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Design and application of nanoparticles as vaccine adjuvants against human corona virus infection

Lichun Mao [a,b], Ziwei Chen [a,b], Yaling Wang [a,c,*], Chunying Chen [a,b,c,d,*]

[a] CAS Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety, CAS Center for Excellence in Nanoscience, National Center for Nanoscience and Technology, Beijing 100190, PR China
[b] University of Chinese Academy of Sciences, Beijing 100049, PR China
[c] GBA National Institute for Nanotechnology Innovation, Guangdong 510700, PR China
[d] Research Unit of Nanoscience and Technology, Chinese Academy of Medical Sciences, Beijing 100021, PR China

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ABSTRACT

In recent years, some viruses have caused a grave crisis to global public health, especially the human coronavirus. A truly effective vaccine is therefore urgently needed. Vaccines should generally have two features: delivering antigens and modulating immunity. Adjuvants have an unshakable position in the battle against the virus. In addition to the perennial use of aluminium adjuvant, nanoparticles have become the developing adjuvant candidates due to their unique properties. Here we introduce several typical nanoparticles and their antivirus vaccine adjuvant applications. Finally, for the combating of the coronavirus, we propose several design points, hoping to provide ideas for the development of personalized vaccines and adjuvants and accelerate the clinical application of adjuvants.

1. Introduction

The coronavirus (CoV) pandemic has caused 116,135,492 infections and 2,581,976 deaths worldwide as of March 7, 2021. In 2019, a novel virus named SARS-CoV-2 caused a global pandemic; this virus shares approximately 80% of its genome with SARS-CoV [1–3]. The early symptoms of SARS-CoV-2 infection are severe acute respiratory infection and other serious complications [4]. This can be partly attributed to the high expression of viral receptors in the medullary respiratory center of the brain [5]. Some infected people do not show obvious symptoms, increasing the difficulties in monitoring the disease and preventing and controlling the epidemic [6].

CoVs are divided into α- and β-coronaviruses, which infect mammals, and γ- and δ-types, which primarily infect birds [7]. Seven strains of human coronaviruses are identified, namely, HCoV-OC43, HCoV-HKU1, HCoV-229E and HCoV-NL63, which are mild-symptom strains, and MERS-CoV, SARS-CoV and SARS-CoV-2, which are severe-symptoms strains. CoVs are positive-sense RNA viruses with a single strand and pleomorphic or spherical shapes [8]. Fig. 1 shows the morphology and diagram of the SARS-CoV-2 and its replication cycle [9–11]. For humans, these viruses cause problems ranging from a mild colds to lethal respiratory tract infections [9]. Currently, there are only several licensed vaccines that prevent the SARS-CoV-2 infection. Numerous antiviral targets have been identified, and plenty of vaccines using different producing methods are still in development [12–16]. SARS-CoV and SARS-CoV-2 infect the human body by binding to a surface

Abbreviations: Ad5, adenoviral type 5; ACE, angiotensin-converting enzyme; APCs, antigen-presenting cells; CaP, calcium phosphate; CD, cluster of differentiation; cdGMP, cyclic diguanylate monophosphate; CpG-B, class B cytosine-guanine; CpG, cytosine-guanine; CoV, coronavirus; DENV, dengue virus; dLN, distal lymph nodes; ds, double-stranded; EV71, enterovirus 71; EDIII, envelope glycoprotein domain III; FNC, flash nano-complexation; GNPs/AuNPs, gold nanoparticles; HEVA, hepatitis E vaccine; HIV, human immunodeficiency virus; Ig, immunoglobulin; IFN, interferon; IL, interleukin; MHC, major histocompatibility complex; MSNs, mesoporous silica nanoparticles; MSRs, mesoporous silica rods; MPLA, monophosphoryl lipid A; NLRP3, nucleotide-binding oligomerization domain-like receptor protein 3; ODN, oligodeoxynucleotide; PAPE, particulate alum via Pickering emulsion; PD, phosphodiester; PEI, poly(ethyleneimine); pLN, proximal lymph nodes; RBD, receptor-binding domain; RSV, respiratory syncytial virus; RM, reverse microemulsion; S protein, spike protein; Th1, T helper type-1; Th2, T helper type-2; TLR, toll-like receptor; TNF, tumor necrosis factor; UV, ultraviolet.

* Corresponding authors at: CAS Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety, CAS Center for Excellence in Nanoscience, National Center for Nanoscience and Technology, Beijing 100190, PR China.

E-mail addresses: wangyl@nanoctr.cn (Y. Wang), chenchy@nanoctr.cn (C. Chen).

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receptor named angiotensin-converting enzyme (ACE) 2 through the spike protein (S protein) of the viruses [17,18]. To speed the vaccine development process, researchers have developed diverse vaccine candidates targeting S protein as main antigen. Six main vaccine platforms, including inactivated viruses, protein subunit vaccines, non-replicating viral vectors, replicating viral vectors, mRNA vaccines and DNA vaccines are developed to prevent viral infections. Despite the initial stage success of the vaccine candidates, some inactivated and subunit vaccines are poorly immunogenic, and adjuvants are needed to accelerate the vaccine application process, boost immune effectiveness and reduce the antigen dosages. Adjuvants reduce the required antigen dose and immune frequency by accelerating, expanding, amplifying and prolonging the required immune responses.

2. Nanoparticles as vaccine adjuvants in antiviral infection

According to the immune response mechanism, an effective vaccine should contain antigens, immune enhancers (also known as adjuvants) or/and delivery systems [19]. The mechanism of action of an antiviral vaccine is shown in Fig. 2 [20]. Adjuvants are pharmacological or immunological agents used to activate the innate immune system, enabling the innate immune system to respond rapidly to infections and stimulating adaptive immune responses that are specific to viral infections. Adjuvants are effective in stabilising antigens conformation, facilitating antigens to target the antigen-presenting cells (APCs), directing antigens presentation and stimulating and enhancing T helper type-1 (Th1) or T helper type-2 (Th2) immune responses. However, few adjuvants are licensed for human vaccines, as shown in Fig. 3 [21–25]. The limited availability of adjuvants has hindered antivirus vaccine development.

Over the years, nanoparticles have attracted attention and have been developed as adjuvants against various viruses. Nanoparticle the structure can be controlled to become a storehouse for loading antigens, to prevent degradation and to prolong the antigen exposure. The distinct sizes and morphology of nanoparticles enable them to selectively induce different types of immune responses and deliver the antigens to specific sites. Through the precise regulation of physical and chemical properties, the immune impact of nanoparticles can be modulated. The two main categories of adjuvants are inorganic and organic adjuvants. Some typical adjuvants have potential uses in antiviral vaccines development.

2.1. Inorganic adjuvants

2.1.1. Aluminium-containing adjuvants

As the first human adjuvant approved by the Food and Drug Administration, aluminium adjuvant is known as the most widely used adjuvant worldwide, having critical importance to mass vaccination programs. Aluminium adjuvants slowly and continuously release antigens, which are processed and presented to T cells as a major histocompatibility complex (MHC) antigen complexes. In terms of immunity, aluminium salt has unique characteristics; for example, aluminium salt-induced immunoglobulin (Ig)E requires interleukin (IL)-4 [26], whereas aluminium adjuvants do not require IL-1, IL-18, or myeloid differentiation primary-response gene 88 [25]. On the one hand, insoluble aluminium salts induce the subpopulations of cluster of differentiation $(CD)4^+$ cells into Th2-like cells [27]. On the other hand, alum has anti-inflammatory properties. The transcription and secretion of IL-10 are strongly enhanced by alum through macrophages and dendritic cells, and such enhancements weaken antigen-specific Th1 responses after vaccination [28]. Moreover, aluminium itself might be antigenic and enhances antigen presentation by APCs (Fig. 4a) [22,29].

Aluminium has been used as a safe adjuvant against viral infections in human beings. An inactivated alum-adjuvant human enterovirus 71 (EV71) vaccine efficacy is 80.4% in treating EV71-related diseases [32]. In the other experiments, a formalin-inactivated Zika virus vaccine candidate with aluminium hydroxide adjuvant was used. The same as the earlier findings, the initial human safety and immunogenicity results showed that the vaccine has mild or moderate adverse effects, such as fatigue (43%) and headache (39%) [33]. In coronavirus treatment, aluminium has played a role. With the aid of aluminium hydroxide gel, an ultraviolet (UV)-inactivated SARS-CoV virion boosted the production of serum IgG to a level similar to that in a hyper-immunised mouse [34]. Different SARS-CoV vaccines with or without alum adjuvants were

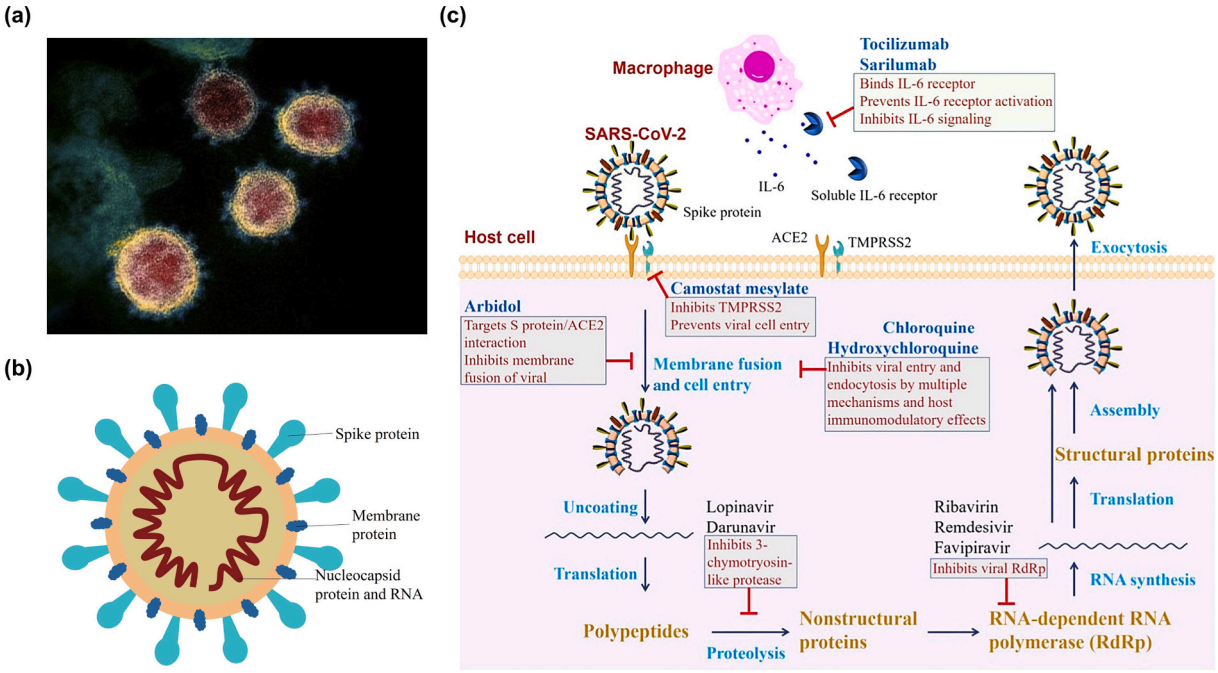

Fig. 1. Morphology and the replication cycle of SARS-CoV-2. (a) Electron micrograph of the SARS-CoV-2. Adapted with permission from ref. [10], copyright © 2020 IMSS. Published by Elsevier Inc. (b) Schematic diagram of the SARS-CoV-2. (c) Schematic representation of the replication cycle of SARS-CoV-2. Adapted with permission from ref. [11], copyright © 2020 Chinese Chemical Society.

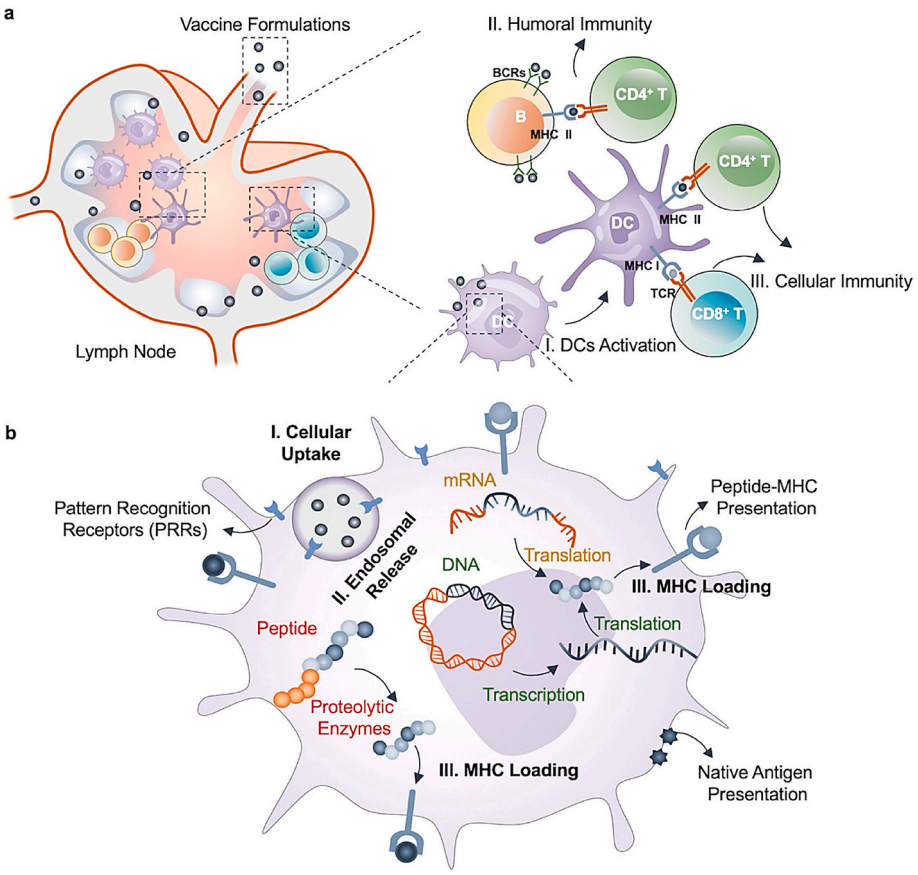

Fig. 2. Schematically illustrate the action mechanism of the antiviral vaccine. Adapted with permission from ref. [20], copyright © 2020 American Chemical Society.

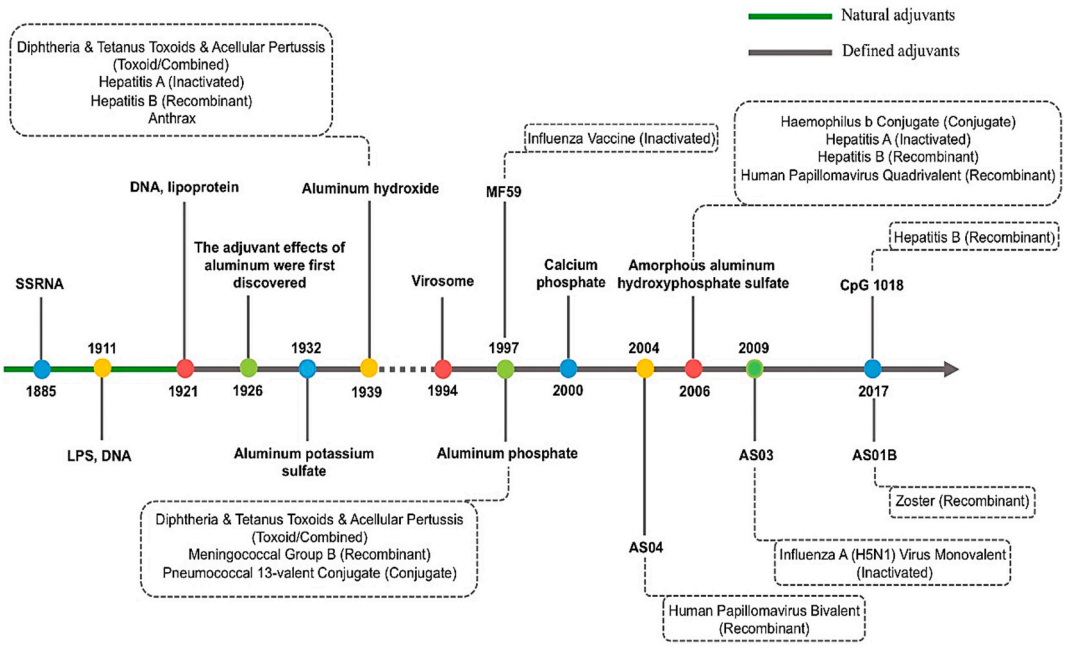

Fig. 3. Timeline of vaccine adjuvants discovery. Adapted with permission from ref. [24], copyright © 2019 Elsevier Ltd.

evaluated using a mouse model. Groups that received double-inactivated (formalin and UV) and S protein alum-adjuvanted vaccines had lower mean percent eosinophils than the corresponding non-adjuvanted group. Although these SARS-CoV vaccines induced antibodies, Th2-type immunopathology was observed in the mice that received the vaccines [35]. Given that Th1 immune responses cannot be strongly induced by alum alone, a combination of alum and other adjuvants can be utilised in enhancing immunogenicity [36]. For instance, glucopyranosyl lipid A was combined with alum to improve the efficacy of a SARS-CoV receptor-binding domain (RBD) protein-based subunit

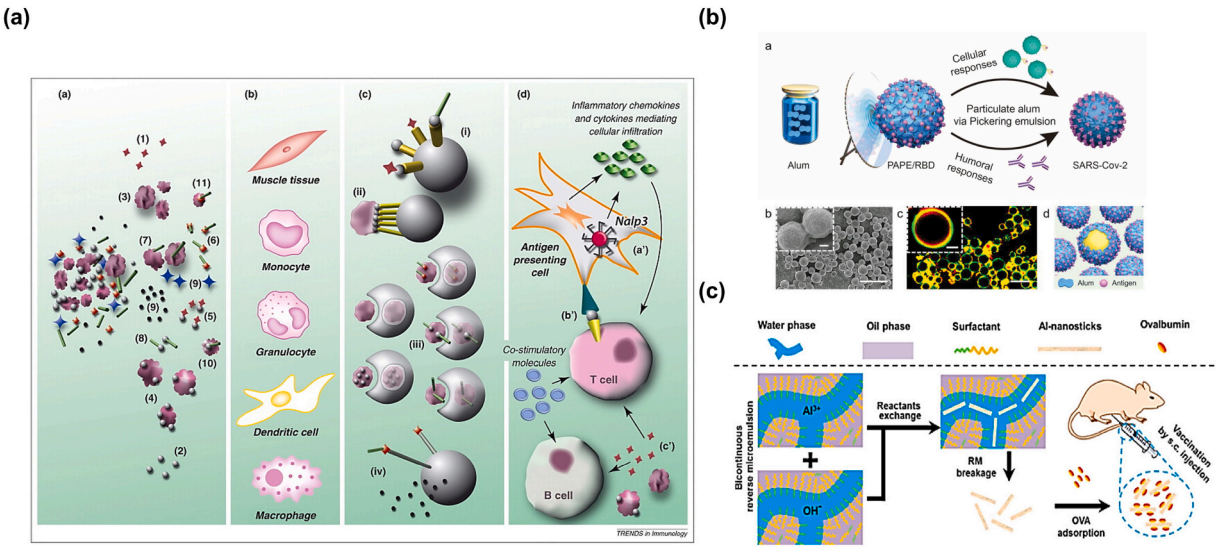

Fig. 4. (a) Schematic illustration of aluminium adjuvant induced innate and adaptive immunity. Adapted with permission from ref. [22], copyright © 2010 Elsevier Ltd. (b) Schematic of particulate alum via Pickering emulsion (PAPE) as a COVID-19 vaccine adjuvant. Scanning electron microscope image of PAPE. Scale bars: 10 μm (inset: 200 nm). Confocal image and schematic illustration of antigen-adsorbed PAPE. Green: antigen, red: the surface alum, Scale bars:10 μm (inset: 1 μm). Adapted with permission from ref. [30], copyright © 2020 Wiley-VCH GmbH. (c) Schematic diagram of the synthesis of the stick-like aluminium (oxy)hydroxide nanoparticles (Al-nanosticks) in a bicontinuous reverse microemulsion (RM). Adapted with permission from ref. [31], copyright © 2017 American Chemical Society.

vaccine [37]. Similarly, a vaccine containing recombinant MERS-CoV RBD, alum and cytosine-guanine (CpG) oligodeoxynucleotide (ODN) optimized RBD-specific humoral and cellular immunity [38]. Aluminium is widely used in the design of drugs or vaccines against SARS-CoV-2. The addition of aluminium adjuvants significantly enhances the production of neutralising antibodies after treatment with RBD recombinant vaccines and SARS-CoV-2 inactivated virus. A SARS-CoV-2 inactivated candidate vaccine containing an aluminium hydroxide adjuvant has reached pilot-scale production and has been used in clinical phase I and phase II trials [39]. Aluminium is expected to be used in the mass production of vaccines.

In addition to the protection provided by antigens, the efficacy and safety of vaccines are strongly affected by the physical and chemical properties of alum adjuvants, the ratio of antigen to adjuvants, the route of administration and other factors. Researchers used nano-alumina as a coating structure to encapsulate EV71, which not only overcomes the size barrier but also simultaneously improves the thermostability and immunogenicity of the vaccine [40]. Aluminium particles as stabilisers were adsorbed on the surfaces of the emulsion and form a PAPE structures, which significantly enhanced the adjuvant activity. PAPE induced a remarkable antibody titer, which was six times that of experimental groups with aluminium alone and stimulated Th1 and Th2 immunity (Fig. 4b) [30]. Xu et al. [31] synthesised stick-like monodisperse aluminium (oxy)hydroxide nanoparticles, which were easily adsorbed and delivered antigens to antigen-presenting cells in a mouse model mainly because of the unique stick-like shapes and nanoscale sizes of the Al-nanosticks, as shown in Fig. 4c. Apart from shape, size is another critical factor for immunogenicity. The weak ability of traditional alumina adjuvants to potentiate cellular immune response may be prevented by reducing their particle sizes into nanometers scale, rather than using traditional microparticles [41]. The possible reason is that nanoscale aluminium salt particles are easily internalised by antigen-presenting cells for the activation of the NLRP3 (nucleotide-binding oligomerization domain-like receptor protein 3) inflammasome [42].

The safety and immune mechanism of aluminium adjuvants should be fully investigated for the improvement of their application. The lowest dose (200 μg Al/kg) of aluminium adjuvant was neurotoxic in mice rather than the higher doses (400 and 800 μg Al/kg), which was not confirming to the dose depend-response profile [43]. Furthermore, the acute exposure and chronic retention of aluminium in vaccines need to be concerned, which can be achieved through modelling [44].

2.1.2. Calcium phosphate

Biocompatible calcium phosphate (CaP) nanoparticles are promising as adjuvants that induce the balanced immune responses of Th1 and Th2 and maybe used as a potential dose-sparing strategy [45–48]. CaP can act as a carrier to activate Th1-type immune response (Fig. 5a-b) [47]. As carriers, CaP nanoparticles can load antigens at different time points through encapsulation during the particle formation or passive adsorption after particle formation [45]. Compared with soluble Toll-like receptor (TLR) ligands, TLR ligand and viral antigen functionalised CaP nanoparticles are effective in inducing the efficient maturation of human APCs [49], as indicated by increase in the levels of co-stimulating molecules and the secretion of proinflammatory cytokines, which cause the remarkable expansion of virus-specific T cells. Another similar design confirmed this conclusion in mice [50]. After the intramuscular injection of nanoparticles, mouse hepatitis B surface antigen expression, antigen-specific T cell response and antigen-specific antibody response (IgG1) in mice were all enhanced (Fig. 5e) [50]. Calcium phosphate has other advantages as a vaccine carrier. Owing to its unique pH-dependent solubility profile, calcium phosphate can control the release of the loaded drugs, such as plasmid DNA (Fig. 5c-d) [51].

The particle size and route of administration of a vaccine adjuvant play important roles in postvaccination antibody response. The nanoscale CaP adjuvant (73 nm) encapsulating human EV71 vaccine displays a higher antibody level than the micro-size or unabsorbed vaccine alone. Meanwhile, the antibody level of the intradermal route is ten times that of the intramuscular route, which is safer and more cost effective [52].

2.1.3. Gold nanoparticles

Gold nanoparticles (GNPs or AuNPs) are extremely useful in drug delivery because of their unparalleled properties, such as stability, low-toxicity, and non-immunogenicity. The sizes of these gold nanoparticles typically range from 10 nm to 100 nm. Gold nanoparticles can stimulate various immune cells and deliver pro-inflammatory cytokines (i.e., IL-1β and tumor necrosis factor [TNF]-α) and Th1 cytokines (IFN-γ and IL-2) to the human body [53]. Owing to these characteristics, GNPs can be used as carriers and adjuvants for viral therapy. The size of a GNP-based

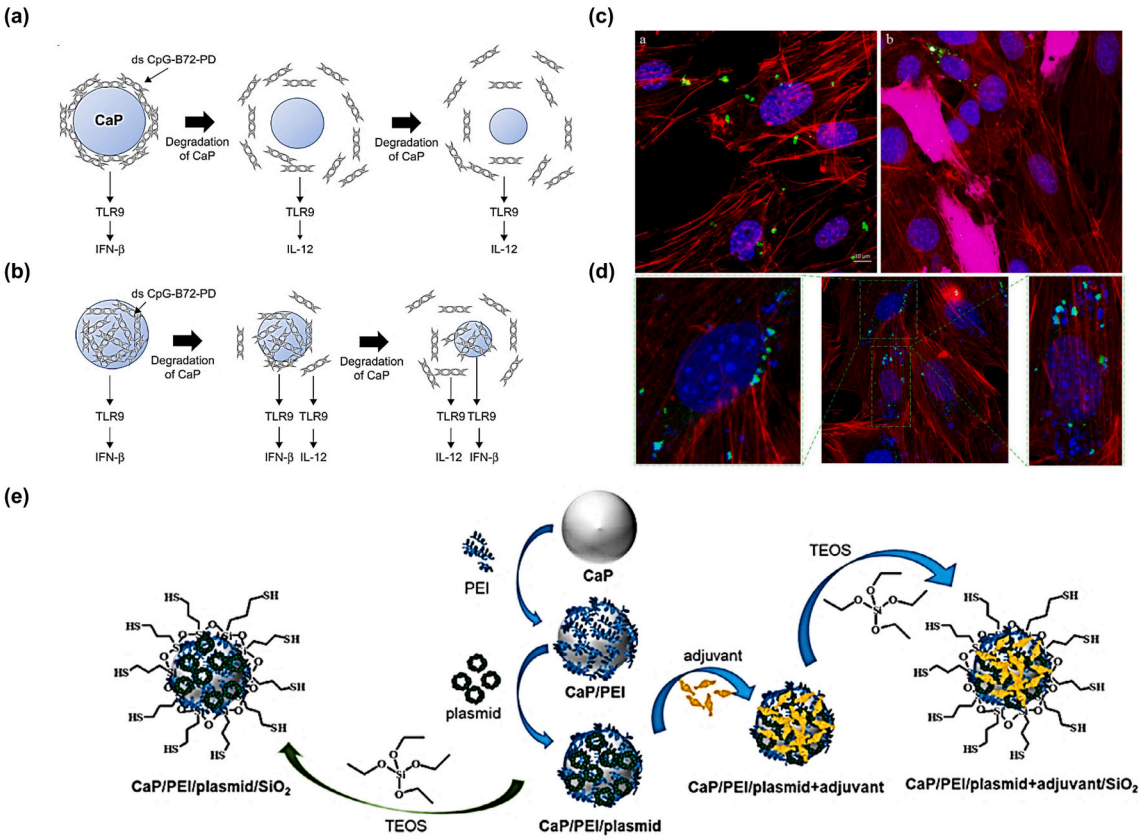

Fig. 5. (a) (b) Proposed model of CaP degradation and ODN release. ds, double-stranded; CpG—B, class B cytosine-guanine; PD, phosphodiester; IFN, interferon. Adapted with permission from ref. [47], copyright © Dove Medical Press Limited. (c) Confocal optical images of MC3T3-E1 cells treated with CaP-pDNA nanoparticle (left) and MC3T3-E1 cells transfected with enhanced green fluorescent protein (right). Green: CaP-pDNA particles. (d) Confocal optical images showing the CaP vector (green) delivered the enhanced green fluorescent protein plasmid (blue) to the MC3T3-E1 nucleus (blue) through the cytoplasm (red f-actin cytoskeleton filaments). Adapted with permission from ref. [51], copyright © 2016 Elsevier Inc. (e) Schematic presentation of the synthesis process for CaP/PEI/plasmid/SiO_2 and CaP/PEI/plasmid + adjuvant/SiO_2 nanoparticles. PEI: poly(ethyleneimine). Adapted with permission from ref. [50], copyright ©2020 Acta Materialia Inc. Published by Elsevier Ltd.

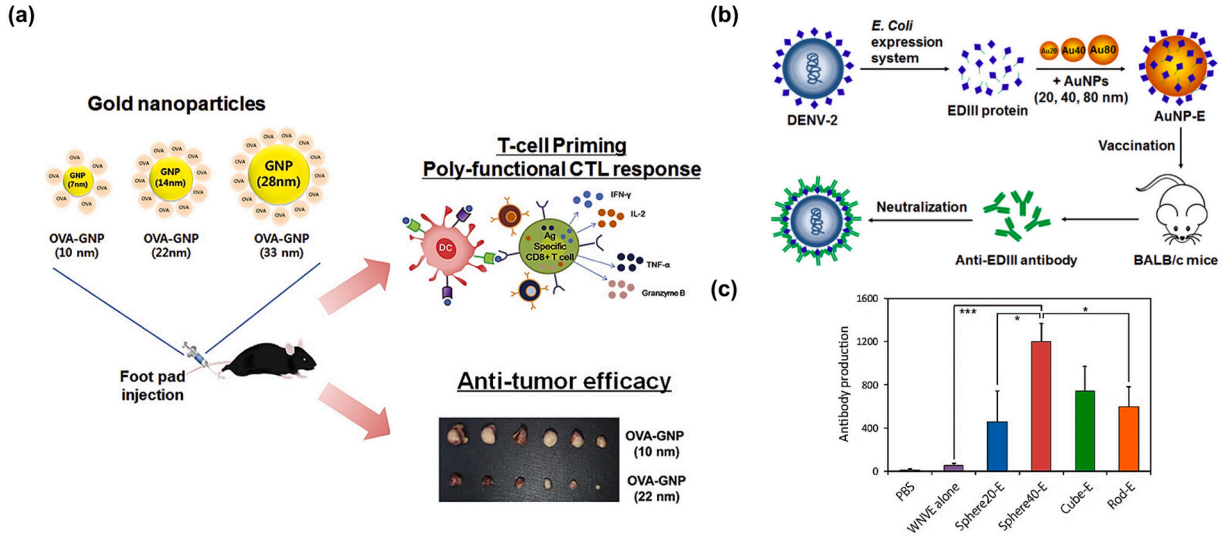

Fig. 6. (a) The effects of the size of GNP-based vaccines on their efficiency of delivery to lymph nodes and induction of $CD8^+$ T-cell responses. Adapted with permission from ref. [54], copyright © 2017 Elsevier B.V. (b) Purified envelope glycoprotein domain III (EDIII) formed a protein corona around gold nanoparticles of three sizes. These complexes (AuNP-E) were then used to induce the production of anti-EDIII antibody which neutralized dengue virus (DENV)-2. Adapted with permission from ref. [55], copyright © 2018 Acta Materialia Inc. (c) Gold nanoparticles induce mice to produce West Nile virus antibodies. Adapted with permission from ref. [56], copyright © 2013 American Chemical Society.

vaccine is an important factor for immune efficiency. The size threshold for the induction of valid cellular and T-cell responses by GNPs lies between 10 and 22 nm (Fig. 6a) [54]. A dengue subunit vaccine was made from a dengue virus enveloped by glycoprotein and gold nanoparticles. The antibody levels induced by this vaccine can be adjusted according to the sizes and concentrations of GNPs (Fig. 6 b) [55]. Spherical (diameters of 20 nm and 40 nm), rod-shaped (40 nm × 10 nm) and cubic (40 nm × 40 nm × 40 nm) GNPs as adjuvants enhance immune response of the West Nile virus vaccine through different cytokine pathways according to GNP size and shape, as shown in Fig. 6c [56].

GNPs boost the immune system. Through simple surface chemical modification (Fig. 7), the transfection ability of the gold rod was improved, and the cellular and humoral immunity were significantly promoted [57]. GNP-adjuvanted S protein can induce antigen-specific IgG response but cannot induce protective antibodies and reduce eosinophilic infiltration in the lungs (Fig. 8a-b) [58]. After different combinations of adjuvants, synergistic enhancement in immune stimulation is permissible and low-dose is allowed. In a layer-by-layer approach, spiky gold nanoparticles were synthesised. Spiky gold nanoparticles delivered polyinosinic-polycytidylic acid and CpG to promote the release of cytokine (IL-6, TNF-α) and the upregulation of co-stimulatory markers (CD40, CD80 and CD86) [59]. GNPs are limited as carriers, and thus appropriate adjuvants development are needed.

As vaccine carriers, GNPs have some particular advantages. A vaccine was obtained by inducing the in situ growth of gold clusters in a hepatitis E vaccine. The process increased immune response in vivo and reduced the potential toxicity. More specifically, HEVA can be tracked due to the intrinsic fluorescence of gold clusters (Fig. 8c) [60]. Owing to the fluorescence of gold clusters in vaccines [61–63], they are helpful in exploring the molecular mechanism of vaccine-induced immune response. GNPs are plasmonic nanoparticles, which can drive vibrational and dipole-like oscillations to destroy cell membranes through electrical excitation. Given this ability, GNPs enhance cell perforation and promote the uptake of DNA vaccine [64]. Gene expression in the mice treated with this method increased 100-fold relative to that in the control group. Furthermore, the model hepatitis C virus DNA vaccine can significantly increase antibody and cellular immune responses. Some new findings can be considered in the design of vaccine adjuvants. A recent study indicated that GNPs are degradable, small GNPs have a faster degradation than large ones and released gold recrystallises into bio-persistent nanostructures [65]. The degradation of GNPs in vitro resulted in the release of bioactive gold ions, which may increase the adjuvant efficiency.

2.1.4. Silica nanoparticles

Vaccines for viruses are often large bioactive molecules, such as proteins or DNA, which can be delivered only by special carriers. Mesoporous silica nanoparticles (MSNs) present the right conditions as vaccine carriers: high colloidal stability, high specific surface area, fantastic surface functionalisation capabilities and large pore volume. Mesoporous silica, composed of amorphous silica, easily degrades in the body over time, and is thus safe in vivo. MSNs potentially regulate the immune system by delivering various cytokines. MSNs with considerably large pores (30 nm) were used to deliver IL-4, a cytokine and had higher capacities for loading proteins than conventional MSNs (3.2 nm; Fig. 9) [66]. IL-4-loaded MSNs trigger M2 macrophage polarization in vivo. Surprisingly, mesoporous silica rods (MSRs) can spontaneously form microporous and three-dimensional scaffolds for growing host immune cells. An injectable MSR-based vaccine enhanced systemic Th1 and Th2 serum antibody and cytotoxic T-cell levels [67]. Dendritic cells were recruited and modulated to the pores between the scaffold rods through the release of inflammatory signals and adjuvants (single-stranded DNA, CpG-ODN).

Mesoporous silica can enhance adjuvant immune activity when used in combination with other inorganic vaccine delivery vectors. For example, SiO_2 with layered double hydroxides core-shell nanoparticles were synthesised to deliver DNA vaccines and activate macrophages. It promoted the expression of IFN-γ, IL-6, MHC II and CD86, thereby enhancing systemic immune responses in animals [68]. Moreover, it facilitated the proliferation of T-cell and skewed the T helper to Th1 polarization.

The physicochemical properties of MSRs are adjustable because of the coupling of different surface ligands. A pH-sensitive antigen and immunostimulant co-carrier system was constructed with mesoporous silica as the nucleus [69]. After coated with the metal organic framework, CpG nucleic acid was adsorbed on the surface. This vector can offset the problems, such as low antigenic endocytosis efficiency, low immune activity and the easy degradation of antigen. Its pH-responsive feature can stabilize the antigen and improve its cellular release efficiency.

2.2. Organic adjuvants

2.2.1. Chitosan nanoparticles

As a natural polysaccharide, chitosan exhibits good biocompatibility. Chitosan is considered a better adjuvant for hepatitis A virus than alum because of the equivalent features of a vaccine containing chitosan solution and alum-adjuvanted suspension [70]. Additionally, chitosan can rebalance Th1 and Th2 levels. With alginate coating, chitosan nanoparticles remarkably increase seroconversion rate (100%), hepatitis A antibody level and splenocyte proliferation rate, considerably improving immunogenicity [70].

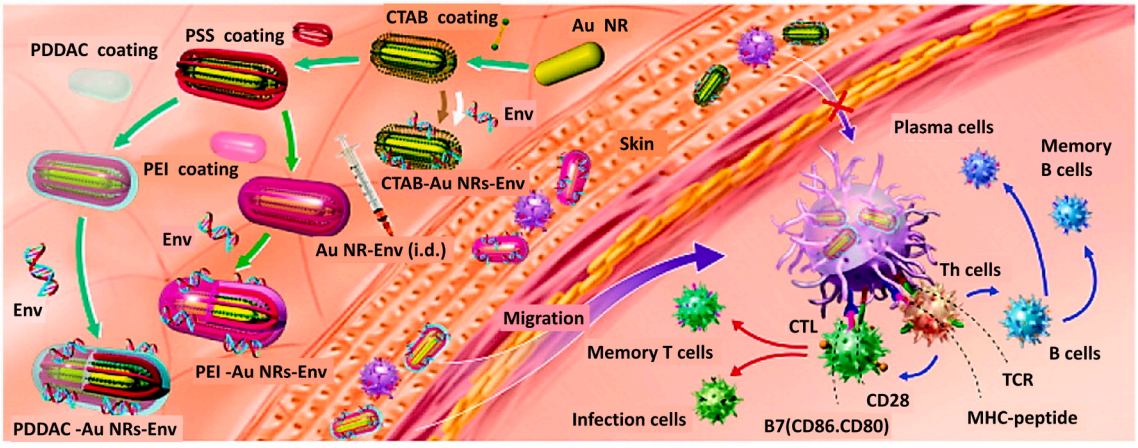

Fig. 7. The proposed mechanism of Au nanorods as vaccine adjuvants. Adapted with permission from ref. [57], copyright © 2012 American Chemical Society.

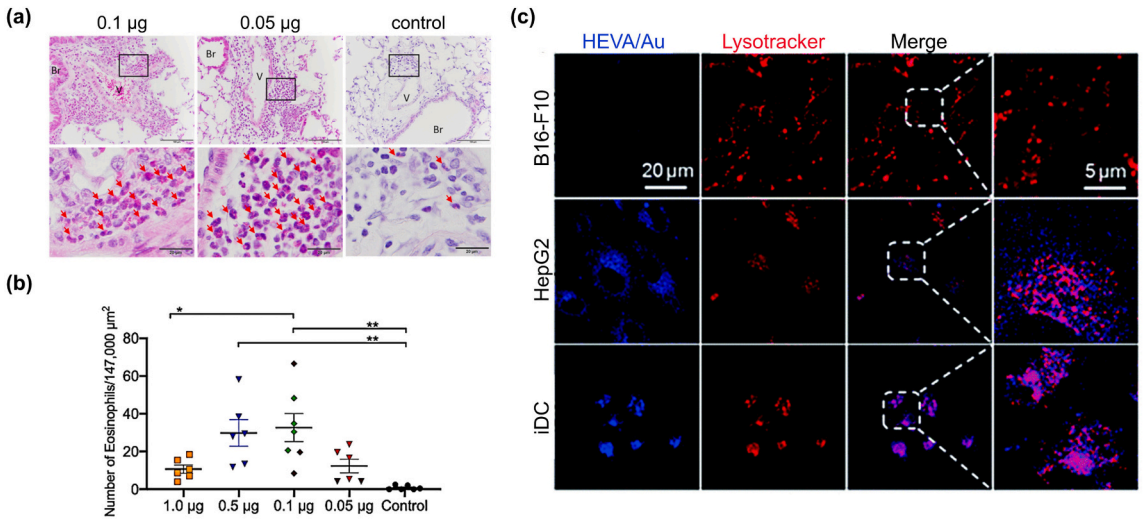

Fig. 8. (a) Lung histopathological study after the challenge of recombinant S protein immunised mice. (b) The number of eosinophils in each lung slice on the 10th day after challenge. Adapted with permission from ref. [58], copyright © 2019 The Societies and John Wiley & Sons Australia, Ltd. (c) Confocal microscopy images of cells after hepatitis E vaccine (HEVA)/Au incubation for 24 h. Adapted with permission from ref. [60], copyright © The Royal Society of Chemistry 2016.

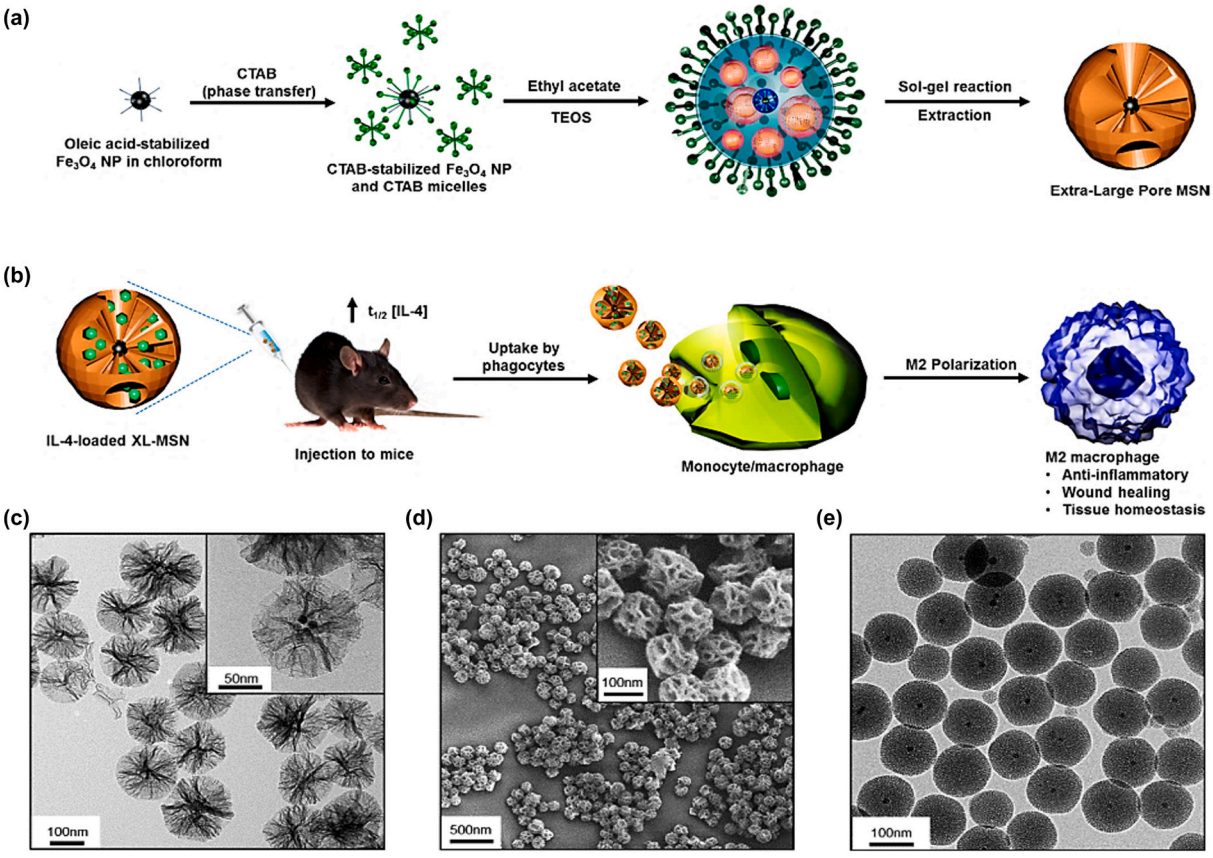

Fig. 9. Schematic statements of (a) synthesis of mesoporous silica nanoparticles with extra-large pores and (b) their application to deliver IL-4 in the polarization of M2 macrophages in vivo. (c) Transmission electron microscope and (d) scanning electron microscope images of 150 nm MSNs. (e) Transmission electron microscope image of conventional MSNs with small pore. Adapted with permission from ref. [66], copyright © 2017 American Chemical Society.

The presence of chitosan's high cationic charge provides a strong affinity for nucleic acids, rendering chitosan an ideal nucleic acid and peptide vector. Biotinylated chitosan nanoparticles loaded with plasmid DNA selectively target dendritic cells and enhance the magnitude of mucosal IgA and systemic IgG levels against nucleocapsid proteins [71]. Thus, they may be excellent materials for designing low-dose vaccines. Trimethyl chitosan complexed with peptide antigen conjugates induce increases in the levels of serum antibodies and antibody titers [72]. This vaccine delivery system can produce vaccine candidates. A novel method for fabricating size-controlled vaccine nanoparticles is essential to ensuring the strength and stability of immune activation. By using the flash nanocomplexation method, the VP1 protein antigen, a structural

protein of EV71, was co-encapsulated with polyelectrolyte complexation of chitosan and heparin. This method controlled the size of the vaccine to nanoscale level (90 nm to 130 nm) and resulted in a high payload capacity [73].

Chitosan nanoparticles are biodegradable, safe and effective adjuvants. Inactivated EV71 adjuvanted with poly-γ-glutamic acid/chitosan enhanced virus-specific humoral (IgG, IgG1 and IgG2a) and cell-mediated immune responses (IFN-γ and IL-4) [74]. Additionally, virus-neutralising antibody responses induced by this adjuvant were much higher than those observed in the virus only group. Combined with other adjuvants (hemokinin-1), the chitosan nanoparticle-based H9N2 influenza vaccine can induce high antibody titers [75]. Chitosan nanoparticles alone can activate mast cells [76]. Incorporating the mast cell activator C48/80 into chitosan nanoparticles is a valuable strategy for improving the efficacy of nasal recombinant vaccines [77].

2.2.2. Lipid-based adjuvant system

Liposomes are spherical vesicles with at least one lipid bilayer. Liposomes can be used as carriers for active therapeutic agents, such as antigens and immunomodulators, and thus liposomes show potential in vaccine adjuvant systems. Liposomes themselves can serve as potential inhibitors of respiratory syncytial virus (RSV), and compared to anti-RSV peptides alone, liposomes with anti-RSV peptides can significantly increase the inhibition of RSV [78]. Glucopyranosyl lipid adjuvant-stable emulsion is particularly effective in enhancing protection against H5N1 virus infection, inducing a continuous versatile (up to 6 months) and cross-reactive hemagglutinin-specific $CD4^+$ T cell response at low doses [79]. The vaccine obtained through the lyophilisation of protein antigens and cationic liposomes triggered immunised mouse antigen-specific multifunctional $CD4^+$ T cells to express significantly high levels of IFN-γ, TNF-α and IL-2 [80].

The covalent binding of antigens to liposomes can optimise the immunogenicity of the humoral immunity of lipid nanoparticles. Compared with immunisation with soluble MD39 trimer (a stabilised version of trimer), immunisation with covalent MD39-conjugated liposomes increased the germinal center and significantly increased antigen-specific T follicular helper cell level and serum MD39-specific IgG responses (Fig. 10a-c) [81]. The trimer maintained the conformational integrity after coupling and induced the production of directed antibodies. The high-density well-ordered covalent attachment of trimers to liposomes not only enhanced the trimers orientation reaction but also eliminated antibodies directed to the C-terminal His tag located at the bottom of the spike, compared with soluble trimers [82].

Monophosphoryl lipid is the third adjuvant material, along with alum and MF59 adjuvant, approved for use in the human body. It exerts an immunostimulatory effect by increasing the expression of proinflammatory cytokines in Th1 immune response, especially IFN-γ and IL-2. As a TLR4 receptor agonist, it can be used in influenza vaccines. In vitro, virions with monophosphoryl lipid A (MPLA) can induce the activation of APCs to a higher degree than virions without adjuvants [83]. The immunisation of mice with MPLA-adjuvanted virosomes with attached nucleoproteins can induce nucleoprotein-specific cytotoxic T lymphocyte and elicit antigen-specific antibody responses and can prevent severe weight loss when the virosomes are inoculated with allotype influenza virus. Monophosphoryl lipid and aluminium hydroxide adjuvants are effective in recruiting monocytes and neutrophils, mediating the production of IgG isotype-switched antibodies and IgG-secreting cell responses and protecting CD4 knockout mice from the influenza virus [84].

2.3. Other adjuvant systems

MF59 is an oil-in-water emulsion composed of metabolizable oil squalene that is less than 250 nm in size. Candidate vaccines containing MF59 adjuvant are extremely safe for young age groups, including infants [85]. Compared with several other commercial adjuvants, MF59 is the most effective in enhancing the immunogenicity of subunit vaccines. IgG antibody responses induced by MF59-adjuvanted MERS-CoV RBD protein resulted in high titers and high levels of IgG1 and IgG2a subtype antibodies in immunised mice [86]. Matrix M1 is a potent adjuvant in clinical trials. When Matrix M1 was used, the level of neutralising antibody was significantly increased to a level 68 times that of a neutralising antibody produced using S nanoparticles only, whereas alum was only increased 15 times. Matrix M1 is more effective especially with MERS-CoV spike than with SARS-CoV spike [87]. With delta inulin-based polysaccharide adjuvants, the formulation of SARS-CoV spike protein or inactivated whole-virus vaccines enhanced the potency of neutralising antibodies and provided protection against lung eosinophilic immunopathology [88]. Furthermore, delta inulin adjuvants resulted in long-term immunity. Fullerols and fullerenes can deliver DNA plasmids and antigen proteins and induce a strong immune response, becoming powerful vaccine adjuvants against human immunodeficiency virus (HIV) and hepatitis C virus (Fig. 11) [89,90]. Some atypical vaccine adjuvant materials, such as polymers, RNA, polysaccharides and carbon dots, are currently available, which will not be described one by one [91–95]. Their physicochemical surface properties, self-assembly and aggregation, and the interaction with biological molecules will influence their immune behavior, toxicities and biological effects [96–100]. More in-situ, high resolution analysis methods should be developing and application to profile the intracellular

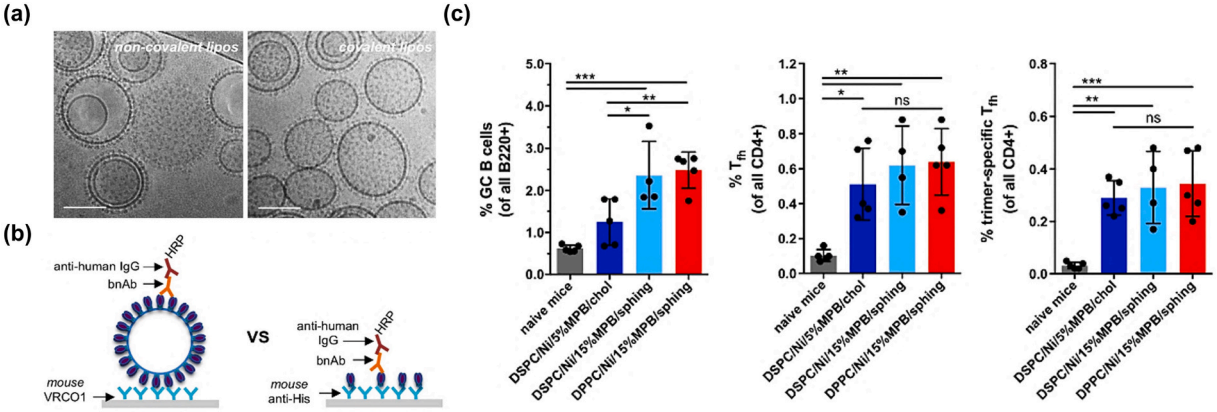

Fig. 10. Covalently-anchored trimer-liposomes. (a) Cryo-electron microscopy images of non-covalent and covalently coupled MD39 liposomes. Ruler: 100 nm. Scale bar: 100 nm. (b) Schematic diagram of antigenic profile analysis of intact liposomes and soluble trimers. bnAb, broadly neutralising antibody. (c) Optimizing the serum stability of liposome trimer in vitro results in enhanced germinal center and IgG response in vivo. Adapted with permission from ref. [81], copyright © 2018, The Author(s).

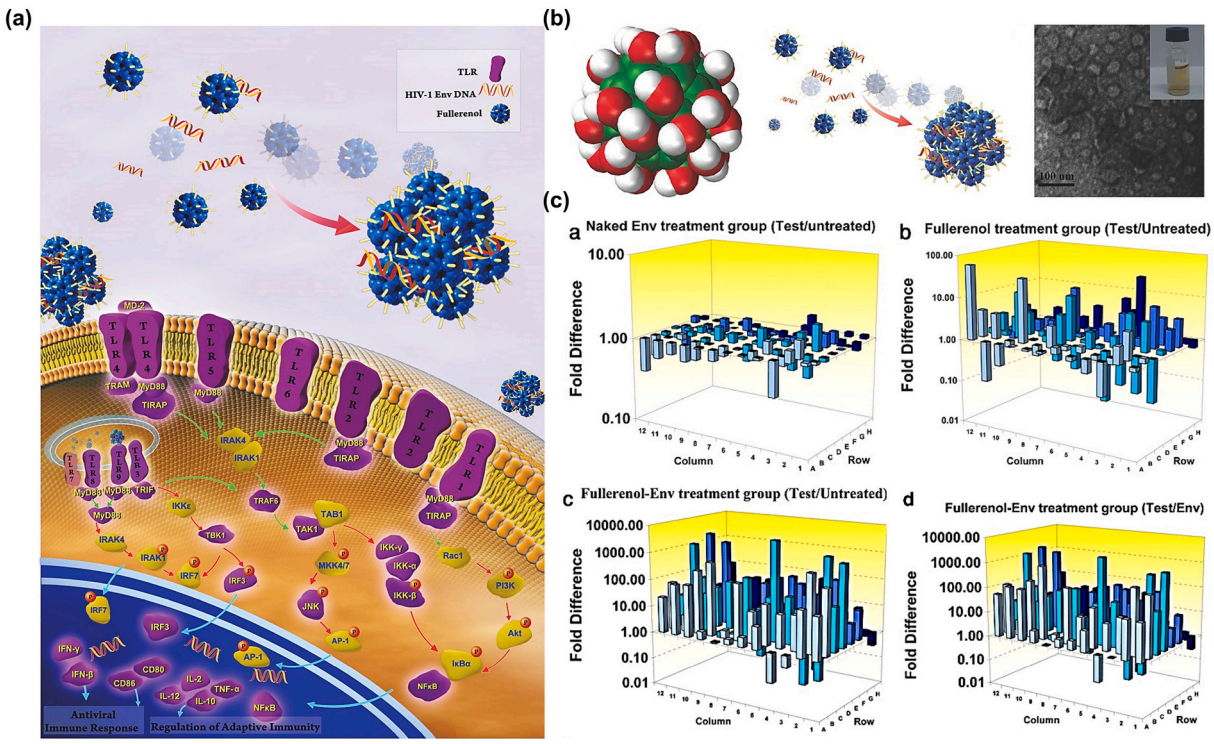

Fig. 11. (a) The possible mechanism of fullerenol as a potent adjuvant. (b) The schematic diagram of fullerenol before and after encapsulate with HIV Env plasmid DNA. (c) The upregulation of the TLR signaling pathway was induced by the formulation in dendritic cells. Adapted with permission from ref. [89], copyright © 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

[63,96,101–105], interface actions [106] and the dissolution and valance states properties [101,107,108] of these nanomaterials.

3. Adjuvants design for coronavirus——SARS, MERS, SARS-CoV-2

In recent years, coronavirus has caused several major epidemics in humans, and the economic and resource losses caused are incalculable. To combating the coronavirus and formulating accurate diagnosis strategies, developing appropriate treatment strategies is necessary. Antiviral treatment can be considered from two aspects: virus-based and host-based anti-CoV treatments [11]. The development of related vaccines and adjuvants is urgently needed. Current adjuvants have the following effects on coronavirus: (i) induce a balanced Th1 and Th2 immune response that prevents eosinophil infiltration; (ii) increase neutralising antibody levels; (iii) induce antigen-specific IgG reactions; (iv) improve mucosal immune induction and enhance the immune response of immature patients, especially infants and the elderly; (v) improve vaccine immunogenicity and reduce vaccination dose; (vi) improve the speed and duration of immune response and provide long-term protection; (vii) as a delivery vehicle, it has a targeting effect and reduces the probability of vaccine degradation, thus improving vaccine delivery efficiency. (viii) Adjuvants can be useful in evaluating vaccine efficacy and safety. The lung eosinophilic pathology can be handled through the following means: developing novel vaccine adjuvants and combining existing adjuvants. By optimizing the ratios of different adjuvants and approaches of administration, the level of neutralising antibodies can be improved. After being modified, adjuvants have the functions of targeting and anti-degradation. Adjuvants still have many undeveloped roles in the treatment of viral infections. These roles should be explored.

3.1. Balanced Th1 and Th2 immune response is induced to prevent eosinophil infiltration

Although coronavirus antigens can induce protective neutralising antibodies, coronavirus vaccines present several problems: the level of protection induced is incomplete [109]. When immunised individuals are infected with the virus, pulmonary eosinophilic lesions may occur, and SARS-CoV virus inactivates vaccines with alum adjuvants [35]. This problem can be solved by combining inactivated virus vaccines or subunit vaccines with different adjuvants. Alum and CpG combined with adjuvants induced the immune responses of Th1 and Th2 in the SARS-CoV S recombinant RBD antigen model [38]. In addition to combining aluminium adjuvants and other adjuvants, selecting appropriate adjuvants is important. The development of extensive eosinophilic immunopathology can be significantly inhibited by vaccines containing delta inulin protein adjuvants after SARS-CoV infection [88]. TLR agonist-adjuvanted S protein prevent eosinophil infiltration in the lungs after SARS coronavirus infection. Moreover, S + TLR-induced Th1 and Th17 biases cytokine in S + TLR immunised mice [58]. By contrast, although the S protein combined with GNPs can induce a specific IgG response, they cannot induce protective antibodies and they limit eosinophil infiltration in the lungs [58]. Immunising mice with a viromimetic stimulator of interferon gene agonist-loaded hollow polymeric nanoparticles can protect mice from lethal MERS-CoV challenge, thereby preventing adverse eosinophilic immunopathology (Fig. 12a-c) [110]. The self-amplified RNA encapsulated by lipid nanoparticles was used to immunise mice. The mice showed increased SARS-CoV-2 specific antibody titers, showing a Th1 bias response (Fig. 12d) [111]. Whether it is the combined use of adjuvants or the selection of new adjuvant materials, the main key is to generate Th1 immunity.

3.2. Increase in neutralising antibody levels

Inactivated virus or live attenuated virus vaccines need adjuvants

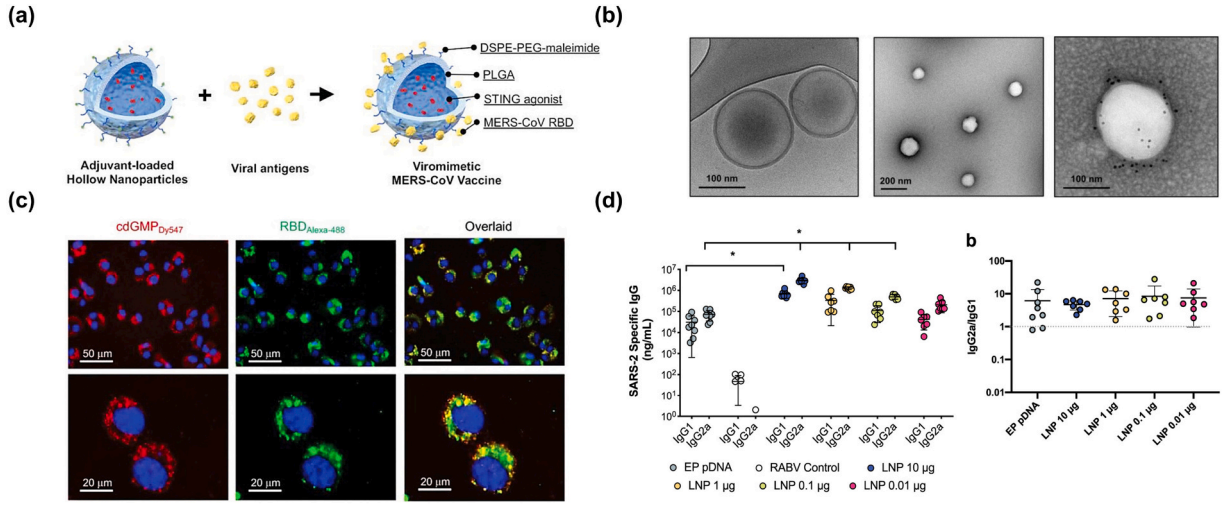

Fig. 12. (a) Schematic illustration of the preparation of adjuvant-loaded viromimetic nanoparticle vaccine. (b) Cryo-electron microscopy (left) and transmission electron microscopy without (middle) and with immunogold staining (right) of MERS-CoV RBD coated nanoparticles. (c) After 24 h of incubation with RBD-NP, the cellular distribution of Dy-547 labeled cyclic diguanylate monophosphate (cdGMP) (red) and AlexaFluor-488 labeled recombinant MERS-CoV RBD antigen (green) in JAWS II cells. Adapted with permission from ref. [110], copyright © 2019 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.) (d) IgG1/IgG2a responses and Th1/Th2 skewing responses in mice vaccinated with SARS-CoV-2 self-amplified RNA lipid nanoparticles vaccine. Adapted with permission from ref. [111], copyright © 2020, The Author(s).

promote immune response. In particular, the immunogenicity of recombinant protein-based subunit vaccines is relatively low, and adjuvants are needed. The basic role of adjuvants is to increase the level of neutralising antibodies. Many existing adjuvants have been proven to have this ability. With recombinant MERS and SARS S protein, the production of two neutralising antibodies was significantly increased in mice, and Matrix M1 outperformed alum (the levels of neutralising antibody produced by S protein with alum was 15-fold and with Matrix M1 was 68-fold the level neutralising antibody produced by S nanoparticles) [87]. A total of 0.1 μg of TLR agonists adjuvanted S protein increased the production of neutralising antibodies sufficiently, reducing the dose of vaccination [58]. The formulation of an inulin-based polysaccharide adjuvant with SARS-CoV spike protein or inactivated whole virus vaccine enhanced the potency of neutralising antibodies and provided protection against clinical diseases [88]. The addition of MF59 remarkably enhanced the immunogenicity of the MERS-CoV RBD subunit vaccine and produced strong IgG and neutralising antibody, which has a protective effect against MERS-CoV infection [86]. The intramuscular immunisation of spike protein formulated with RNA adjuvant can result in the high humoral immune response of the MERS, which is characterised by the production of IgG1 and neutralising antibodies [112].

Over and above being used alone, the combination of different adjuvants is another way to increase the level of neutralising antibodies. A strong anti-MERS-CoV neutralising antibody was induced in response to the immunisation with rASP-1 (an immunopotentiating adjuvant) and alum-adjuvanted RBD vaccine in two separate injection sites. The binding of RBD to cell-related receptors was significantly inhibited by the produced antibodies [113]. After immunisation with MERS-CoV S recombinant RBD protein, alum and CpG, mice were induced to produce robust neutralising antibody responses [38]. The above examples demonstrate the importance of using combined adjuvants to induce a synergistic protective immune response. In each combination, the ratio, mixing order and additional site of various immune-stimulating adjuvants should be optimized in order that adjuvanted vaccine can stimulate the best protective immune response [114].

Neutralising antibodies can be enhanced through several modes of administration. Using intranasal and intraperitoneal immunisation methods may be an ideal solution to provide serum and mucosal neutralising activity against SARS-CoV [115]. Based on the nucleocapsid protein co-administered with a derivative of the mucosal adjuvant macrophage-activating lipopeptide, a heterologous prime-boost vaccination protocol can elicit strong immune responses [116].

3.3. Induction of antigen-specific IgG reactions and IgA

The S protein added by GNP induced a high level of SARS coronavirus antigen-specific IgG response, however, virus-specific IgG and neutralising antibodies are insufficient [58]. After recombinant RBD protein was co-immunised with incomplete Freund's adjuvant and CpG ODN, mice were induced to produce potent RBD-specific antibodies and T-cell responses. However, the production of neutralising antibodies was low [38]. Combined with protein-based and inactivated MERS-CoV vaccines, the level of antigen-specific IgA induced by the RNA adjuvant immunisation pathway was higher, especially the level of IgA induced by intramuscular injection [112]. MERS-CoV S protein, expressed by an adenoviral type 5 (Ad5) vaccine, induced a high level of antigen-specific IgG and neutralising antibodies in mice [117]. After immunising mice with a virus-like nanoparticle vaccine, RBD-specific IgG2a antibody levels were higher, showing a balanced response of Th1 and Th2 [110]. In contrast, the free MF59 and RBD nanoparticles adjuvanted RBD antigen induced a weaker IgG1 response, with very limited IgG2a levels. Recombinant protein S377–588-Fc formulated with MF59 adjuvant was the only group that induced a very low level of IgG antibody response [118]. Compared with antigen immunisation alone, the adjuvanted formulation significantly enhanced spike protein-specific immunoglobulin response. After 2 weeks of immunisation, Advax-1 (a new delta inulin-based polysaccharide adjuvant) significantly enhanced the IgG1 response, while Advax-2 significantly increased the broad antibody isotypes, namely IgG1, IgG2a, IgG2b and IgG3, while CpG adjuvant significantly increased IgG2a, IgG2b and IgG3 without increasing IgG1, all three stimuli can be maintained for one year [88]. According to the CpG ODN 2006-assisted intranasal immunisation protocol, inactivated SARS-CoV induced large amounts of SARS-CoV-specific IgG antibodies in the serum, and a detectable amount of IgA antibodies in sera and all tested mucosal secretions and tissues [115].

3.4. Activation of T cells

The T cell response induced by MERS-CoV RBD protein cannot be

significantly enhanced by some of the commercial adjuvants, including aluminium, Freund's adjuvant, monophosphoryl lipid A, MF59 and Montanide ISA51 [86]. rCRT/39–272, a recombinant fragment of murine calreticulin, can induce $CD4^+$ helper T cell responses and also has stimulatory effects on B cells and dendritic cells in mice, which makes it a potential molecular adjuvant in the preparation of human SARS-CoV vaccines [119]. Different routes of administration for vaccines and adjuvants lead to different immune effects. The intramuscular administration of spike protein formulated with RNA adjuvant was a considerable route for inducing humoral immune responses. In comparison, the intranasal route may be more suitable in T cell activation and IgA induction [112]. A recombinant adenoviral type 5 or type 41 vector-based vaccines express the MERS-CoV spike protein. After a single intramuscular injection of Ad41-S or Ad5-S, the mouse spleen and lung lymphocytes triggered a functional antigen-specific T cell response that lasted for several months, but intragastric administration failed to induce this reaction [117].

By using delta inulin adjuvant to form SARS-CoV antigen, T cell long-acting IFN-γ response was generated [88]. Immune responses to mice, endogenous, exogenous or chimeric immunogens vary and are influenced by the forms of SARS coronavirus nucleocapsid. Immunised with the exogenous nucleocapsid protein and Freund's adjuvant can significantly enhance the antibody response of mice, causing a high T cell IL-4 to IFN-γ response ratio to the antigen peptides [120].

3.5. *Providing long-term protection or preventing the vaccine from being degraded*

The β-propiolactone-inactivated whole virion SARS-CoV was applied to immunise mice in the combination of adjuvant, $AS01_B$ and $AS03_A$. Mice have better and stronger protection against late challenges (16–18 weeks) that animals vaccinated without adjuvanted vaccines [121]. Another experiment showed that the immunity obtained with delta inulin adjuvant is long-term [88]. Over a long period, the titer of the neutralising antibody was high, and the vaccine added by the whole UV-inactivated SARS-CoV and TLR agonists had sufficient protection against SARS-CoV infection [109]. A vaccine based on attenuated vesicular stomatitis virus expresses SARS-CoV S protein. Mice vaccinated can produce SARS neutralising antibodies and can resist SARS-CoV challenge one or four months post single vaccination [122].

In view of the characteristics of SARS transmission through the respiratory pathway, dendritic cells were designed to target plasmid DNA loaded with biotinylated chitosan nanoparticles. The preparation not only can induce the systemic and mucosal nucleocapsid protein-specific reaction but also resist the digestion of nuclease in mice [71].

4. Summary and future prospective

The cunning virus may mutate to evade the broad-effect antibodies caused by a universal vaccine [123]. A truly universal vaccine faces huge challenges, which also makes us look forward to the role of adjuvants. Materials for adjuvants have been rapidly developed [57,89,90,124,125], especially adjuvants used for tumor treatment and the research and development of antiviral vaccine adjuvants. In addition to traditional aluminium, MF59 and other adjuvants are approved for human treatment. Other adjuvants, such as gold nanoparticles and chitosan, have been extensively researched in recent years. The size of gold nanoparticles is controllable, and the gold clusters also have intrinsic fluorescence, so that they can be used as adjuvants to improve the immune efficiency of viral vaccines and track the vaccines. As a degradable biological material, chitosan is used as an adjuvant for various viral vaccines, such as hepatitis A, SARS, enterovirus 71 and H9N2 influenza.

The new coronavirus vaccines currently being developed include DNA vaccines [126], mRNA vaccines [16], inactivated candidate vaccines [39] and small molecule drugs [127]. As of March 5, 2021, a total of 79 vaccines have entered clinical evaluation and 182 candidate vaccines have entered preclinical evaluation [128]. Two broad categories of these antiviral vaccines are classified: gene-based vaccines and protein-based vaccines. The first type of vaccine includes live virus vaccines, nucleic acid vaccines and recombinant vaccine vectors and mainly encodes the gene sequences of protein antigens produced by host cells. The second type of vaccine includes single viral proteins or subdomains, completely inactivated viruses and viral proteins assembled in the form of particles [129,130]. However, these vaccines may have shortcomings, such as insufficient immunogenicity and side effects. As mentioned earlier, nanoadjuvants are useful in regulating immune responses by controlling the timing and location of vaccine, increasing the strength and persistence of antibody responses and affecting T cell-derived cytokine patterns.

Based on the above summary, we have put forward the following design suggestions for the new coronavirus vaccine adjuvant, hoping to help the relevant researchers to develop an effective, safety and useful vaccine on a large scale to prevent the virus from repeating or continuing to spread.

i. Selection of different adjuvant combinations for different types of vaccines, improve the immunogenicity of the vaccine and optimise the physical and chemical properties of the vaccine, such as permeability, stability and toxicity and improve the availability of the vaccine.
ii. The adjuvant can improve the electrostatic complementarity and hydrophobic interaction of the vaccine against the virus, which is crucial for enhancing the receptor binding of RBD in SARS-CoV-2 and evading antibody recognition.
iii. Target the design of the adjuvant according to the virus's action site and action environment so that the adjuvant works from the beginning of infection.
iv. To enhance the immune effects of the vaccine, it is necessary to optimise both antigen dose and adjuvant ratio, and then optimise the frequency of immunisation, time interval and route of administration for long-term immunity and the prevention of side effects, such as eosinophilic pathology.
v. Use the less toxic nanomaterials as adjuvant carriers to reduce side effects on the human body.
vi. Development of a cold chain-free vaccine formulation to improve the stability of vaccines and reduce the cost of vaccine delivery.

Author statement

C. Chen and Y. Wang desiged the outline and provided critical suggestions. L. Mao and Y. Wang conceived and revised the manuscript. Z. Chen collecting and classified reference. All authors contributed to the article and approved the submitted version. All authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Declaration of Competing Interest

We declare that we have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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