of nanovaccines co-delivery VP1 subunits and TNF-α or CpG by using FNC which is scalable and highly batch-to-batch reproducibility. The nanovaccines of small size target LNs and elicit strong cellular and humoral immune response [41]. Reprinted from [41] Copyright 2018 with permission from American Chemical Society.
immunity for treating melanoma tumors.

3.2 Preparation of nanovaccines based on hydrogen bonds and hydrophobic interaction

Hydrogen bonding is a weak physical interaction, and it plays a critical role in shaping protein hierarchical structure. Although single one is weak, multiple hydrogen-bonding interaction becomes strong enough to capture different molecular species into one particle. Tannic acid (TA), a polyphenol from plants, contains many galloyl groups that form many hydrogen bonds with various bioactive molecules using its hydroxy group as a hydrogen bonding donor. Besides hydrogen bonding, its phenol OH, a weak acid, may also form ionic interaction with a weak base. Moreover, its phenyl may form hydrophobic interaction with the phenyl groups of other molecules. Therefore, TA is a natural material that has multiple physical interaction and may bind guest molecules universally. These physical interactions are reversible and thus TA complex allows a sustainable release. Also, this material is biocompatible and has been approved in application of skin and oral administration by FDA. Thus, TA has shown very promising application in delivery of various drugs. For example, TA interacts with some chemical drugs like paclitaxel and doxorubicin to form drug nanoparticles by hydrogen bonding [49,50]. It may also allow the hydrogen interaction with nucleic acids and it was confirmed that the interaction occurred between TA phenol and phosphodiester bonds of DNA backbones [51]. For proteins and peptides, the phenol OH interacts with carbonyl -C(=O)NH of peptide linkage and OH groups of serine in the structures [52]. TA may interact with amine group of lysine and phenyl of phenyl alanine, respectively (Figure 5). This property has been applied to deliver peptide drugs by this group [53–55]. Also it has been applied recently to prepare microsized vaccines by mixing of TA, poly(N-vinylpyrrolidone) (PVP), and OVA and hydrogel interaction between three components drove to form particulate physical networks and it was applied to prepare [56]. Herein, PVP as a hydrogen bond acceptor interacts with TA forming networks, wherein OVA was also trapped by multiple physical interaction.

This group has developed this strategy to use the unique properties of TA for preparation of subunit nanovaccines (Figure 6) [57]. The antigen is the EBNA1, a protein expressed in all EBV-associated tumors. We simply mixed aqueous solutions of TA, EBNA1, CpG or IFN-α (as adjuvants), and also PF127 using the FNC facility. The nanovaccines of 100 nm size with a low size distribution were prepared efficiently. Herein, the hydrogel bonding as well as ionic and hydrophobic interactions between TA and EBNA1, also TA and CpG or IFN-α drove the formation of nanoparticles. At the meantime, the hydrogen bonding between TA phenols and PF127 ether units efforted a PEG corona to the nanovaccines for improving pharmokinetics. The loading efficiency of antigen and adjuvant was 91% and 99%, re-
spectively. Uniform and small nanovaccines demonstrated efficient targeting LNs and site retention thereof. This simple nanovaccines elicited strong responses of humoral and cellular immunity in animals, inhibiting tumorigenesis. Moreover, the vaccines broke tolerance of the immune system by decrease of infiltration of regulatory T cells to the tumor lesion to remodel the tumor microenvironment. Thus, by combining with anti-PD-L1 this vaccine reversed the resistance of immune checkpoint inhibitor and demonstrated remarkable decrease in tumor size and prolonged survival of animal model. Attributed to use of TA and FNC fabrication, present nanovaccines may have promising application in treating EBV-associated tumors.

In above examples, we chose the literature reports with a clear interaction mechanism. In other cases, multiple interactions, including electrostatic charge interaction, hydrophobic interaction, metal coordination, and pi-pi interaction have been involved in the design of subunit vaccines. These vaccines have been studied in the prevention and treatment of infectious diseases and tumor treatment [58–61]. These nanovaccines are developed in order to obtain the functions of protection of antigens and adjuvants, effective transportation to LNs, high uptake by APCs, adjustable antigen release and effective cross-presentation, result a potent and controllable immune response.

### 3.3 Formation of nanovaccines by physical encapsulation into hollow particles

Hollow nanoparticles that have hydrophilic internal cavities may deliver hydrophilic biomacromolecules by physical encapsulation without changing the structure of guest molecules. This way has been applied extensively in delivering protein drugs and nucleic acids. The hollow particles are normally easily generated by self-assembly of lipids or amphiphilic copolymers and double emulsion of hydrophobic polymers with the aids of surfactants. Subunits of proteins/peptide and some adjuvants like CpG, poly I:C, and cytokines are hydrophilic and thus are suitable by encapsulation within these hollow nanomaterials. The release of contents is highly depended by the diffusion of molecules to cross the materials or degradation of materials.

Vesicles by molecular self-assembly have a double layer, which has a uniform thickness from few to ten nanometers depending upon molecular weights. Lipids have a defined molecular structure and the risk of materials in application to deliver drugs may be low. Common lipids, like 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), may be applied to generate vesicles, also called liposomes. Since lipids are small molecules, liposomes have a very thin membrane and become vehicles of vaccines [62]. Recently, we applied liposomes of DOTAP and DOPE to load spike proteins, the subunits of SARS-CoV-2 [59]. To strengthen the potency, two adjuvants, MPLA (agonist of TLR4, co-assembled with liposome) and CpG (agonist of TLR9, encapsulated in hollow cavity), were also applied. Such nanovaccine elicited a humoral immune response and strong IgA antibodies in mice.

However, liposomes are easily destroyed during body traffic and show risk of leakage before arriving targets. As a contrast, polymeric vesicles, also named polymersomes, are much more stable during trafficking [63]. Very recently, polybutadiene-b-PEG self-assembled into polymersomes with diameters of 100–200 nm and thickness of 10 nm. The spike protein 1 of SARS-CoV-2 and CpG was incorporated during vesicle formation. The size of vesicular vaccines may be decreased and uniformed by extruding with porous membrane [64]. Such nanovaccines demonstrated neutralizing titers in mice that persisted for at least 40 days and elicited of memory CD4+ and CD8+ T cells that produced Th1 cytokines. By this way of fabrication, it is convenient to modify the surface charge property by introducing lipids, like DOTAP, during self-assembling.

However, the solutes are partitioned inside as well as outside of liposomes or polymersomes, and thus the outside free one needs to be removed during vaccine preparation before administration. In terms of release, the hydrophilic subunits and/or adjuvants need to penetrate through the hydrophobic membranes, which is unfavorable in thermodynamics. It may be easier for liposomes because the membrane is very thin and could be easily disrupted during transportation in the body. But the polymersomes have a thicker membrane and it could be a difficult task only by penetration.

Another common strategy of hollow particulation is, with the aid of surfactants, to firstly form water droplets containing solutes in organic solvent with delivered materials (water in oil, w/o), and secondly to redisperse the oil phase in water (w/o/w). This is so-called double emulsion approach, widely applied to prepare hollow particles to load various hydrophobic drugs. This approach is simple and specifically suitable for large hollow particles. Biogradable polymers, like FDA approved poly(lactide-co-glycolide) (PLGA), are mostly applied for this case. Demento et al. [65] prepared particulate vaccines by double emulsion loading PLGA to load OVA. The particle size was around 240 nm. It is very informative that the lysosomes of phosphatidylcholine (PC) were applied for comparison in terms of delivery materials. Attributed to a much slower release profile, the PLGA vaccines elicited more robust cellular immune response and a higher frequency of effector-like memory T cell phenotype (Figure 7). Such a system also may be easily functionalized by further loading agonist adjuvants and introducing active targeting immune cells [66]. Hydrophobic imiquimod (IMQ, TLR7/8 agonist) was loaded into PLGA domains of double...
emulsion vaccines, meanwhile the amphiphilic MPLA (TLR4 agonist) was incorporated along the particle surface. Multifunctionalized vaccines may be tuned programmably for tumor immunotherapy. In a similar work, Chen et al. [67] used PLGA to encapsulate indocyanine green (ICG, photothermal agent) and imiquimod (R837, TLR7 agonist) by oil-in-water (o/w) emulsion method to form PLGA-ICG-R837 nanoparticles of ca. 100 nm. PLGA-ICG-R837 nanoparticles can be used for the near-infrared laser to trigger photothermal ablation of tumors, releasing tumor-associated antigens and combining with the adjuvant R837 to show a vaccine-like function. By adding PEG-b-PLGA during the second emulsion, a PEG corona can be introduced to the surface of vaccines to improve retention by escaping recognition of the reticuloendothelial system (RES) [68].

Though there are many pieces of research, the release kinetics of bioactive molecules from these polymeric hollow particles by double emulsion still lack a depth understanding. It could be difficult for the hydrophilic biomacromolecules to penetrate from the hydrophilic internal cavities through the hydrophobic wall to the outside. Many reports vaguely claimed that a gradual degradation of materials may allow a sustained release. Actually even for the easiest degradable PLGA (L:G=50:50), its nanoparticle (ca. 190 nm) still needs more than 100 days to degrade completely [69], which is much longer than the clearance time. Therefore, there still needs a precise understanding between degradation rate, release profile, and window of immune stimulation.

4 Summary and prospect

Sudden and highly pathogenic viruses are always a long-term threat to human beings. The outbreak of SARS-CoV-2 at the end of 2019 has changed the world so greatly and it is still unclear how long it can last. Some vaccines, including subunit ones, against COVID-19 have been shotted during a large ratio of the world population. However, the performance of these vaccines is still far from satisfaction. Therefore, the development of safe and efficient vaccine technology for virus infection and treating tumor always is an urgent task. The accumulated research has shown that nanoparticulate vaccines combining molecular adjuvants may significantly improve the immunogenicity of subunits, revealing very promising application. This paper highlights the physical approach to generate the nanovaccines by using weak intermolecular physical interaction between functional species and delivery materials as well as encapsulation within the hollow nanoparticles. By the ways, no chemical modification of protein or peptide antigens is involved, thus they are simple for formulation fabrication and more feasible for application. It could be more suitable for vaccination to large population against virus spreading.

Since delivery materials are involved, the choice of safe yet functional materials becomes a critical issue. The materials of small molecules have a defined molecular structure and their quality control during production is much easier. They are also easily cleared away from body via kidney in a short time. In general, these materials are lipids or analogues. They have formulated in the mRNA vaccines against COVID-19. In the so-called LNP as the delivery system of mRNA, ionizable lipids and helper lipids have been applied and they have been injected to the population for more than thousand million. So far, these lipid materials are safe in such a large population. However, the rapid clearance also becomes the weakness of this system as some vaccines need a relatively longer retention.

The synthetic materials like PLGA that have been approved in clinical application for implantation and delivery of cancer drugs. Regarding the tiny amount vaccines of ad-
ministration, the safety of materials should not be a big concern. Although so far there is no application in large population for synthetic macromolecules, their application in tumor vaccines is predictable in near future. To decrease the risk of materials, a better understanding on material degradation and antigen release in vaccination needs to be explored. For new synthetic materials, the research on tailoring materials structure in order to show better targeting to LNPs and defined release pathway in immune cells is worthwhile and the application may be more focused on tumor immune therapy. In terms of the natural occurred materials, like CS, TA, heparin, alginate and even protamine, albumin, and ferritin, in general they are safe delivery materials for vaccines. The challenges are their uncontrolled interaction to subunits and adjuvants attributing to their complex structure as well as purity and quality control regarding the immunogenetic impurities.

We need to emphasize the delivery materials from natural resources and the FNC process. As mentioned above, these materials are hydrophilic and charged and they are suitable to load proteins and some adjuvants by trapping into nanonet-works through physical interaction. However, it was painful since the formation of particulate vaccines by batch mixing is poorly controlled in size and composition, dragging technology translation. Instead, by continuous and vortex mixing with the FNC, the formation of particles becomes kinetically controlled and thus the quality of particles may be improved greatly. One may learn that the LNPs of mRNA vaccines from Moderna and BioNtech were produced by a two-inlet mixing technology. In this case, some ingredients must be mixed in advance to give two stock solutions, leading to complexity and uncertainty. In contrast, the FNC with multiple inlets allows a direct mixing of several individual solutions. Combining the scalable character and high batch-to-batch reproducibility, the FNC is a powerful technique not only for subunit vaccines but also for nucleic acid vaccines.

Subunit vaccines are showing more and more applications. In response to the current coronavirus disease (Coronavirus disease 2019, COVID-19) pandemic, many subunit vaccines have been on the pipelines of development. According to the data compiled by WHO, as of 4 January 2022, there are 47 subunit vaccines in clinical trials worldwide, taking 35% among all the vaccines for COVID-19 [70]. These subunit vaccines are mainly the recombinant SARS-CoV-2 spike protein or receptor binding domain (RBD) protein combining with adjuvants, and few are the dimer or trimer of subunits. Because of emergent application, these subunit vaccines against COVID-19 have not been formulated as particles. However, one may find some nanoparticulated subunit vaccines on the list of those pre-clinical trials, demonstrating their great potential.

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