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Antigen Delivery Systems: Past, Present, and Future

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Abstract

The COVID-19 pandemic has increased demand for safe and effective vaccines. Research to develop vaccines against diseases including Middle East respiratory syndrome, Ebolavirus, human immunodeficiency virus, and various cancers would also contribute to global well-being. For successful vaccine development, the advancement of technologies such as antigen (Ag) screening, Ag delivery systems and adjuvants, and manufacturing processes is essential. Ag delivery systems are required not only to deliver a sufficient amount of Ag for vaccination, but also to enhance immune response. In addition, Ag types and their delivery systems determine the manufacturing processes of the vaccine product. Here, we analyze the characteristics of various Ag delivery systems: plasmids, viral vectors, bacterial vectors, nanoparticles, self-assembled particles, natural and artificial cells, and extracellular vesicles. This review provides insight into the current vaccine landscape and highlights promising avenues of research for the development and improvement of Ag delivery systems.

Key Words: Vaccine, Antigen delivery system, Vector, Nanoparticle, Self-assembled particles, Extracellular vesicle

INTRODUCTION

The recent coronavirus (COVID-19) pandemic has increased the demand for infectious disease prevention strategies, especially vaccine development. In addition to COVID-19, the World Health Organization (WHO) has created a list of high priority infectious diseases requiring research and development efforts to improve diagnostic tools, vaccines, and related drugs. The list includes Crimean-Congo haemorrhagic fever, Ebola virus/Marburg virus disease, Lassa fever, Middle East respiratory syndrome coronavirus (MERS-CoV), severe acute respiratory syndrome (SARS), Nipah and henipaviral diseases, Rift Valley fever, and Zika (Mehand *et al*., 2018). Vaccines have also attracted attention as a strategy to improve the effects of conventional cancer therapies in the form of therapeutic cancer vaccines (Saxena *et al*., 2021). For successful vaccine development, improved technologies are required; this can be achieved through improving Ag screening and the Ag delivery system to overcome the limitations of solitary Ags, selecting appropriate adjuvants, and overcoming challenges posed by manufacturing processes. With such advances, we can more quickly control the emergence of new infectious diseases and overcome the limitations of current preventive and therapeutic vaccines.

Ag delivery systems can function as adjuvants that enhance immunogenicity. In addition, the physical and chemical properties of the final vaccine may be dependent on the delivery system. In this review, we analyze the different Ag types, modes of action, immunogenicity, and safety issues of current Ag delivery systems, and introduce the research trends of new systems that have potential for clinical application in the future.

THE CURRENT VACCINE LANDSCAPE

The traditional global vaccine market consisted of the whole-cell vaccines and subunit vaccines. Whole-cell vaccines include inactivated vaccines and live attenuated vaccines, while subunit vaccines include recombinant protein subunit vaccines, polysaccharide vaccines, and conjugate vaccines. COVID-19, an infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was declared a pandemic in March 2020. The urgent demand

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Table 1. Different types of vaccine delivery systems

Delivery system	Features	Advantages	Disadvantages.
Plasmids	They deliver the target gene into the host cell. Target Ags are expressed in host cells.	• Safety • Stability • Simple development process • Activation of both cellular and humoral immunity	• Weak immunogenicity • Requirement of administration device
Viral vectors	They can serve as a vector to deliver genetic/protein Ags and also as an adjuvant capable of activating the host immune system.	• Strong immunogenicity	• Safety concerns • Vector-originated immune response
Bacterial vectors	Non-pathogenic commensal/mutual/ attenuated bacteria can be used as delivery vectors for genetic and protein Ags.	• Simple genetic modification • Easy production on a large scale • Suitable for mucosal vaccines	• Safety concerns • May need to detoxification
Particle-based vectors	They are engulfed and processed/ presented by antigen presenting cells. They include liposomes, lipid nanoparticles, and polymeric particles.	• Safety • Suitable for multivalent vaccines	• Stability issues • Low delivery efficiency
Self-assembled nanoparticles	Some proteins/peptides can self-assemble and form nanoparticles, and provides the protein structure to generate Ag-specific potent immunity.	• Safety • Suitable for multivalent vaccines	• Difficulty in assembly • Challenges in the manufacture
Cells	Antigen presenting cells present Ags and costimulations to host T cells.	• Strong T cell immune responses	• Necessity of isolating cells from patients • High cost of production
Extracellular vesicles	They are natural cell-derived vesicles with various lipids and surface proteins.	• Biocompatibility • High targeting efficiency	• Low efficiency of production • Lack of characterization

for COVID-19 vaccines was an opportunity for exploring vaccines developed using innovative platforms, which previously had a higher barrier to clinical entry than traditional vaccines. Several papers have reviewed the development processes, efficacies, and adverse effects of COVID-19 vaccines developed using both traditional and innovative platforms (Shin *et al*., 2020; Sharif *et al*., 2021; Bates *et al*., 2022).

Here, we focus the development trends of Ag delivery systems of vaccines for various diseases. Table 1 shows the types of Ag delivery systems and their features. We also summarize the advantage and disadvantage of Ag delivery systems of vaccines to compare different types of Ag delivery systems. In the case of whole-cell vaccines, an additional Ag delivery system is not required. Subunit vaccines can also induce immune responses without an Ag delivery system, depending on the Ag type. However, it is necessary to improve immunogenicity through an appropriate Ag delivery system or adjuvant in the case of a subunit Ag with relatively low immunogenicity. In particular, immunogenicity can be enhanced if Ags can form particles such as virus-like particles (VLPs) or self-assembled nanoparticles (Kushnir *et al*., 2012). An appropriate Ag delivery system is essential in the case of genetic vaccines, such as DNA and mRNA vaccines, due to the low delivery efficiency of Ag to host cells, weak immunogenicity, and/or physicochemical instability (Hou *et al*., 2021).

Table 2 presents a representational list of Ag delivery systems of vaccines that have ongoing or completed clinical trials for various indications. Table 2 includes the current status of clinical trials and their information, such as number of participants, dose and administration route. COVID-19 vaccines have been developed using almost all types of vaccine platforms. In addition, vaccines against viruses including human immunodeficiency virus (HIV)-1, MERS-coronavirus, Zika virus, and Ebola virus have been developed using various platforms. Based on the VLP system, hepatitis B virus (HBV), human papilloma virus (HPV), and COVID-19 vaccines have been approved (Gao *et al*., 2018; Ho *et al*., 2020; Stander *et al*., 2022), and rotavirus vaccines are currently being evaluated in clinical trials (NCT03507738). Cell-based vaccines could be applied to treat incurable diseases such as cancer (Cheever and Higano, 2011) and HIV-1 (da Silva *et al*., 2018), and are currently being evaluated in clinical trials for COVID-19 (NCT04299724). Tumor-derived exosomes and bacterial-derived outer-membrane vesicles (OMV), are in clinical trials as vaccines against cancer (Santos and Almeida, 2021) and *Shigella* infections (NCT02676895), respectively.

Collectively, Ag delivery systems are being widely applied to vaccine development for various diseases. In this review, we aim to introduce the mechanisms of action and characteristics of these Ag delivery systems and discuss the recent research that explores new systems with high potential for future clinical application.

Table 2. Ag delivery systems of approved vaccines and vaccines in clinical trials

Delivery system		Status	Target organism[1)]	The number of participants/dose/administration route	Recruitment status (*Last verified date*)	Reference
Plasmid		Phase 1	Ebola virus	46 participants/1 mg of INO-4201/Intradermal injection followed by electroporation	Completed (*May, 2022*)	NCT04906629[3)]
		Phase 1	HIV[a]	1668 participants/4 mg of DNA-HIV-PT123/ Intramuscular injection	Completed (*October 2021*)	NCT02207920
		Phase 2	MERS-CoV[b]	192 participants/0.6 mg of INO-4700/Intradermally injection followed by electroporation	Completed (*January, 2023*)	NCT04588428
		Phase 1	Zika virus	45 participants/4 mg of ZIKV DNA vaccine/ Intramuscularly injection by needle and syringe or needle-free injection device	Completed (*September, 2019*)	NCT02996461
		Various	Cancer	Various	Various	(Lopes *et al*., 2019)
		Approval	SARS-CoV-2[c] (2021)[2)]			(Sheridan, 2021)
Virus	Non-replicating	Phase 1	Ebola virus	120 participants/4×10^{10} viral particles or 1.6×10^{11} viral particles of Ad5-EBOV/Intramuscular injection	Completed (*August, 2015*)	NCT02326194
		Phase 1	HCV[d]	548 participants/2.5×10^{10} total virus particles of AdCh3NSmut1 followed by 1.8×10^{8} plaque forming units of MVA-NSmut/ Intramuscular injection	Completed (*April, 2017*)	NCT01436357
		Phase 1	HIV[e]	31 participants/10^{10} particle units of rAd5 vaccine/Intramuscular injection	Completed (*May, 2014*)	NCT00709605
		Phase 1	Influenza virus	15 participants/5×10^{8}, 5×10^{9}, 2.5×10^{10}, 5×10^{10} virus particles of ChAdOx1-NP+M1/ data not available	Completed (*November 2014*)	NCT01623518
		Phase 1	MERS-CoV	26 participants/10^{8} plaque forming units of MVA-MERS-S/Intramuscular injection	Completed (*October 2020*)	NCT03615911
		Phase 1	Norovirus	66 participants/1×10^{10} or 1×10^{11} infectious units of VXA-G1.1-NN/Oral administration	Completed (*May 2018*)	NCT02868073
		Phase 1	RSV[f]	66 participants/data not available/Oral administration	Completed (*August 2018*)	NCT02830932
		Phase 1	Zika virus	24 participants/5×10^{9}, 2.5×10^{10}, 5×10^{10} virus particles of ChAdOx1 Zika/Intramuscular injection	Completed (*November 2021*)	NCT04015648
		Phase 1	*Mycobacterium tuberculosis*	72 participants/5×10^{9}, 2.5×10^{10} virus particles of ChAdOx185A/Intramuscular injection	Completed (*August 2022*)	NCT03681860
		Phase 1	*Plasmodium Falciparum*	26 participants/5×10^{9} or 5×10^{10} virus particles of ChAd63 RH5 with 1×10^{8} or 2×10^{8} plaque forming units of MVA RH5/ Intramuscular injection	Completed (*December 2015*)	NCT02181088
		Approval	*SARS-CoV-2 (2021)*			(Tregoning *et al*., 2021)

TYPES OF VACCINE DELIVERY SYSTEMS

Recently, genetic vaccines, which consist of either DNA or mRNA, have been used clinically. The efficiency of gene delivery determines vaccine efficacy (Liu *et al*., 2021). An adjuvant may be added to genetic vaccines to increase vaccine efficacy. Otherwise, the adjuvant effects originated from Ag delivery systems can contribute to the immunogenicity of vaccines. Gene delivery systems can be divided into categories such as plasmids, viral vectors, and lipid-based particles. Recently, bacterial vectors have also been studied (Yurina, 2018).

Table 2. Continued 1

Delivery system		Status	Target organism[1)]	The number of participants/dose/administration route	Recruitment status (*Last verified date*)	Reference
	Replicating	Phase 1	Ebola virus	30 participants/3×10^5, 3×10^6, or 2×10^7 pfu plaque forming units of rVSVΔG-ZEBOV-GP/Intramuscular injection	Completed (*May 2017*)	NCT02283099
		Phase 1	Influenza virus	166 participants/1×10^7, 1×10^8, 1×10^9, 1×10^{10}, 1×10^{11} virus particles of replication-competent Ad4-H5-Vtn/Oral administration	Completed (*March 2020*)	NCT01006798
		Phase 3	RSV	63 participants/3×10^8 infectious units of MVA-BN-RSV vaccine/Intramuscular injection	Active, not recruiting (*January 2023*)	NCT05238025
		Phase 1	SARS-CoV-2	35 participants/3.3×10^8, or 1×10^9 egg infectious dose 50 of NDV-HXP-S/Intranasal and/or intramuscular administration	Recruiting (*December 2022*)	NCT05181709
		Phase 1	Zika virus	48 participants/1×10^5, or 2.5×10^4 of MV-ZIKA-RSP/Injection	Completed (*October 2021*)	NCT04033068
		Phase 1	*Bacillus anthracis*	120 participants/1×10^9, 1×10^{10}, 1×10^{11} virus particles of replication-competent Ad4-PA and Ad4-PA-GPI/Intramuscular injection	Completed (*March 2020*)	NCT01979406
		Approval	Ebola virus (2019)			(Wolf *et al*., 2021)
Bacteria	Live	Phase 1	SARS-CoV-2	24 participants/1×10^9, 3×10^9, 1×10^{10} colony forming units of bacTRL-Spike/Oral administration	Completed (*May 2022*)	NCT04334980
	Bacteria-like particle	Phase 1	RSV	48 participants/140 μg F-protein/2mg or 350 μg F-protein/5mg of SynGEM/Intranasal administration	Unknown (*September 2017*)	NCT02958540
Particle-based	Liposome	Phase 1	HIV	24 participants/500, or 2000 μg of HIV-1 gp41 MPER-656 liposome vaccine/Intramuscular injection	Completed (*June 2022*)	NCT03934541
		Phase 1	Ovarian cancer	10 participants/ Liposome formulated mRNA vaccine in combination with adjuvant chemotherapy/Intravenous injection	Active, not recruiting (*June 2022*)	NCT04163094
		Approval	HAV[g] (1994)			(Tretiakova and Vodovozova, 2022)
			Influenza virus (1997)			(Schaad *et al*., 2000)
			VZV[h] (2017)			(Tretiakova and Vodovozova, 2022)
			Plasmodium falciparum and HBV[i] (2015)			(Tretiakova and Vodovozova, 2022)

Subunit vaccines have the advantage of selectively inducing desired Ag-specific immune responses, but also have the disadvantage of relatively weak immunogenicity, which can be improved by administering them in combination with an adjuvant. Another strategy for improving immunogenicity is to develop recombinant protein vaccines that form particles, including VLPs, which have improved immunogenicity compared to soluble Ag proteins administered alone (Kushnir *et al*., 2012).

Antigen presenting cell (APC)-based vaccines can induce strong T-cell immune responses. One limitation of ACP-based vaccines is that they are difficult to use generally. Recently efforts have been made in the development of artificial cell-based vaccines in order to overcome the limitations of APC-based vaccines (Perica *et al*., 2014). Extracellular vesicles

Table 2. Continued 2

Delivery system		Status	Target organism[1)]	The number of participants/dose/administration route	Recruitment status (*Last verified date*)	Reference
	Lipid nanoparticle	Phase 1	Influenza virus	201 participants/25, 50, 75, 100, and 400 μg of lipid nanoparticle-based H10N8 mRNA vaccine/Intramuscular or intradermal injection	Completed (*April 2022*)	NCT03076385
		Phase 1	RSV	651 participants/Escalating doses of lipid nanoparticle-based mRNA-1345/Injection	Active, not recruiting (*November 2022*)	NCT04528719
		Phase 1	Zika virus	90 participants/Escalating doses of lipid nanoparticle-based mRNA-1325/Injection	Completed (*December 2019*)	NCT03014089
		Phase 1/2	Cancer	5 participants/0.13 mg or 0.39 mg of lipid nanoparticle-based National Cancer Institute-4650 mRNA vaccine/Intramuscular injection	Terminated (*May 2020*)	NCT03480152
		Approval	SARS-CoV-2 (2020)			(Lamb, 2021)
Self-assembled nanoparticle		Phase 1	Influenza virus	280 participants/15 μg or 45 μg of HA included in H7N9 virus-like particle, or 5 μg or 15 μg of HA included in H7N9 virus-like particle combined with 30 or 60 units of the saponin-based ISCOMATRIX adjuvant/Intramuscular injection	Completed (*October 2014*)	NCT01897701
		Phase 1	Norovirus	102 participants/5/5 μg, 15/15 μg, or 50/50 μg of norovirus GI.1/GII.4 bivalent virus-like particle vaccine/Intramuscular injection	Completed (*April 2018*)	NCT01168401
		Phase 1	Rotavirus	110 participants/2.5 μg, 7 μg, or 21 μg of rotavirus-like particle vaccine/Intramuscular injection	Completed (*August 2019*)	NCT03507738
		Approval	HBV (1986)			(Ho *et al*., 2020)
			HEV[J] (2011)			(Gao *et al*., 2018)
			HPV[K] (2006)			(Gao *et al*., 2018)
			SARS-CoV-2 (2022)			(Stander *et al*., 2022)
Cell-based	Cell	Phase 1	CMV[I]	42 participants/2×10^7 of CMV pp65-LAMP mRNA-loaded dendritic cells/Intradermal injection	Active, not recruiting (*August 2022*)	NCT00639639
		Phase 1/2	HCV	10 participants/5×10^6 of autologous dendritic cells pulsed with recombinant HCV core and NS3 antigen/Intracutaneous injection	Completed (*February 2019*)	NCT03119025
		Phase 1/2	HIV	11 participants/10^7 of autologous dendritic cells pulsed with inactivated HIV-1 infected apoptotic cells/Subcutaneous injection	Completed (*May 2016*)	NCT00510497
		Phase 1/2	SARS-CoV-2	175 participants/Dose exploration of autologous dendritic cells loaded with SARS-CoV-2 spike protein/Subcutaneous injection	Not yet recruiting (*December 2021*)	NCT04386252
		Approval	Cancer (2010)			(Cheever and Higano, 2011)

Table 2. Continued 3

Delivery system		Status	Target organism[1)]	The number of participants/dose/administration route	Recruitment status (*Last verified date*)	Reference
	Artificial cell	Phase 1	SARS-CoV-2	100 participants/5×10^6 Covid-19 artificial APC vaccine/Subcutaneous injection	Recruiting (*March 2020*)	NCT04299724
Extracellular vesicle	Exosome	Phase 2	Cancer	41 participants/53–2,422 μg of exosomal protein quantity/Injection of tumor antigen-loaded dendritic cell-derived exosomes/Intradermal injection	Completed (*March 2018*)	NCT01159288
	Outer-membrane vesicle	Phase 2	*Shigella sonnei*	74 participants/Generalized modules for membrane antigens based 1790GAHB vaccine containing 25 μg or 100 μg of *Shigella sonnei*/Intramuscular injection	Completed (*October 2018*)	NCT02676895
		Approval	*Neisseria meningitides* serogroup B (2013)			(van der Pol *et al*., 2015)

[1)]The representative target organism of each Ag delivery system. This list does not include all vaccines in clinical trials or licensed for use. [2)]The number in parentheses indicates the year of approval in the representative country. [3)]Found on ClinicalTrials.gov. [a]Human immunodeficiency virus. [b]Middle East respiratory syndrome-related coronavirus. [c]Severe acute respiratory syndrome coronavirus-2. [d]Hepatitis C virus. [e]Human immunodeficiency virus. [f]Respiratory syncytial virus. [g]Hepatitis A virus. [h]Varicella-zoster virus. [i]Hepatitis B virus. [j]Hepatitis E virus. [k]Human papillomavirus. [l]Cytomegalovirus.

(EVs), which include cell-derived exosomes (Ward *et al*., 2021) and OMVs of gram-negative bacteria (Mancini *et al*., 2021), can be used as vaccines.

Plasmids

A plasmid is self-replicating DNA and can be exist independently of the chromosome. It can be used as vector that delivers the target gene into the host cell. Therefore, plasmids have functioned as vectors for gene therapy (Pena *et al*., 2020) and also for genetic vaccines (Hobernik and Bros, 2018). Plasmids can be used as vectors to deliver target genes into the host cells for gene therapy and genetic vaccines (Fig. 1A). While the protein Ag acts as an exogenous Ag and mainly induces an antibody (Ab) response, Ag from plasmid expressed in the cytosol efficiently induces T-cell responses by acting as an endogenous Ag. In addition, the persistent expression of Ags can be an advantage in terms of long-lasting effects (Yang *et al*., 2014). The simple development process for genetic vaccine is another advantage they have over whole-cell vaccines and protein vaccines (Liu *et al*., 2021).

One characteristic of plasmid-based genetic vaccines is that they activate both cellular and humoral immunity (Shedlock and Weiner, 2000). T cells are important for inducing memory responses and contribute to the protective immunity against re-infection (Jung *et al*., 2021; Moss, 2022). Recently, it was reported that cross-reactive T cell responses were induced by vaccination against the SARS-CoV-2 omicron variant, which has multiple Ag mutations (Moss, 2022). Previously, immunogenicity of plasmid vectors alone is relatively low, but a low level of Ag expression has been a concern. Techniques such as electroporation to increase transfection efficiency have been applied to address this low expression, and plasmid vaccines are currently in clinical trials for various cancers (Gnjatic *et al*., 2009), MERS (NCT04588428), and Zika virus (NCT02996461). Recently, the first plasmid vaccine for COVID-19 was approved for use in India (Sheridan, 2021).

As a way to overcome the weak immunogenicity of plasmid DNA vaccines, multiple booster shots can be administered. Due to the low immunogenicity of vectors, plasmids may be suitable for vaccines requiring multiple administrations. The strategy of boosting with a plasmid vaccine after priming with a viral vector vaccine has been demonstrated and it was shown that this regimen efficiently induced a long-lasting memory immune response (Yang *et al*., 2014). In the future, it is possible to utilize plasmid vaccines as booster shots in heterogeneous prime-boost vaccination approaches.

Viral vectors

Naturally, viruses have a life cycle of attachment, entry, production of viral nucleic acids and proteins, assembly inside the host cells, and finally release from the host cells. Therefore, viruses can serve as a vector to deliver Ags and also as an adjuvant capable of activating the host immune system. However, there is a safety issue due to the concern of pathogenicity caused by the virus.

Adenoviruses are common viruses that the majority of humans are exposed to throughout their lifetime and are characterized by low pathogenicity. To address safety concerns and allow for wider application, adenovirus vectors have been developed that lack their replication ability (Fig. 1B) (Mendonça *et al*., 2021). Because the viral genomes of adenoviruses are not inserted into the host chromosome, the long-term maintenance of the target genes is difficult, but there are no concerns of insertional mutagenesis. Adenoviruses are used in the licensed COVID-19 vaccines of several groups, including Johnson & Johnson and Oxford–AstraZeneca, as replication-defective viral vectors (Tregoning *et al*., 2021). To overcome the barriers of pre-existing adenovirus-specific neutralizing

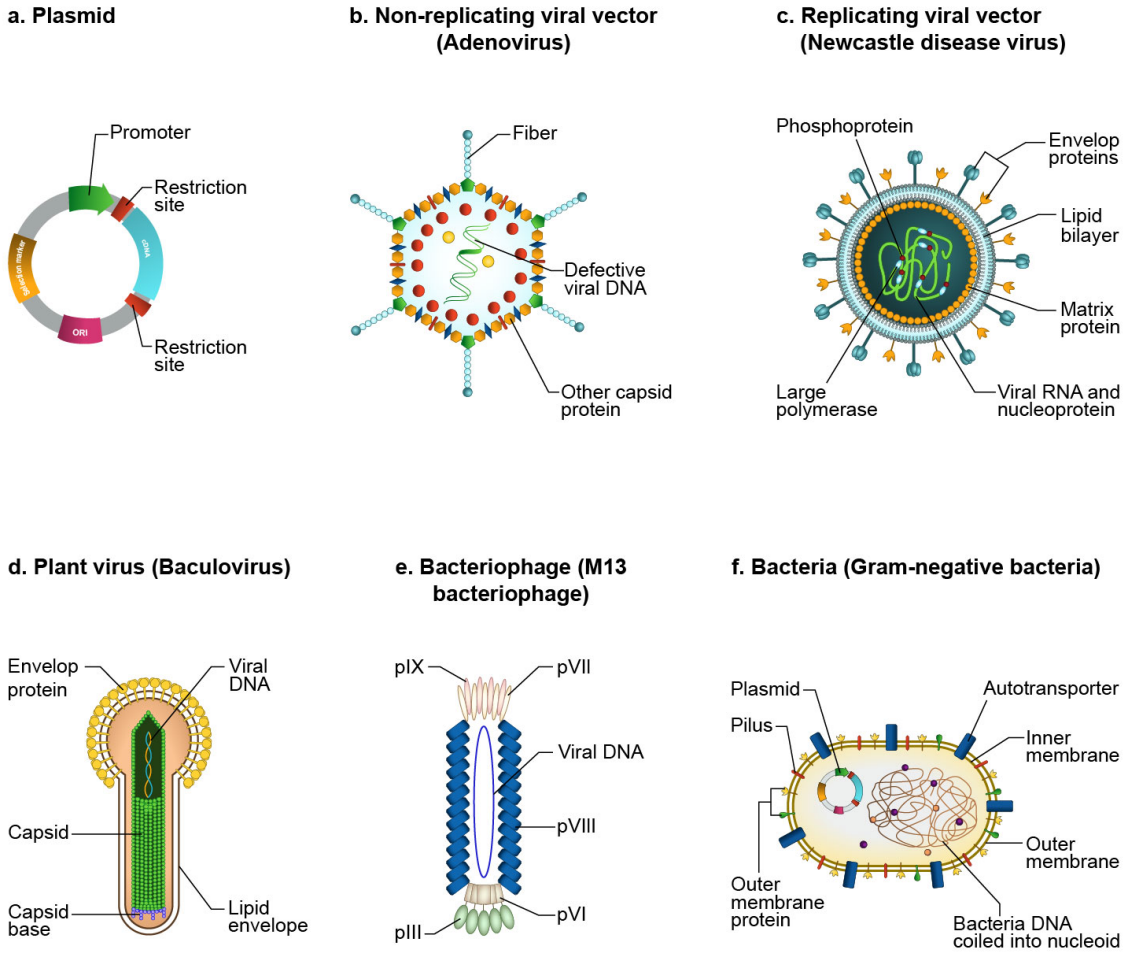

Fig. 1. Structures of genetic Ag delivery systems. (A) Plasmid vectors include an origin of replication, an antibiotic resistance gene, a promotor, and a gene of interest. (B) Representative non-replicating viral vector, adenovirus consisting of a double-stranded DNA genome and capsid proteins including fibers. (C) Representative replicating viral vector, NDV consisting of nuclear proteins, phosphoproteins, and large polymerases combined with viral RNA. The enveloped proteins protrude from the viral envelope. Matrix proteins are attached to the inner surface of envelope. (D) Baculovirus consisting of circular double stranded DNA genomes and enveloped rod-shaped nucleocapsids. (E) Filamentous phage M13 comprising of a single stranded DNA genome, major coat proteins (pVIII), and minor coat proteins (pIII, pVI, pVII and pIX). (F) Gram-negative bacteria with surface structural components on the outer-membrane including pili, autotransporters, and other outer-membrane proteins.

Abs, modified adenoviruses or chimpanzee adenoviruses can be used (Antrobus *et al*., 2014; Gebre *et al*., 2021). The "Sputnik V" COVID-19 vaccine developed by the Gamaleya National Research Centre, uses adenovirus type 26 and adenovirus type 5 as vectors that are administrated 21 days apart (Jones and Roy, 2021). A recent study showed that the repeated administration of viral vector-based vaccines was effective (Dolzhikova *et al*., 2021). The "Sputnik Light" vaccine contains recombinant adenovirus type 26, one of the components of the Sputnik V, and has been approved for use as a booster after Sputnik V or other vaccine administration. Further research is required to elucidate the mechanism for the induction of neutralizing Abs against viral vectors after vaccination, and their role in the immunogenicity of vaccines.

Replicating viral vectors contribute to prolonged Ag expression and activate strong immune responses. Some replicating virus-based vaccines are in clinical trials and a Vesicular stomatitis virus (VSV)-based Ebola vaccine has been recently approved by the European Medicines Authority (EMA) and the United States Food and Drug Administration (FDA) (Wolf *et al*., 2021).

VSV elicits strong T cell- and Ab-responses and there has been concern about pre-existing vector-specific immunity. According to a study of vaccination with both a VSV-based Lassa vaccine and a VSV-based Ebola vaccine, vector-specific immunity did not inhibit immunogenicity of the vaccines (Marzi *et al*., 2015). A VSV-based COVID-19 vaccine, BriLife®, which is currently in phase II of clinical trials, contains cDNA encoding the SARS-CoV-2 spike (S) protein and also carries Ag protein on its membrane (Yahalom-Ronen *et al*., 2020). This replicating viral vector also may contain spontaneously acquired Ag mutations, as the mutations found in SARS-CoV-2. As a result, BriLife® can induce neutralizing Abs towards alpha, gamma, and delta variants of COVID-19 at levels comparable to the wild type virus (Yahalom-Ronen *et al*., 2022).

Newcastle disease virus (NDV), an avian paramyxovirus,

is known as a safe virus for humans due to host restriction (Fig. 1C) (Kim and Samal, 2016). Several clinical trials using the oncolytic properties of NDV are underway (Zamarin and Palese, 2012). Recently, an NDV-based COVID-19 vaccine (Patria®) was evaluated in clinical trials. This vaccine can be produced at a low cost and can be administrated intramuscularly or intranasally (Ponce-de-León *et al*., 2022).

To protect from respiratory syncytial virus (RSV) infection, various vaccine types, including live-attenuated vaccines and virus-vector based vaccines, have been developed and are in the clinical trial stage (Biagi *et al*., 2020). Meissia Vaccines, Inc. developed a live-attenuated RSV vaccine using their AttenuBlock™ technology based on codon de-optimization for attenuation, thermal stability, and immunogenicity (Stobart *et al*., 2016). Recently, the same group developed a recombinant RSV expressing a chimeric SARS-CoV-2 S protein on its envelope; clinical trials with it are in progress.

Baculoviruses are insect viruses and have the advantage of being safe for humans, unlike animal viruses (Fig. 1D); replication and insertion into the genome do not occur and there is no pre-existing baculovirus-specific immune response (Lu *et al*., 2012). Baculovirus vectors have the advantage of being easy to manipulate and mass-production is possible at a low cost. Using baculovirus systems, efficient gene silencing through the delivery of microRNA has been reported in mammalian cells (Chen *et al*., 2015). They are currently used in vaccine development as an expression system to produce VLPs and recombinant protein vaccines. Baculovirus have the potential to serve as a safe delivery system for gene therapy and vaccines in the future.

Bacteriophages, which infect bacteria, are not directly pathogenic in humans (Fig. 1E); they are safe for use in humans and can be easily mass-produced using bacteria as production cells. Bacteriophages can display protein Ags fused with phage coat proteins and can deliver plasmid DNA encoding a target Ag (González-Mora *et al*., 2020). Research on bacteriophage-based vaccines is being conducted in experimental animals (Mascolo *et al*., 2007). Ag-specific immune responses might be induced by the administration of Ag-expressing bacteriophages, with or without adjuvants. The efficiency of bacteriophage uptake by endocytosis into APCs determines the capacity of bacteriophages as a delivery system. Targeting bacteriophages to dendritic cells (DCs) using anti-DEC205, showed improved immunogenicity of bacteriophage-based vaccine in the absence of adjuvants (Sartorius *et al*., 2011).

Recent bacteriophage-based clinical trials have used bacteriophages as a biological antibiotic for the targeted treatment of bacterial infections, but did not demonstrated their efficacy (Górski *et al*., 2020). Nevertheless, it was confirmed that the immunogenicity and safety issues of the bacteriophage itself were not significantly problematic in humans. The mechanisms of action and adjuvant effects originating from bacteriophage vectors in humans should be studied further.

Bacterial vectors

Majority of the preparations made with live bacteria are probiotics, which may be administered orally, and they can regulate abnormal intestinal fermentation (Plaza-Diaz *et al*., 2019). Generally, they are used to relieve diarrhea or constipation, and improve the intestinal environment. In addition, it has been reported that probiotics contribute to the prevention and treatment of infectious diarrhea (Ahmadipour *et al*., 2019) and reduction of serum cholesterol, and prevention of cancer (Kechagia *et al*., 2013).

Non-pathogenic commensal or mutual bacteria and attenuated microorganisms such as *Salmonella* or *Listeria* can be used as delivery vectors for genetic and protein Ags (Fig. 1F). As a delivery system, bacterial vectors have several advantages; genetic modification is relatively simple and they are easy to produce on a large-scale (Cao and Liu, 2020). Bacteria may function as an adjuvant with bacterial components. Additionally, they can adhere to or colonize on host cells and some bacteria are able to deliver their cargo into host cells.

Bacterial vectors can deliver genes expressing Ags, and are particularly good at doing this through mucosal routes. The mechanism of DNA transfer is not fully elucidated, but it has been proposed that bacteria are phagocytized by immune cells and the released DNA plasmids are taken up by APCs (Yurina, 2018). BacTRL™ is a technology for gene delivery that is based on commensal bacteria. This vaccine can be administered orally or through systemic injection. Currently, the oral COVID-19 vaccine using this delivery system is in the clinical trial stage (Sheridan, 2021). It contains live *Bifidobacterium longum* carrying a plasmid encoding the SARS-CoV-2 S protein (NCT04334980). Other vaccines for influenza and rotavirus infection using this delivery system are in the discovery stage.

Ags delivered by microbes may be expressed in intracellular, secreted, or surface-displayed forms. For Ag display on the surface of bacteria, autotransporters such as AIDA-I, adhesion involved in diffuse adherence-I of enteropathogenic *Escherichia coli*, and outer membrane proteins such as ice nucleation protein from *Pseudomonas syringae*, have been used as carrier proteins (Lee *et al*., 2003; Nicchi *et al*., 2021). A live *E. coli* surface display vaccine containing a *Mycobacterium avium* subsp. Paratuberculosis 3061c gene was developed using an ice nucleation protein surface display system and has been shown to induce strong humoral and cellular immune responses in intraperitoneally immunized mice (Xu *et al*., 2021). Genetically engineered bacteria for display of multiple Ags can be applied to develop the multivalent vaccines (Jong *et al*., 2014).

Stability during storage and transport is also important issues in the development of vaccines. One of the most resistant forms of bacteria is as a spore. Probiotics containing *Bacillus* spores are currently available in the market (Elshaghabee *et al*., 2017). *Bacillus subtilis* spores fused with the tetanus toxin fragment C (TTFC) via CotB, a coat protein of *B. subtilis*, have been used for vaccine development (Duc le *et al*., 2003).

To obtain a safe vaccine based on bacterial systems, bacteria-like particles (BLP) can be used in a mucosal vaccine. When food-grade *Lactococcus lactis* are killed under hot acidic conditions, their surface proteins and intracellular components are lost, however, spherical particles with peptidoglycan cell walls remain (Van Braeckel-Budimir *et al*., 2013). These particles can be used as an Ag delivery system with immune-stimulating activity through toll-like receptor-2. BLP-based vaccines prepared by binding RSV Ags to the surface of BLPs can improve the weak immunogenicity of recombinant protein vaccines (Van Braeckel-Budimir *et al*., 2013). FluGEM-A, a inactivated influenza nasal vaccine mixed with BLPs as an adjuvant has been evaluated in a phase I clinical trial (Van Braeckel-Budimir *et al*., 2013). The intranasal FluGEM-A vac-

cine was well tolerated and induced mucosal Ab and T cell responses in the immunized subjects. An intranasal SynGEM vaccine comprising of BLPs linked with the RSV F protein was reported to be safe and immunogenic in human volunteers (Ascough *et al*., 2019).

When attenuated pathogenic bacteria are used as vectors, their potential to regain pathogenicity needs to be evaluated. In addition, it is necessary to analyze the adjuvant capacity of the bacterial vector itself and the possibility of vector-induced adverse effects. Certain immune responses against the vectors may reduce immunogenicity to the target Ag and lead to safety issues. To develop safe bacteria-based vaccines without the risk of severe adverse effects including septic shock, genetic modification of bacterial lipopolysaccharides (LPS) to be expressed at low levels may be necessary.

Particle-based antigen delivery systems

Each particle may have different physicochemical, biological, and immunogenic properties depending on its shape, size, composition, chemical functional group, and so on (Bachmann and Jennings, 2010). Particulated Ags have a higher immunogenicity than the soluble form. The biodistribution is affected by the diameter of particle. In particular, nanoparticles of between 20 and 45 nm size are suitable for targeting DCs in lymphoid tissues (Reddy *et al*., 2006). Their shapes can affect not only biodistribution, but also the efficiency of uptake into macrophage (Xie *et al*., 2017). The surface molecules of the particles can be modified with various compounds such as polyethylene glycol (PEG), hyaluronic acid, and polysarcosine. Surface modification may affect the hydrophilicity of particles, half-life *in vivo*, and phagocytosis into macrophages (Gustafson *et al*., 2015; Mitchell *et al*., 2021). After endocytosis, they are processed and presented by APCs, which can result in cytotoxic T lymphocyte (CTL)- and Ab-responses. In addition, a particle-based delivery system is an appropriate platform for multivalent vaccines that can deliver several Ags together or a single Ag along with adjuvants. The strategy of lymphoid organ or DC targeting can help enhance the activity of nanoparticle-based vaccines (Campbell *et al*., 2018).

According to their chemical components, nanoparticles are classified into organic and inorganic categories. Further, organic particles can be classified as lipidic and polymeric particles (Fig. 2A, 2B). Lipidic particles include liposome, lipid nanoparticles (LNPs), lipoproteins, and microbubbles. Liposomes have the structure of one or more stacked lipid bilayers, containing an interior aqueous space (Corthesy and Bioley, 2018). Liposome-based gene delivery has advantages over viral delivery systems such as a large-size gene delivery capacity, biological safety, and easy purification. However, one disadvantage is that delivery efficiency is low with liposome-based gene delivery, compared to that with viral vectors (Elsana *et al*., 2019). Currently, several licensed vaccines, such as influenza vaccine (Schaad *et al*., 2000) and Herpes zoster (Tretiakova and Vodovozova, 2022), use liposomes as their Ag delivery systems.

Unlike liposomes, LNPs contain lipids in the core, allowing for drug encapsulation in a non-aqueous region (Tenchov *et al*., 2021). In particular, LNPs composed of cationic lipids are suitable as gene carriers because they form a complex with anionic nucleic acids and bind well to the cell membrane (Guevara *et al*., 2020). LNPs are composed of relatively solid lipids and have improved physical stability. Currently, LNPs are used clinically as mRNA delivery systems in COVID-19 vaccines (Lamb, 2021).

Recently, Entos Pharmaceuticals has introduced the Fusogenix proteo-lipid vehicle (PLV) platform (Sheridan, 2021). The Fusogenix PLV consists of neutral lipids and fusion-associated small transmembrane (FAST) proteins, which fuse with the target cell membrane. As a result, cargo is transferred directly into the cytoplasm, rather than being transferred by endocytosis. A PLV-formulated DNA vaccine encoding the SARS-CoV-2 S protein and genetic adjuvants has entered a phase II clinical trial (Sheridan, 2021).

Physiologically, high-density lipoproteins (HDLs) deliver cholesterol from the periphery to the liver. The mechanisms of scavenger receptor type B1-mediated lipid uptake for HDL have been reported (Raut *et al*., 2018). When nucleic acids or chemical drugs are encapsulated and administered using HDLs, they are delivered into the cytoplasm using an independent endocytic pathway. The use of HDLs would allow for an efficient, safe, and targeted vaccine delivery system.

Gas-filled microbubbles can also be used as an Ag delivery system (Corthésy and Bioley, 2017). Microbubbles are typically 1-10 μm in size and consist of a shell and a high molecular weight gas core. Shell materials can be proteins, polymers, or lipids. Microbubbles are taken up by DCs where they efficiently deliver Ags to induce an Ag-specific immune response. Microbubbles also possess the immune-stimulating ability to induce DC activation; they induced efficacy at levels comparable to conventional lipids or polymer-based Ag delivery systems. In addition, intranasal mucosal vaccination with a formulation of gas-filled microbubbles and protein Ags induced mucosal and systemic immune responses and protection against *Salmonella* infection in mice (Pigny *et al*., 2016).

Cationic polymers have been used to deliver DNA, siRNA, and mRNA (Lou *et al*., 2019). Anionic nucleic acids bind to cationic polymers to form nanoparticles called polyplexes (Iqbal *et al*., 2020). It has bene shown that synthetic peptides with a glutamic acid-alanine-leucine-alanine repeat could selectively enter DCs via endocytosis and subsequent endosomal escape (Lou *et al*., 2019).

Chitosan is a natural cationic polymer obtained from chitin. Chitosan can form complexes with DNA and contribute to improved DNA stability and transfection efficiency (dos Santos Rodrigues *et al*., 2019). In addition to genetic Ags, protein Ags can be delivered by nanoparticle systems. Chitosan can interact with negatively charged Ags and produce Ag-encapsulated chitosan. Intranasal administration of HIV gp140-chitosan nanoparticles in female volunteers induced $CD4^+$ T cell and humoral immune responses following intramuscular boosting (Cosgrove *et al*., 2016).

Dendrimers are composed of hyper-branched monomers with central core. They are characterized by their homogeneous size, various functionalities through surface functional groups, multivalency, water solubility, and ability to contain drugs (Chowdhury *et al*., 2022). Some dendrimer-based chemotherapeutics, including docetaxel, are currently being tested in clinical trials (Alven and Aderibigbe, 2020). When used as a gene delivery system, the dendrimer structure can protect internal mRNA from enzyme degradation. A polyamidoamine dendrimer-based mRNA vaccine was developed and shown to be effective in preclinical studies (Nitika *et al*., 2022). Due to the stable multivalency of dendrimers, they have been recognized as a useful vaccine platform.

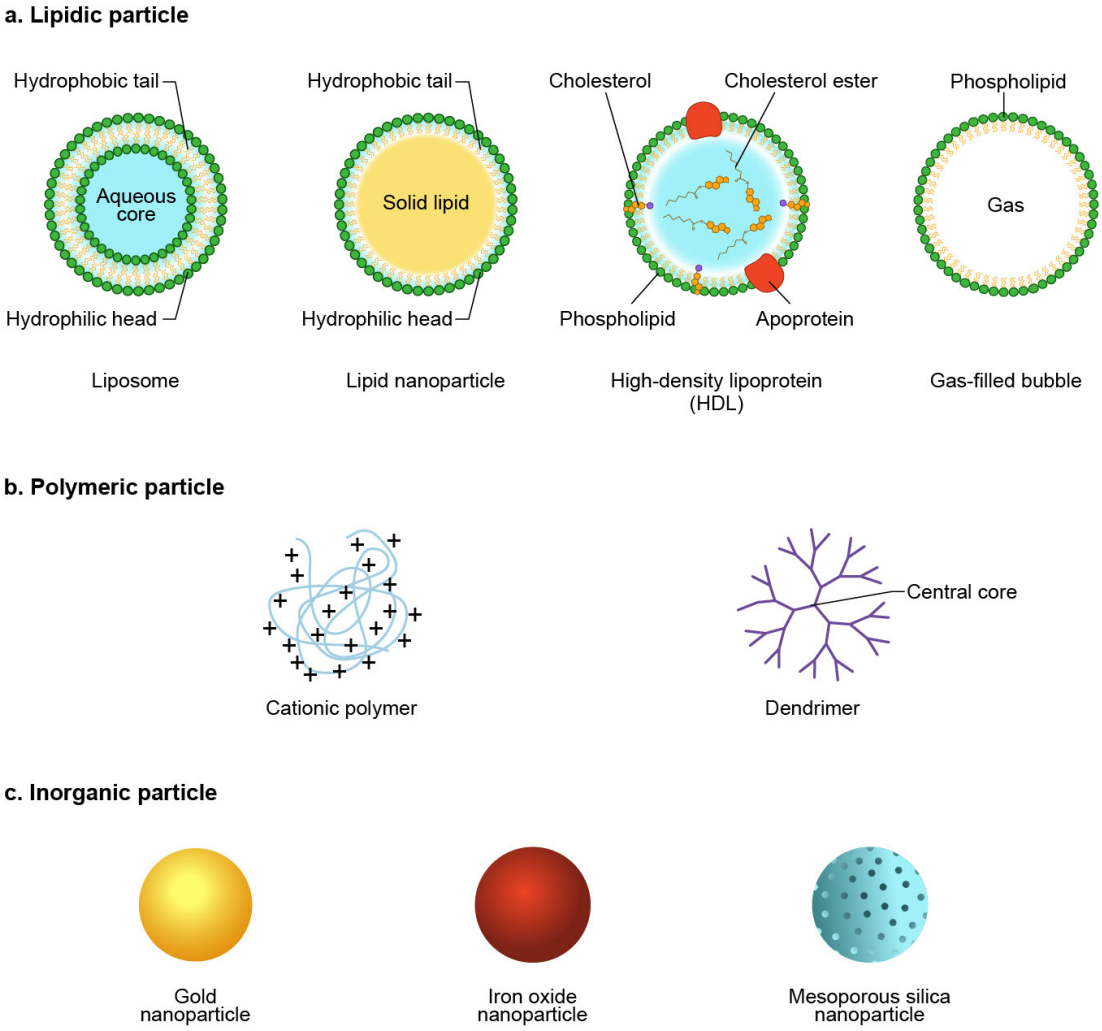

Fig. 2. Structures of nanoparticle-based delivery systems. (A) Lipidic particles. Liposomes are enclosed with one or more lipid bilayers and contain an interior aqueous space. Lipid nanoparticles are composed of a lipid layer with solid lipids in the core. High-density lipoprotein (HDL) is complex and composed of phospholid and apoproteins. Microbubbles are gas-filled spheres with shells. (B) Polymeric nanoparticles include cationic polymers and dendrimers. (C) Inorganic nanoparticles include gold nanoparticles, iron-oxide nanoparticles, and mesoporous silica nanoparticles.

In addition to lipid- and polymer-based particles, their hybrid forms are attracting attention as non-viral gene delivery systems. Lipopolyplexes (LPP) are complex compositions of lipids, polycations, and nucleic acids at a nanoscale size (Rezaee *et al*., 2016). LPP have advantages of both liposomes-nucleic acids complexes, including high stability and low cytotoxicity, and polymers-nucleic acids complexes, including high transfection efficiency and homogenous particle size. An LPP-based mRNA vaccine for COVID-19 was developed that was highly immunogenic and generated protection against SARS-CoV-2 infection in mice and monkeys without systemic adverse effects (Yang *et al*., 2021). This vaccine is currently being evaluated in a clinical trial.

For use in inorganic particle-based Ag delivery systems, gold nanoparticles (GNPs), iron-oxide nanoparticles, and mesoporous silica nanoparticles (MSNs) have been developed (Fig. 2C). Currently, GNP are being evaluated in clinical trials for the treatment of cancers using photothermal therapy or cancer diagnostics (Singh *et al*., 2018). GNP were also studied for their ability to deliver genes via gene gun bombardment (Chang *et al*., 2008). Iron-oxide nanoparticles have been approved for cancer diagnosis and photothermal therapy (Soetaert *et al*., 2020). While these are promising developments, the biocompatibility of inorganic nanoparticles is relatively poor compared to that of organic nanoparticles.

MSNs are recognized as one of the most efficient drug delivery system (DDS) and gene delivery systems (Porrang *et al*., 2022). Their pore structures provide them with the advantages of easy synthesis and manipulation, and improved biocompatibility, stability, and loading efficiency. As an RNAi therapeutic delivery system, a porous silica nanoparticle named DegradaBALL (Degradable nanoBALL) has been tested, showing durable and effective gene silencing (Kang *et al*., 2020). It can protect RNA from enzymatic degradation and allow for endosomal escape after endocytosis into cells. This system has been applied for the delivery of proteins, including

recombinant interleukin-2, and this strategy has been shown to increase therapeutic half-life and reduce systemic adverse effects (Kim *et al*., 2022a).

It is important to develop stable nanoparticles of appropriate sizes. Because small particles have a larger surface area to volume ratio, the release of drugs on or close to the particle surface is faster. However, small particles have a greater risk of particle aggregation during storage. In addition, the early release of cargo from particles before cellular uptake may result in decreased delivery efficiency. The degree of biodegradation of the Ag delivery system affects cargo release. Thus, studies of the release patterns, including after storage and administration to the patient, are necessary.

Self-assembled nanoparticles

Self-assembled molecules exist in various organisms including viral capsids, enzymes, and cytoskeletons (Fig. 3A). Ferritin, an iron storage protein, naturally self-assembles and forms nanoparticles, and provides the protein structure to generate potent immunity as an Ag delivery system (Kim *et al*., 2022b). These particles can contribute to immunogenicity because their size and shape are suitable for Ag presentation. They have advantages of multivalency and biocompatibility. Additionally, they can be stored for several months in a refrigerated state.

In one study, genetically fused influenza virus Ag and *Helicobacter pylori* ferritin was expressed in mammalian cells and spontaneously assembled into nanoparticles (Kanekiyo *et al*., 2013). Immunization with this nanoparticle influenza vaccine resulted in high levels of neutralizing Abs and increased protection in virus-challenged ferrets. In clinical trials, influenza-ferritin nanoparticle vaccines elicited immunogenicity (Powell *et al*., 2021). A recent study reported the protective immune responses against SARS-CoV-2 infection in rhesus monkeys immunized with a vaccine containing SARS-CoV-2 RBD S proteins displayed on ferritin nanoparticles (Li *et al*., 2021). Furthermore, a modifiable sortase-A-tagged ferritin based platform has been introduced (Saunders *et al*., 2021); SARS-CoV-2 RBD S protein was conjugated to ferritin nanoparticles by sortase A. This vaccine elicited a potent cross-reactive neutralizing Abs against SARS-CoV-1, SARS-CoV-2, and mutants of the SARS-CoV-2 in monkeys.

Computationally designed, self-assembling, two-component protein complexes, I53-50A and I53-50B, have been recently reported (Bale *et al*., 2016). Trimeric I53-50A and pentameric I53-50B can be assembled into 120-subunit complexes (I53-50) with icosahedral symmetry. SARS-CoV-2 RBD fused with I53-50A was expressed in mammalian cells to obtain proper glycosylated proteins, while I53-50B was produced in an *E. coli* system. These methodologies have the advantages of high production efficiency and high stability. This vaccine was approved for the prevention of COVID-19 by the Ministry of Food and Drug Safety, Republic of Korea (available at covid19.trackvaccines.org).

VLPs are one type of self-assembled nanoparticles, but they may be classified separately from other self-assembled protein/peptide nanoparticles due to their viral origin (Fig. 3B). Particles with a similar size and shape as VLPs, but made with completely synthetic particles are called synthetic VLPs (Ghasparian *et al*., 2011). VLPs can deliver Ag without the risk of viral infection because they do not have a viral genome. The viral capsid proteins in which Ag proteins are fused are expressed via genetic recombination.

Previously, plant-based manufacturing system for VLP vaccine against influenza had been described (Landry *et al*., 2010). Whole plants were transfected with *Agrobacterium* containing hemagglutinin expression cassette, and six days after infection, plants were harvested and VLP could be purified. This VLP influenza vaccination induced both humoral and cellular immune responses and good safety profiles in humans (Landry *et al*., 2010, 2014). This system has advantages of quick production and scalability, therefore, it may be preferred in pandemic situations.

VLPs are currently used for Hepatitis B (Engerix-B®) (Ho *et al*., 2020), Hepatitis E (Hecolin®), HPV (Cervarix®, Gardasil®, and Gardasil 9®) (Gebre *et al*., 2021), and SARS-CoV-2 (Stander *et al*., 2022) as a preventive vaccine platform. A recently developed VLP vaccine against COVID-19 was immunogenic and well-tolerated in heathy volunteers (Ward *et al*., 2021) and was approved in Canada in 2022 (Stander *et al*., 2022). In addition, studies applying VLPs as anti-cancer vaccines, gene therapy, and as DDSs are ongoing (Shirbaghaee and Bolhassani, 2016).

As a recombinant protein production system, various cells

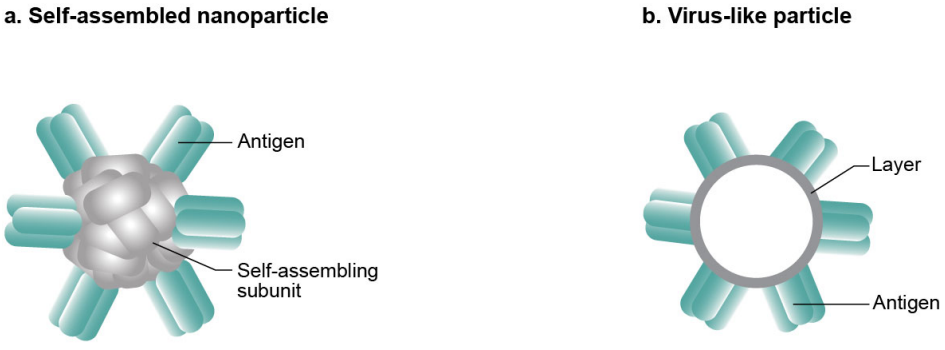

Fig. 3. Structures of self-assembled nanoparticles and virus-like particles. (A) Self-assembled nanoparticles, including ferritin, provide surfaces to display Ag. Ferritin self-assembles into highly ordered nanoparticle. Ags can be genetically fused or conjugated to ferritin. (B) Viral-like particles (VLP) consist of assembled viral proteins and lack viral genetic material. Capsid proteins fused with Ag proteins can be produced through genetic engineering, and self-assemble into VLPs. Ag can be attached to the surface of pre-formed particles. Enveloped VLPs have a lipid membrane, while non-enveloped VLPs have a capsid protein layer.

such as plant cells, mammalian cells, bacteria, insect cells, and yeast can be utilized. Cervarix® used insect cells, and Gardasil® and Gardasil 9® use *Saccharomyces cerevisiae* as an expression system (Stander *et al*., 2022). Although the mammalian cell expression system is relatively expensive and complicated, it may be necessary to obtain protein Ags with the characteristic glycosylation and folding patterns for vaccine development.

Cells and artificial cells

To generate protective immune responses, it may be essential to activate T cells through presentation of Ags via professional APCs. Compared to the T-independent immune response, the T-dependent immune response results in more efficient class switching or affinity maturation of Abs (Weinstein *et al*., 2012) and contributes to establishing a memory response (Ahrends *et al*., 2019). Furthermore, it is known that depending on the host, particularly in the case of immunosuppressed patients with cancer or infection, Ag uptake/processing/presentation by APCs may not occur efficiently (Bashaw *et al*., 2017; Bandola-Simon and Roche, 2019). Therefore, to optimize the induction of an Ag-specific immune response, *ex vivo* APC modulation may be necessary.

There have been various approaches to elicit strong cell-mediate immunity for HIV-1 eradication using DC-based vaccines (da Silva *et al*., 2018) (Fig. 4A). When mRNA encoding HIV-1 Ags was delivered to autologous DCs and then administered to patients with HIV-1, Ag-specific T-cell responses were induced without severe adverse effects, but these effects were transient (Gandhi *et al*., 2016) . In addition, a DC vaccine loaded with cytomegalovirus (CMV) pp65 mRNA was designed to target CMV proteins that are expressed in glioblastoma (Batich *et al*., 2020). Clinical trials using DC vaccines demonstrated prolonged long-term survival in patients. Recently, phase I and II clinical trials of autologous DCs loaded *ex vivo* with SARS-CoV-2 S protein were conducted for safety and quality testing (NCT04386252).

There is a limitation to the application of APC-based vaccines, especially as general-purpose vaccines, due to the necessity of isolating cells from patients, the risk of contamination in the cell culture process, and consequentially, the high cost of production. An artificial cell structure that can mimic APCs, induce immunogenicity at a relatively low cost, and activating T cells as efficiently as APCs could be beneficial (Perica *et al*., 2014). Recently, their potential as drug carriers has been highlighted, due to their stability and biocompatibility (Emir Diltemiz *et al*., 2021). As APCs, artificial cells present Ags and costimulatory signals to T cells. Recently, novel iron-oxide dextran-coated particles displaying major histocompatibility complex (MHC)-Ig dimers and B7.1-Ig via direct chemical coupling have been developed (Fig. 4B) (Perica *et al*., 2014). This nanoparticle could present epitope peptides to T cells and has been shown to induce T cell expansion *in vitro* and result in tumor rejection in a murine tumor model. Currently, a COVID-19 vaccine using artificial APCs is in clinical trials (NCT04299724).

Extracellular vesicles

Cell-derived EVs are a group of small-sized, lipid-based vesicles, involved in various cellular processes. EVs are comparable to liposomes, however, they are natural cell-derived vesicles with various lipids and surface proteins. There are several types of EVs such as exosomes, microvesicles, and apoptotic bodies (Herrmann *et al*., 2021). In the physiological process, they may function as signal carriers for cell-to-cell communication.

Exosomes are originated from intraluminal vesicles in endosomes (Chen *et al*., 2021). Secreted intraluminal vesicles are exosomes that consist of lipid layers, proteins, and nucleic acids such as DNA and RNA (Fig. 5A). Exosomes have high cellular uptake efficiency and biocompatibility; however, they are less immunogenic as an Ag delivery system. Therefore, they have attracted attention as a DDS capable of delivering various substances with little toxicity. Recently, there have been several studies investigating the function of exosomes as a delivery system for genes or Ags (Chen *et al*., 2021).

DC-derived exosomes (Dex) include MHC class I and II and costimulatory and adhesion molecules, and can be loaded

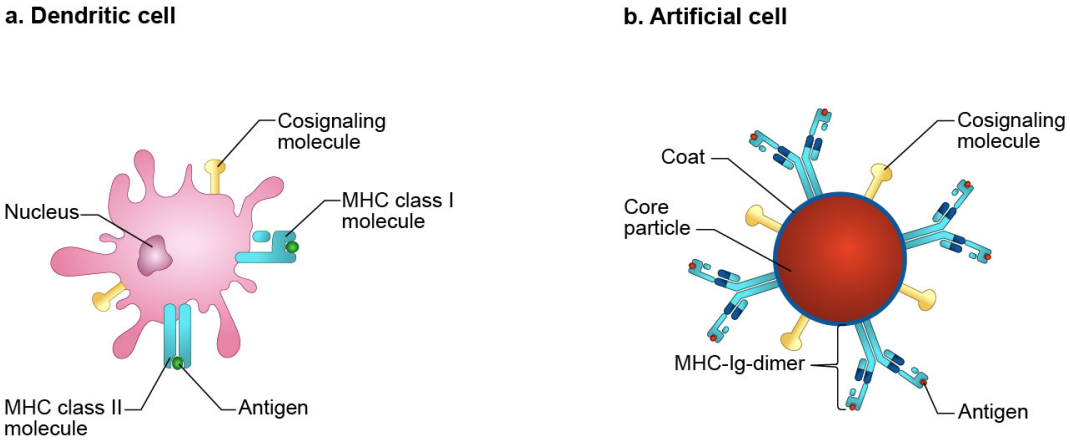

Fig. 4. Structures of natural and artificial cell-based delivery systems. (A) On the surface of dendritic cells, there are peptide-loaded MHC class I/II molecules and cosignaling molecules. Dendritic cells acquire Ag through introduction of genetic Ag or pulsing of antigenic peptides/proteins. Binding of T cell receptor to peptide-MHC complex and interaction of costimulatory molecules result in T cell activation. (B) Artificial cells consist of coated particles, surface-displayed peptide-loaded MHC-Ig dimers, and molecules for co-stimulation. They can present antigenic peptide-MHC complex and deliver the activation signal of costimulatory molecules to T cells.

with multiple Ags (Xia *et al*., 2022). It has been reported that Dex can activate T cell responses directly or indirectly via endogenous DCs. Most studies on Dex have been conducted on patients with various cancers (Xia *et al*., 2022), but initial clinical studies showed limited anti-cancer therapeutic responses.

EVs may originate from gram-positive or gram-negative bacteria. One of the EVs, OMVs, are released from gram-negative bacteria via a budding process, and may be used as a DDS or Ag-delivery system (Fig. 5B). OMVs contain lipids, proteins, DNA, and various pathogen-associated molecular patterns (PAMPs), such as LPS, which can function as adjuvants. Engineered OMVs have been applied in diagnosis and treatments (Huang *et al*., 2022). As an Ag delivery system, OMVs can improve Ag-specific immunity with their immunostimulatory molecules and are easily produced and purified in large scales. Due to the endotoxicity of LPS, detergent extraction or genetic modification to reduce this may be performed; however, during this process immunogenicity of the vaccine may be decreased.

OMV-based vaccines for protection against *Neisseria meningitides* serogroup B have been licensed for use. Besides, there are several types of multivalent OMV group B meningococcal vaccines, including detergent-treated OMVs and native OMVs, currently in clinical trials (van der Pol *et al*., 2015). Recently, a genetically modified OMV vaccine against *Shigella sonnei* has entered clinical trials (Mancini *et al*., 2021). This genetically modified bacteria-produced OMV contains penta-acylated LPS with low endotoxicity and immunodominant O-Ag. The application of this vaccine induced protection in animal models and was well-tolerated and immunogenic in human studies (van der Pol *et al*., 2015). Several OMV-based vaccines against other bacteria, including *Salmonella* and *Bordetella pertussi*s, have been investigated in animal studies (van der Pol *et al*., 2015).

A heterologous, Ag containing OMV vaccine has been developed. OMVs with surface Ags could deliver Ags to APCs, which could activate Ag-specific T cell hybridoma (Daleke-Schermerhorn *et al*., 2014). Surface-bound Ags on OMVs could elicit Ag-specific Ab responses efficiently, compared with luminal Ags (van der Pol *et al*., 2015). In addition, OMV-based vaccines presenting virus protein Ags have been reported (Shehata *et al*., 2019). *E. coli* transformed with virus Ags produced OMVs comprised of chimeric Ags of MERS-CoV and H1N1 influenza. Therefore, OMV-based vaccine approaches are not limited to OMV-producing bacteria, but can be applied to viral infection and cancers as well.

CONCLUSIONS

With the advent of new infectious diseases, new types of vaccines have been developed and licensed. It is necessary to identify the types of vaccines not yet in clinical use to understand the future of vaccine trends.

Various Ag delivery systems can be classified into subcellular and entity/cellular level systems (Fig. 6). Considering subcellular level systems, there are macromolecules, cell-inspired lipidic particles, and virus-inspired VLPs. These have advantages including simple development and production processes. Organelle-like EVs are originated from cells and can deliver various cargos instead of cells. Inorganic particles have the advantages of easy synthesis and manipulation, and high stability. As entity/cellular level systems, microorganisms, such as viruses and bacteria, are efficient systems to deliver genetic and/or protein Ags, and adjuvant effects are expected due to the presence of PAMPs. APCs are specialized to stimulate Ag-specific immune responses, but the complex manufacturing processes of cell-based vaccines are a barrier to widespread use.

An ideal vaccine can maintain protective efficacy against various mutant strains of the targeted pathogen. To obtain this cross-protection, conserved Ag selection is essential. In addition, the approach of constructing a multivalent vaccine combining various mutation-specific Ags may be beneficial. In the development of COVID-19 vaccines, multivalent vaccines showed an efficacy against several variants (Kim *et al*., 2022b). Multivalent vaccines containing the Ags of different pathogens have been developed to induce protective efficacy against multiple diseases simultaneously.

Optimal Ag delivery systems can improve the efficacy, safe-

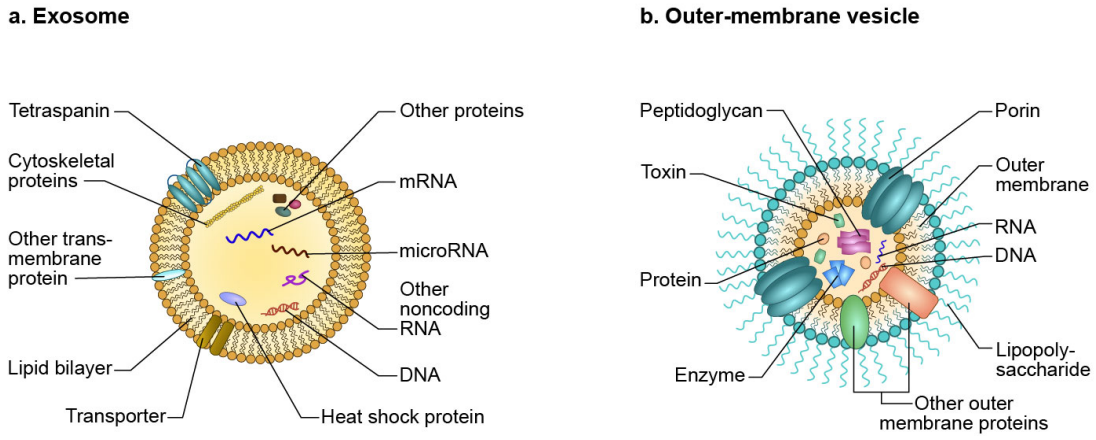

Fig. 5. Structures of exosome and outer membrane vesicles. (A) Exosomes consist of lipid layers and several membrane proteins. They contain various cargo including proteins and nucleic acids, such as DNA and RNA. (B) Outer-membrane vesicles (OMV) are enclosed by bacterial outer-membranes that are embedded with proteins and LPS. There are nucleic acids, proteins, enzymes, toxins, and peptidoglycan in OMVs.

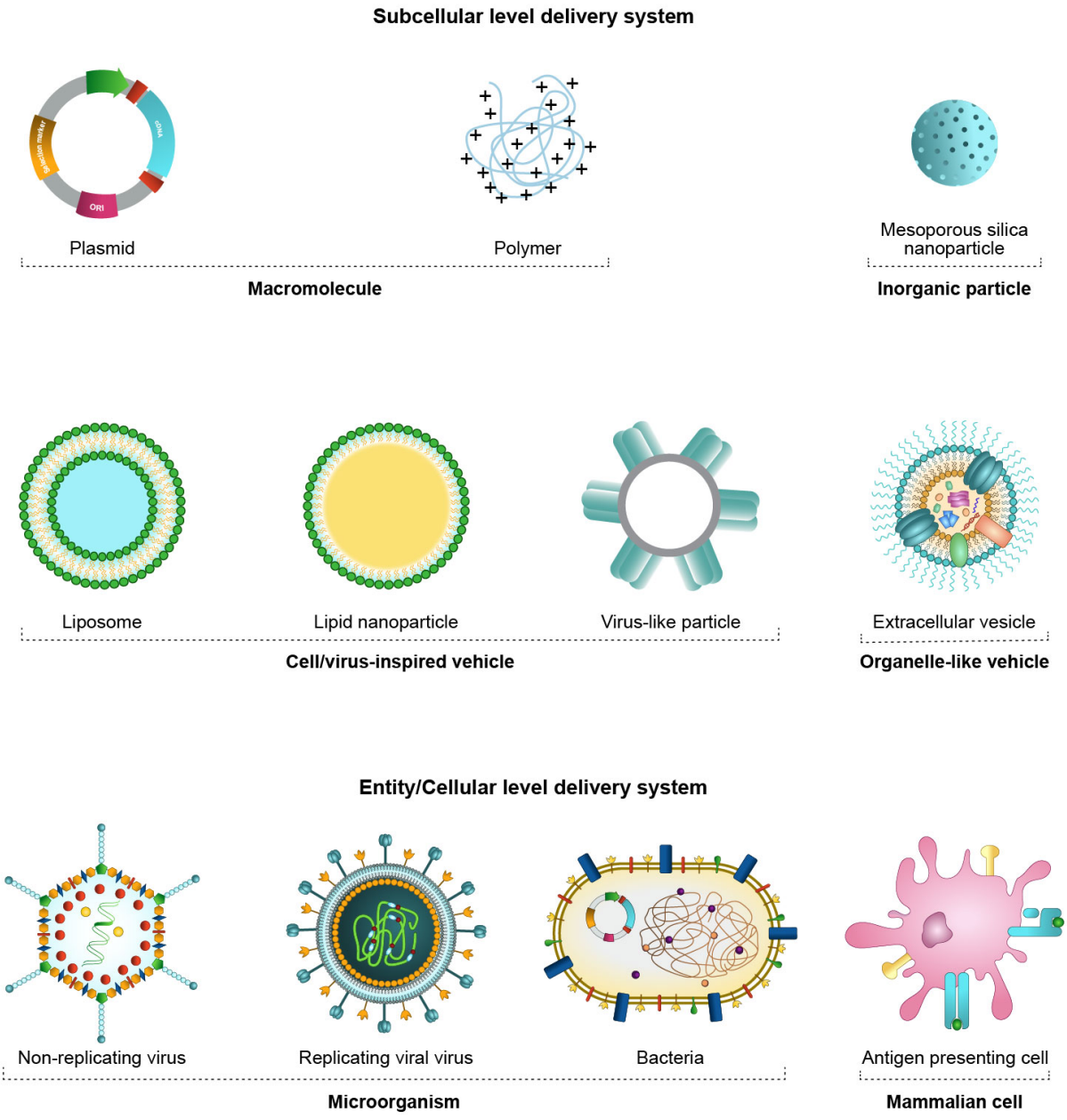

Fig. 6. Schematic illustration of subcellular and entity/cellular level antigen delivery systems. Subcellular level systems include macromolecules (nucleic acids and polymers), bio-inspired vehicles (liposomes, lipid nanoparticles, and virus-like particles), organelle-like extracellular vesicles, and bio-irrelevant inorganic particles. Microorganisms, such as viruses and bacteria, and mammalian cells can be used to deliver antigens.

ty, and stability of vaccines and, together with technological advances concerning Ags and adjuvants, may be the key to overcoming the limitations of current vaccines.

CONFLICT OF INTEREST

The 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.

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