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Vaccine formulation design: challenges and opportunities

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The rise in activity and multi-faceted impact of infectious agents such as human immunodeficiency virus and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused an unprecedented increase in morbidity and mortality around the globe. The spread of infectious diseases at an alarming rate has led to accelerated research on vaccine therapeutics, which can be further exemplified with COVID (coronavirus disease) vaccine development as a global emergency. This review aims to provide insights into vaccine development, components, manufacturing processes, types/platforms and strategies to improve their efficacy. The development of vaccines comprises four stages: (1) exploratory and preclinical, (2) clinical, (3) approval and (4) manufacturing and post-marketing surveillance. Vaccine formulations comprise antigens, adjuvants, preservatives, stabilizers, antibiotics, diluents and trace components. Vaccine manufacturing is a multi-step process involving antigen generation, release, purification, addition of other ingredients (*e.g.*, adjuvants, preservatives, stabilizers, *etc.*), quality control testing and filling. Conventional vaccine platforms include live attenuated, inactivated/killed, toxoid, polysaccharide and polysaccharide conjugate, synthetic peptide and virus-like particles. Advanced technologies include viral vectors, bacterial vectors, DNA (deoxyribonucleic acid) and RNA (ribonucleic acid) vaccines. These platforms provide rapid development of vaccines at a relatively low cost compared to conventional counterparts. Several approaches have been adopted for improving vaccine efficacy such as the inclusion of adjuvants and delivery of vaccines *via* mucosal and transcutaneous routes. Efficient uptake of vaccine antigens by microfold cells (found in the epithelium covering mucosa-associated lymphoid tissues) with subsequent transfer to the underlying antigen-presenting cells provides an efficient vaccine delivery route. In the case of the transcutaneous route, abundant antigen presenting cells found in the skin layer (*e.g.*, Langerhans) ensure efficient vaccine delivery and induction of potent immune responses. Additionally, both these routes can overcome limitations associated with traditionally employed parenteral routes, such as risk of disease transmission in unhygienic conditions and reuse of contaminated needles, production of biohazardous waste, requirement of trained personnel for administration, invasiveness and poor patient compliance. Identification of conserved pathogenic sequences using advanced genetic engineering methods, machine learning, and artificial intelligence can help in developing efficient vaccines. Moreover, global partnerships, funding and provision of resources from the World Health Organization (WHO) can ensure vaccine development, testing and research activities for developing countries.

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1. Introduction

Recent outbreaks such as the Ebola epidemic (2014)[1] and coronavirus disease 2019 (COVID-19)[2] pandemic indicate that infectious diseases remain unpredictable and pose a real and serious threat to public health as well as the global economy. In addition to infectious diseases, chronic non-communicable disorders are considered the leading cause of morbidity and mortality around the globe. Chronic non-communicable diseases including cancer, diabetes, Alzheimer's, cardiovascular and respiratory diseases are responsible for ~71% of all deaths. Vaccination is the most effective technique to prevent or eradicate serious infectious diseases and treat non-communicable ailments.[2–6] Vaccines, whether applied as a prophylactic or therapeutic modality, can limit infection transmission, prevent clinical manifestation of disease and establish herd immunity. Mass vaccination campaigns have led to reduced morbidity and mortality associated with several infections

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such as smallpox, poliomyelitis, hepatitis, diphtheria, tetanus and COVID-19. Various therapeutic vaccines are currently in clinical trials against chronic non-communicable diseases including cancer (*e.g.*, lipid nanoparticle-based mRNA-4157),[7] diabetes (*e.g.*, interleukin-1β-targeted epitope peptide (1βEPP))[8] and hypertension (*e.g.*, angiotensin II vaccine AGMG0201).[9] As per World Health Organization (WHO) reports, approximately 2–3 million deaths are prevented annually through vaccination programs.[2,10] This review provides insights about vaccine development, components, manufacturing, safety, efficacy, types, techniques, delivery platforms, current challenges and strategies to improve their efficiency.

2. Vaccine development and manufacturing

2.1. Vaccine development stages

The development of a new vaccine will typically take up to ~10–15 years. The process of vaccine development comprises four stages including (1) exploratory and preclinical, (2) clinical, (3) approval and (4) manufacturing and post-marketing surveillance (Fig. 1). The first stage takes ~2–3 years and involves basic laboratory research as well as reverse vaccinology/computational modelling to identify antigens capable of serving as vaccine candidates. Trials are conducted using cell/tissue-culture systems, organ chips and animal models to assess vaccine safety, efficacy and immunogenicity.[11,12] The second stage takes approximately 6–8 years and involves human clinical trials in three different phases *i.e.*, I, II and III. During phase I, a small number of healthy and immunocompetent individuals are administered developed vaccine primarily to assess safety. Additionally, appropriate dose and immune responses are evaluated as secondary effects. During phase II, the developed vaccine is administered to a large number (hundreds) of individuals divided into different groups (*e.g.*, elderly *versus* young) to evaluate safety, dose, interval between doses and immunogenicity. During phase III, the vaccine is administered to thousands of individuals to assess efficacy measured as the percentage of reduction in the rate of incidence of infection in the vaccinated group compared to the placebo counterpart. The data is assessed by artificial intelligence and machine learning tools.[11,13,14] After completion of human trials and confirmation of vaccine safety, efficacy and immunogenicity, the developed vaccine moves to the third stage which takes approximately 1–2 years. During the approval stage, the concerned regulatory bodies (*e.g.*, Food and Drug Administration (FDA), European Medicines Agency) review the results of preclinical and clinical trials and approve the developed vaccine for the fourth stage which takes ~1–2 years. During the manufacturing and post-marketing surveillance stage, the vaccine is produced on a large scale, marketed and monitored for effectiveness within the population. During this stage, adverse effects are also recorded.[11,14]

During recent years, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) vaccine was developed quite rapidly (within ~1 year) compared to the conventional time duration. Several factors accelerated the development of SARS-CoV-2 vaccine, including hefty financial investment, past research experience, rapid regulatory review and clinical testing. Globally, billions of dollars were invested from different public and private sources due to the pandemic urgency and massive demand; this accelerated the research and development of SARS-CoV-2 vaccine. Previous research related to innovative vaccine platform technologies, including viral vectors and messenger ribonucleic acid (mRNA), with other coronaviruses like severe acute respiratory syndrome coronavirus 1 (SARS-CoV-1) and Middle East respiratory syndrome (MERS) providing a background to build upon. The previous research experience offered developers with a better understanding of virus structure, transmission mechanism and target (*e.g.*, the spike protein in mRNA-, viral vector- and protein-based vaccines) for inducing an immune response. Moreover, advanced genome editing technologies such as clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 reduced the timeline for developing the vaccine.[15,16] Regulatory bodies adopted a proactive approach by setting minimum manufacturing

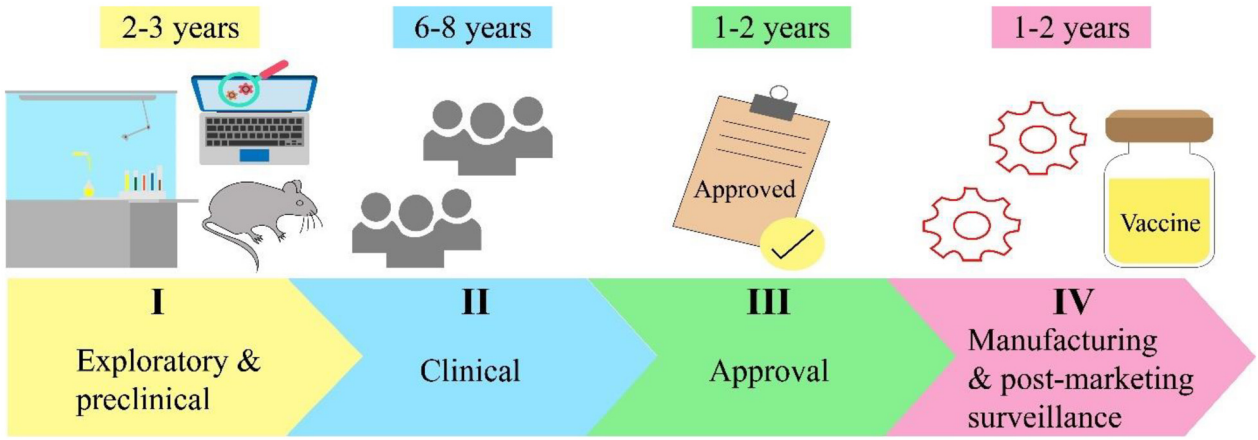

Fig. 1 Stages of vaccine development.

requirements of clinical and non-clinical data. In addition, the regulators prioritized SARS-CoV-2 vaccine reviews over other medicinal products which improved the timeline. Clinical trials in countries with a high burden of SARS-CoV-2 and at sites with adjustable research capacity as well as infrastructure led to a rapid development of vaccine. This indicates that the conventional time consuming barriers to the development and commercialization of vaccines can be effectively addressed; this will lead to their rapid manufacturing in the future.[15]

2.2. Vaccine components

Generally, vaccines comprise active ingredients, adjuvants, preservatives, stabilizers, antibiotics, diluents and other trace components (Fig. 2). The active ingredient is an antigen that is a modified or partial form of disease-causing microbe and stimulates the immune system without causing disease. Vaccines are divided into several categories based on the types of antigens *e.g.*, live attenuated,[17,18] inactivated/killed,[19] toxoid,[20] polysaccharide and polysaccharide conjugate,[10,21] synthetic peptide,[22,23] virus-like particles,[24] bacterial vectors,[25] viral vectors,[26] DNA[27,28] and RNA.[10,29] These vaccine types will be discussed later in the manuscript.

Adjuvants or enhancers, also known as immune potentiators or modulators, improve efficacy and immunogenicity of vaccines. The addition of adjuvants helps induce a rapid, robust and long-lasting immune response. Adjuvants exert their action through different mechanisms. The first mechanism involves an enhanced antigen presentation and depot

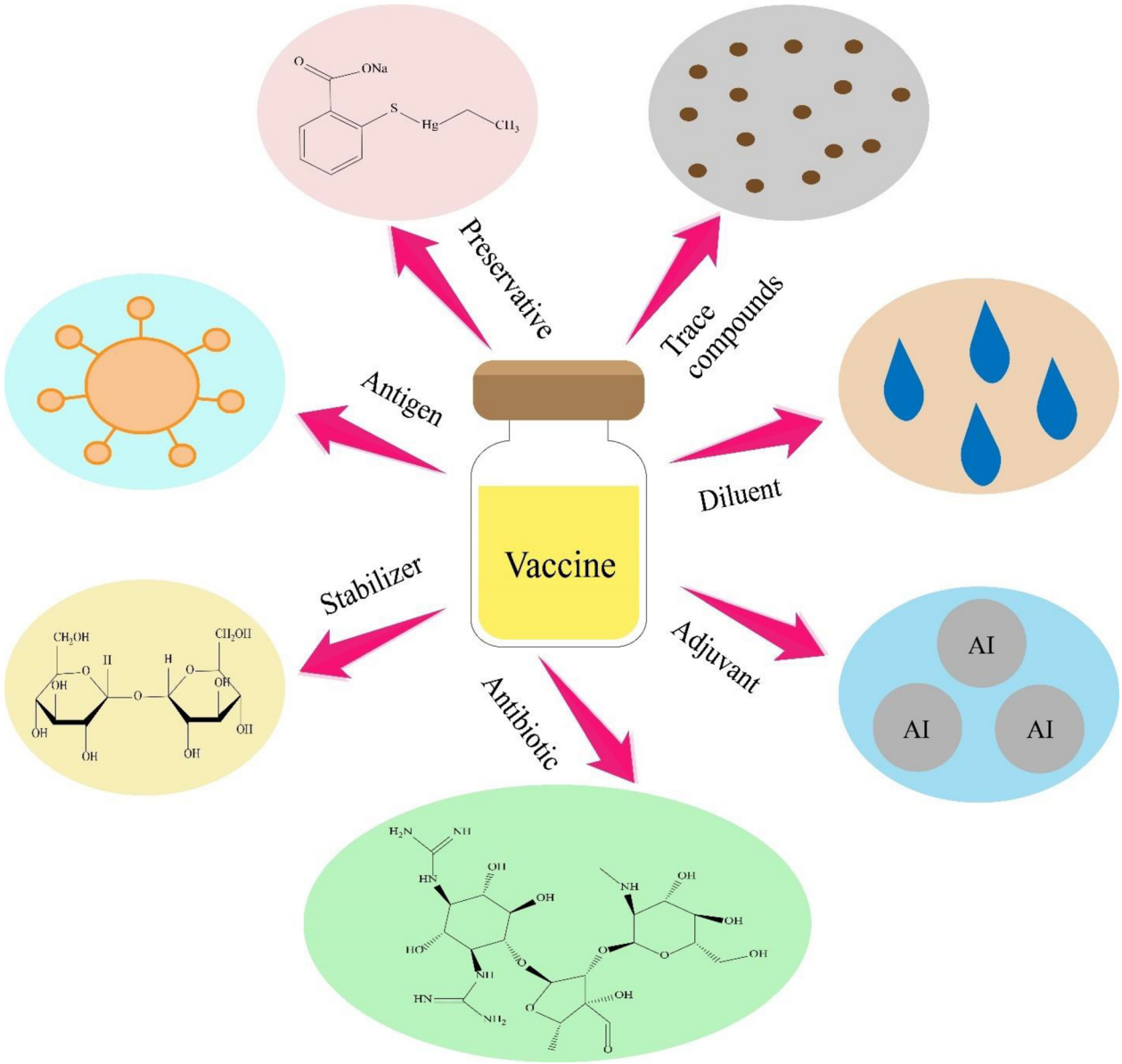

Fig. 2 Common constituents of vaccine formulation.

formation resulting in a high antigen concentration at the site of injection; in turn, this leads to an improved uptake by antigen presenting cells such as macrophages, dendritic cells and B cells.[30] Examples of adjuvants reported to enhance antigen presentation at the site of injection include aluminum salts (*e.g.*, aluminum hydroxide, aluminum phosphate, and aluminum hydroxy phosphate sulphate), liposomes (*e.g.*, monophosphoryl lipid A-based liposomal adjuvants), emulsions (*e.g.*, MF-59, a squalene-based oil-in-water emulsion) and nanoparticles (*e.g.*, polylactic-*co*-glycolic acid-based nanoparticles).[30–32] A series of four different adjuvants has been developed by GlaxoSmithKline (GSK), Brentford, UK:[2,38] AS01 (monophosphoryl lipid A and a saponin derivative QS-21 combination-based liposomal adjuvant),[33] AS02 (monophosphoryl lipid A and QS-21-based oil-in-water emulsion),[34,35] AS03 (alpha-tocopherol, squalene and polysorbate 80-based oil-in-water emulsifier)[36] and AS04 (comprising a combination of monophosphoryl lipid A and an aluminum salt).[37] The second mechanism involves transient secretion of chemokines and cytokines that recruit various immune cells at the site of injection, leading to the generation of a local pro-inflammatory environment. The recruited cells express several pathogen-recognition receptors both on the surface (*e.g.*, toll-like receptors and C-type lectin receptors) and intracellularly (*e.g.*, nucleotide-binding and leucine-rich repeat receptors and retinoic acid-inducible gene I-like receptor). These pathogen-recognition receptors are recognized by the adjuvants resulting in the maturation and activation of recruited cells. The activated cells then migrate to draining lymph nodes, interact with the antigen-specific B or T cells and activate immune response.[39] An example includes activation of monocytes and macrophages by aluminum salts.[30] In recent years, several polysaccharides (*e.g.*, saponins,[40] chitosan,[41] glucans,[42] fructans,[43] mannans,[44] hyaluronic acid,[45,46] alginate,[47] polyglutamic acid,[48] polyethyleneimine,[49] polylactic acid[50] and polymethyl methacrylate[51]) have been investigated as adjuvants. Other recently explored new adjuvants include CAF01 (combination of dimethyldioctadecylammonium and trehalose dibehenate),[52] JVRS-100 (combination of octadecenolyoxy[ethyl-2-heptadecenyl-3 hydroxyethyl] imidazolinium chloride-based liposome, cholesterol and a DNA plasmid),[53] SAF (squalene, Tween 80 and Pluronic L121-based oil-in-water emulsion),[30,54] ISCOMs (based on phospholipids, cholesterol and QS-21)[55,56] and IC31 (comprises 11-mer antibacterial cationic peptide (KLK) and ODN1a, which is a synthetic oligodeoxynucleotide).[30,56,57]

Several preservatives have been added to vaccine formulations to prevent bacterial or fungal contamination, such as phenol, 2-phenoxyethanol and thiomersal. Stabilizers protect vaccines from adverse conditions including heat, freeze drying, *etc*. Amino acids (*e.g.*, glycine, monosodium salt of glutamic acid), proteins (*e.g.*, gelatine, human serum albumin), sugars (*e.g.*, sucrose, lactose, trehalose) and silk have been employed to stabilize vaccine formulations. Antibiotics are added during the manufacturing stage to prevent microbial contamination. These antibiotics are not part of final vaccine formulations. Some examples of antibiotics added in vaccine formulations include neomycin, streptomycin, polymyxin B, gentamicin and amphotericin B. Diluents or suspending fluids used in vaccine preparations include sterile water, saline and protein containing fluids. Other trace constituents may include residual inactivators (*e.g.*, formaldehyde, glutaraldehyde) and cell-culture materials (*e.g.*, chicken egg protein).[10,30]

2.3. Vaccine manufacturing steps

Vaccine manufacturing is a multi-step process including antigen generation, release, purification, addition of other ingredients (*e.g.*, adjuvants, preservatives, stabilizers *etc*.), quality control testing and filling (Fig. 3).[58,59]

The first step involves generation of antigen (followed by inactivation or subunit isolation) or a recombinant protein derived from the disease-causing agent. Viruses are grown on primary cells (*e.g.*, chicken fibroblasts) or continuous cell lines (*e.g.*, MRC-5, a diploid cell-culture line comprising of fibroblasts). Bacteria are commonly grown in bioreactors. Recombinant proteins are produced in bacteria, yeast or cell culture. Initially, a master cell bank (collection of vialed cells) is established. The master bank is extensively characterized with subsequent generation of working cell banks.[60]

The second step involves release of antigen from the substrate and its isolation from the culture medium. The third step involves purifying the produced antigen. This step varies with vaccine type; *e.g.*, inactivation of antigen is performed for an inactivated viral vaccine without further purification. In contrast, the purification step involves multiple unit operations of column chromatography and ultrafiltration in the

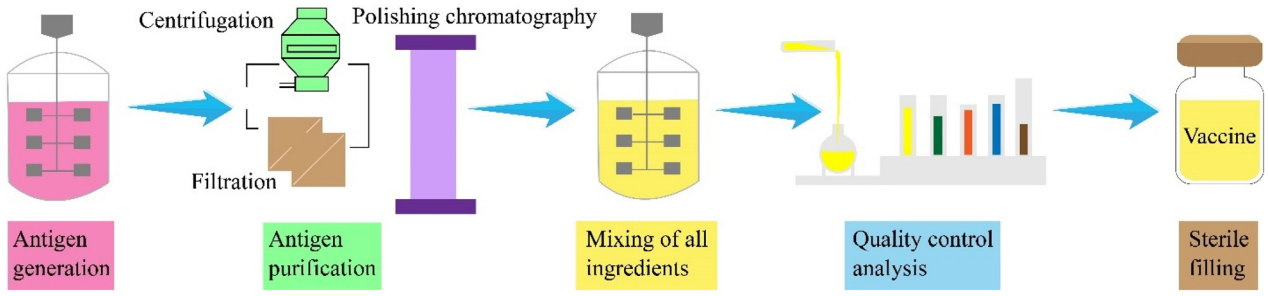

Fig. 3 Basic steps of vaccine manufacturing.

case of recombinant proteins.[60,61] In the next step, other formulation constituents are added, such as adjuvants, preservatives, and stabilizers. All formulation components are thoroughly mixed in a container.

The fifth step includes quality control tests including potency, sterility, purity, safety or other product-specific assays. During this step, sterile single or multiple-dose vials are filled with formulation followed by sealing with de-pyrogenated stoppers/plungers. If the formulation requires freeze-drying, the stoppers are partially inserted to permit removal of moisture during the drying process. Finally, outer caps are placed to secure stoppers. All containers are then inspected critically to detect any minute cosmetic or physical defects. As with the formulation phase of the vaccine manufacturing operation, extensive control and monitoring of the environment and critical surfaces are conducted during operations. Quality control testing is also done at this stage. Throughout all these stages, extensive control and environmental monitoring are conducted to avoid any contamination.[59–63]

3. Safety and efficacy of vaccines

3.1. Induction of immune responses by vaccines

A brief presentation of the immune system responses is shown in Fig. 4. Vaccines are aimed to induce a rapid immunity and long-term antigen-specific immune memory. Upon administration, host immune cells (antigen-presenting cells (APCs) comprising macrophages, B-lymphocytes, dendritic cells and Langerhans cells) recognize vaccine antigens. This recognition is mediated by proteins that can identify pathogen-associated molecular patterns. These pattern recognition receptors exist on the surface (*e.g.*, toll-like receptors) and in the cytoplasm (*e.g.*, retinoic inducible gene I) of antigen-presenting cells. The interaction between antigens and recognition receptors on the antigen presenting cells stimulates intracellular signaling events that can lead to phagocytosis, maturation and secretion of cytokines.[64,65] The internalized antigens are displayed on cell surface receptors known as major histocompatibility complex (MHC). Antigens that are generated in or move into

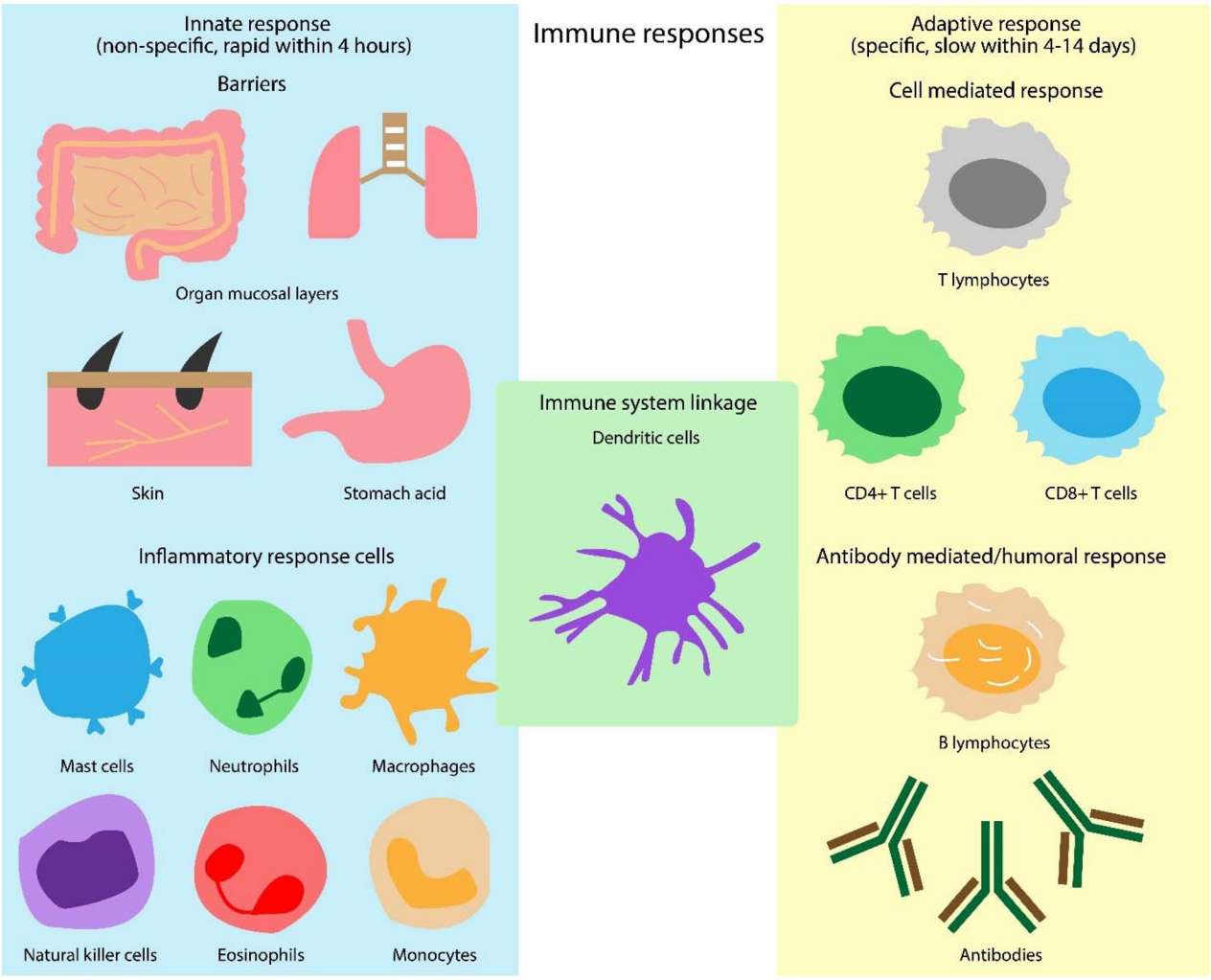

Fig. 4 Representation of immune system responses.

the cytoplasm (*e.g.*, live attenuated viruses) are exhibited by MHC-I and are recognized by the T-cell receptor of $CD8^+$ T cells. Antigens which move into cells *via* phagocytosis (*e.g.*, inactivated viruses or antigens that are shed from infected cells) are exhibited by MHC-II and are recognized by $CD4^+$ T cells.[66] Extracellular antigens are displayed on MHC-I through vacuolar or endosome-to-cytoplasm cross-presentation pathways.[67]

Activated antigen-presenting cells displaying antigens on MHCs move to the draining lymph nodes and spleen to encounter naïve T-cells.[68] These naïve cells differentiate and proliferate into effector cells following interaction between antigen-presenting cells and T-cells through MHC and T cell receptor binding. $CD4^+$ and $CD8^+$ cells need additional signals from co-stimulation and cytokines for activation and differentiation. The interaction between ligands and co-receptors on antigen presenting and T-cells (*e.g.*, CD80 and CD28, respectively) governs co-stimulation. Cytokines exist in the microenvironment or are secreted by the antigen-presenting cells. $CD4^+$ T-helper cells differentiate into effector T helper lineages as a result of MHC-II and T cell receptor binding, ligand–receptor interaction and signals by cytokines.

Cues from T helper 1 cells (*e.g.*, interferon-gamma) and interaction between T cell receptors and MHC-I leads to the differentiation of $CD8^+$ T cells into cytotoxic (killer) cells that recognize and eliminate infected cells. The effector cells, $CD4^+$ and $CD8^+$ lymphocytes, differentiate into memory cells (*e.g.*, effector and central memory). T helper 1 cells promote the production of Immunoglobulin (Ig) G1 and G3 antibodies by B cells.[66] The production, maturation and differentiation of B cells into memory B and antibody-secreting plasma cells is facilitated by Interleukins (IL) 4, 5 and 13 which are secreted by T helper 2 cells. Follicular T helper and T helper 17 cells are responsible for producing high-affinity antibodies and mucosal immune responses, respectively. Follicular T helper cells regulate the affinity maturation of B cells and selection of high-affinity germinal center B cells which differentiate into high-affinity memory B cells. These memory cells differentiate into antibody-secreting plasma cells, which move from the draining lymph nodes germinal centers to the bone marrow, followed by the generation of antibodies over a few months to decades. These antibodies provide protection against reinfection.[10,69–71]

B cells can also identify and respond to antigens before T helper cells are involved. Upon vaccine delivery, B cells recognize and internalize antigens followed by differentiation into short-lived antibody-secreting cells (plasmablasts). These cells generate the first wave of antibodies: IgM.[66]

A thorough understanding of immune responses can help in the development of efficient vaccines. For example, vaccines capable of promoting the endogenous antigen processing pathway or cross-presentation and generating a robust cytotoxic T-cell response can serve as suitable candidates against intracellular pathogenic organisms.[10]

3.2. Strategies for improving the efficacy of vaccines

The route of administration is a key factor that significantly influences the efficacy of vaccines, just like the inclusion of adjuvants. Currently, most vaccines are delivered *via* the parenteral route. However, several disadvantages are associated with this traditional vaccine administration route such as the requirement of trained personnel, production of biohazardous waste, risk of transmission of blood–borne diseases by the reuse of contaminated syringes (particularly in developing countries), needle-prick injuries/invasiveness and poor patient compliance. During recent years, needle-free approaches have been extensively explored to overcome these drawbacks. Needle-free delivery ensures relatively improved safety for the vaccinator, vaccinee and community, compliance with vaccination schedules, minimal pain, rapid administration and reduced cost. More recently, mucosal and transcutaneous immunizations have emerged as promising needle-free alternatives.[2,45,72,73]

Mucosal surfaces serve as entry points for the majority of pathogens due to their higher permeability. The vaccines delivered at mucosal surfaces can mimic natural infectious agents and act more efficiently. Vaccine antigens are taken up by the microfold (M) cells residing in the epithelium covering gastrointestinal-associated lymphoid tissues, bronchus-associated lymphoid tissues and nasal-associated lymphoid tissues. The antigens are then transferred to the underlying antigen-presenting cells such as macrophages, dendritic cells and B cells (Fig. 5). Vaccines capable of specifically targeting microfold cells (*e.g.*, live attenuated) after mucosal delivery induce robust and potent immune responses.[2,74–76] Some benefits associated with mucosal vaccination include the induction of mucosal and systemic immunity. However, the coating of a mucus layer on all mucosal surfaces works against successful immunization. Other drawbacks associated with this mode include the risk of antigen instability/dilution before absorption at mucosal surfaces and difficulty in stimulating a robust IgA antibody response (practically).[77–79] An efficient immune response can be successfully elicited by the addition of strong mucosal adjuvants (*e.g.*, *Escherichia coli* heat-labile enterotoxin, cholera toxin and their nontoxic mutants) to the formulation. However, the internal use of these adjuvants raises safety concerns due to the ganglioside-binding property of the cholera toxin B subunit, which permits the migration of these molecules along the olfactory nerve fibers and finally into the olfactory lobes of the brain. A novel adjuvant (CTA1-DD) comprising of cholera toxin A (ADP ribosylating) subunit fused with a peptide (DD) and capable of targeting B lymphocytes acts as a strong mucosal adjuvant with a high level of safety. Another promising adjuvant for mucosal vaccines is chitosan; a polycationic polysaccharide.[74,75]

Transcutaneous vaccines are also capable of inducing robust and potent immune responses because the epidermis serves as a chief immunological organ containing antigen-presenting cells such as Langerhans cells, which are dendritic cell variants that cover ~25% surface area of the skin (Fig. 6). However, the keratinized stratum corneum must be breached to ensure successful vaccine delivery. Disruption of the top skin layer not only increases permeation of antigens but also results in the secretion of proinflammatory cytokines; in turn, this activates the immune system.[74,80–82]

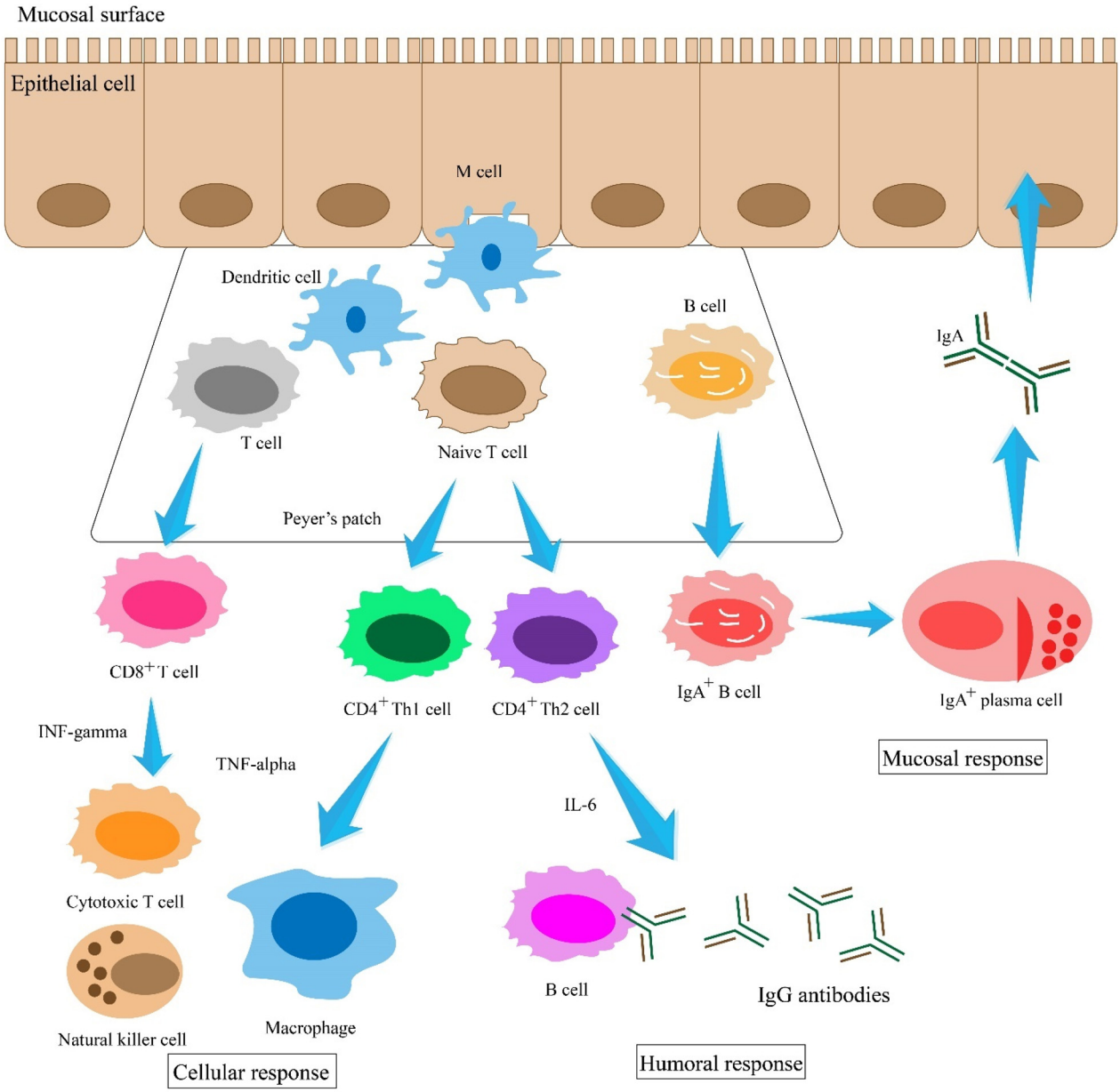

Fig. 5 Representation of immune responses by the mucosal route.

Several methods used to make stratum corneum permeable for efficient vaccine delivery include hydration, abrasion, electroporation, iontophoresis, microneedle patches, needle free jets and powder injections (Table 1).

Stratum corneum hydration is typically achieved by occlusion and wetting of the skin, which leads to the swelling of keratinocytes that facilitates diffusion of the vaccine formulation into the skin though the intercellular spaces. Abrasion, commonly achieved by tape striping, result in disruption of the outer skin layer and an increased permeation of vaccines through the skin.[85,97,98] Electroporation involves the application of a high voltage (>50 V) electric pulse for a short time (micro or milli seconds), generating transient and reversible aqueous pores in the lipid bilayer of the stratum corneum. This process results in increased antigen permeation.[99–101] In iontophoresis, a relatively low voltage (≤10 V) electric pulse is continuously applied to push the antigens into the deeper skin tissues.[45,102]

A microneedle (MN) patch comprises an array of sequentially positioned micron-scaled needles that generate transient pathways across the stratum corneum without activating the underlying nociceptors.[103–105] The MNs directly deliver vaccines into the cutaneous layers and achieve stronger immune responses than the intramuscular injections. However, the diameter, length, tip geometry, and density of MNs may influence their skin perforation efficiency, thereby affecting antigen

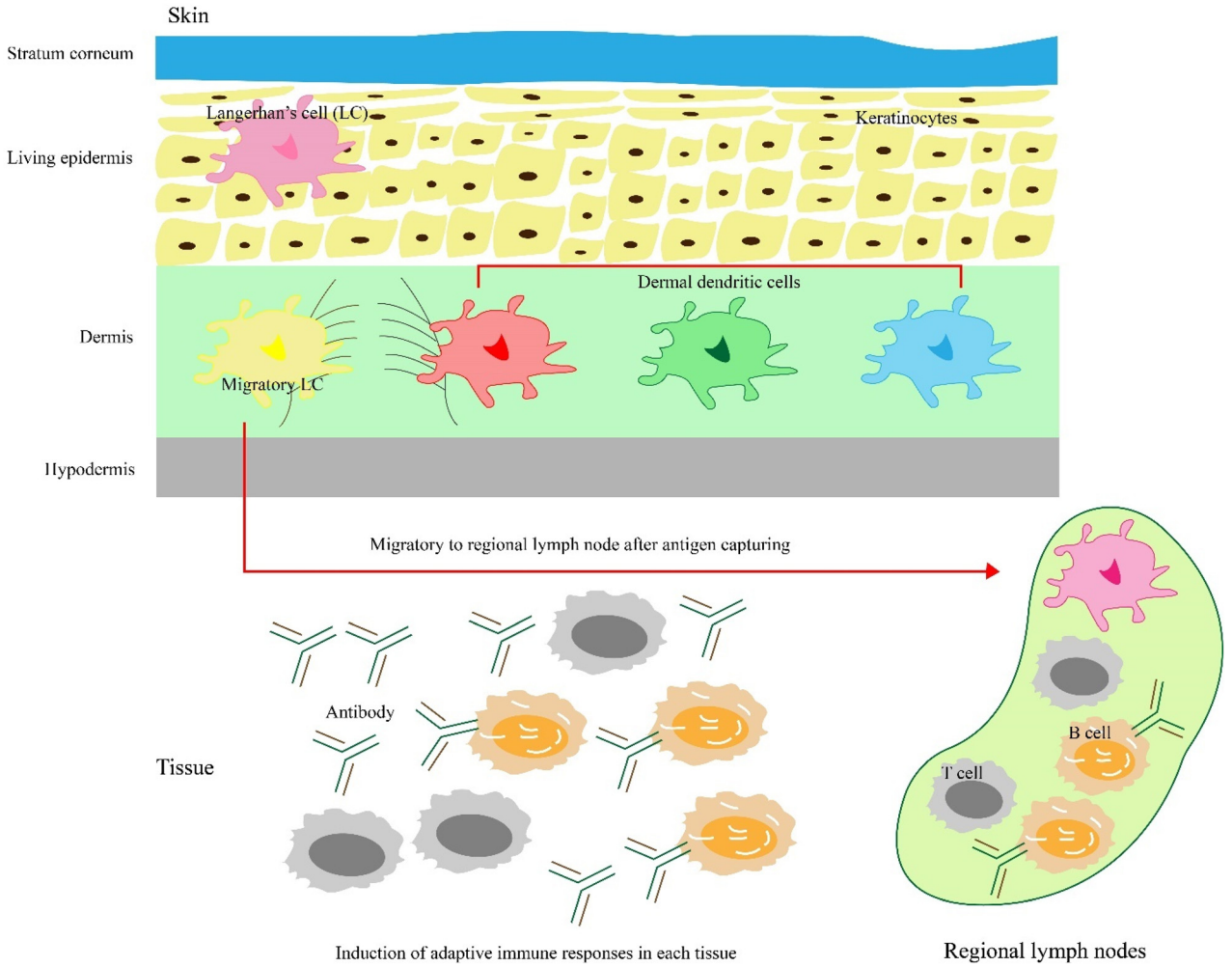

Fig. 6 Representation of immune responses by the transcutaneous route.

delivery and the elicited immune response. The MNs are fabricated in four different designs; solid, coated, soluble/dissolving and hollow. Solid MNs commonly prepared using metals pierce the skin, resulting in the generation of micropores. They are removed after piercing followed by the application of topical formulation that easily diffuses into the deep skin layers through micron-sized pathways. In case of coated MNs, vaccines are coated onto the needle surface and administered/deposited directly into the skin upon piercing. MNs commonly fabricated using soluble polymers (*e.g.*, polyvinyl pyrrolidone, carboxymethyl cellulose, and hyaluronic acid) and sugars (*e.g.*, maltose and dextrin) dissolve, leading to the rapid distribution of antigens into the surrounding environment. This polymer/sugar-based MN design efficiently addresses safety concerns associated with metal-based counterparts, such as fracture and the retention of sharp, biohazardous materials in the skin. Hollow MNs create conduits for the diffusion of liquid vaccine formulations into the skin. This MN design permits precise control over the flow rate and the effective concentration of antigen to be delivered.[99,106–108]

Jet injectors operate by forcing fluids through a nozzle to produce high pressure jets at a velocity of 100–200 m s^{-1} using compressed gas or a spring. The high impact jet leads to piercing of fluid into the skin. A powder injector accelerates dry vaccine formulations deep in the cutaneous layers by using fast expansion of helium gas. However, some literature reports suggest that there is a risk of transmission of blood–borne infections using these injectors because of the contamination with body fluids.[85,97–99]

In addition to the above-mentioned needle-free and minimally invasive approaches, biocompatible elastic lipidic vesicles (*e.g.*, liposomes) and micro- or nano-scaled polymeric particles have been extensively used for efficient, pain-free delivery of vaccines. However, literature reports suggest that the lipidic or polymeric carrier mediated vaccine delivery elicited relatively weak immune responses compared with the traditional intramuscular counterpart due to their insufficient transdermal penetration of nanoparticles (NPs). Therefore, efficient penetration of these carriers requires skin pretreatment to mitigate the stratum corneum barrier.[99,109,110] Several litera-

Table 1 Transcutaneous delivery of vaccines by various permeation enhancing/stratum corneum barrier disrupting techniques

Approach	Vaccine formulation	Observations	Ref.
Hydration	Heat-labile enterotoxin (LT, obtained from *Escherichia coli*) absorbed on a single-ply polyester-rayon gauze pad with polyethylene backing covered by a dressing	The application of the prepared formulation to the skin in wet gauze elicited robust LT-antibody responses in humans	83
Abrasion	Influenza vaccine	Antibody response following stratum corneum disruption using emery paper, D-Squame tape or 3 M tape were ~100 to ~300 times higher than that elicited by hydration alone	84 and 85
	Heat-labile enterotoxin (LT, obtained from *Escherichia coli*)	Pretreatment with emery paper or tape stripping followed by application of LT containing gauze patch resulted in a relatively anti-LT antibody titre compared to hydration alone in human participants	84 and 85
Electroporation	Orthopoxvirus DNA vaccine	Immunogen-specific neutralizing antibodies were induced in rabbits, which protected the animals against aerosolizes rabbitpox virus	86
	DNA vaccine against Middle East respiratory syndrome coronavirus (MERS-CoV)	DNA vaccine delivery using higher voltage (200 V) led to 10-fold greater cellular responses than that induced by lower voltage (100 V) in Guinea pigs	87
Iontophoresis	Rabies vaccine loaded hyaluronic acid, PVP, D-sorbitol-based iontophoresis-microneedle (MN) patch combination	Iontophoresis-MN patch combination treatment led to a ~206% increase in the IgG titre of dogs compared to the untreated counterpart The virus specific neutralizing antibody titre was increased by ~2.2 times (compared to threshold value) following iontophoresis-MN patch combination treatment	45
	mRNA encoding tumour-associated antigen gp100	In the disease model (melanoma-bearing mice), iontophoresis mediated vaccine delivery led to a significant reduction in tumour volume *via* generation of cytokines and stimulation of cytotoxic $CD8^+$ T cells	88
	Cancer antigen gp-100 peptide KVPRNQDWL, methoxy-polyethylene glycol, 2-(*N*,*N*-diethylamino) ethyl methacrylate	Accumulation of gp-100 peptide and nanogels in the epidermis Increase in the number of Langerhans cells in the epidermis A significant reduction in tumor growth	89
Microneedles	Dissolving tetanus toxoid vaccine loaded MN patches comprising of sodium carboxymethyl cellulose (CMC), polyvinyl pyrrolidone (PVP), D-sorbitol	A significant increase in IgG (~21%), interferon-γ (~30%), $CD4^+$ (~41.5%), and $CD8^+$ (~48.5%) cell counts was observed in tetanus vaccine containing MN patches treated albino rats with respect to the untreated group	72
	Influenza virus vaccine, CMC and trehalose comprising formulation coated on solid stainless steel MNs	Haemagglutination inhibition titres in mice following vaccine coated MN treatment were comparable with the intramuscular counterpart	90
	SARS-CoV-2-S1 subunit vaccine loaded dissolving MN patches fabricated using CMC	Potent antigen specific antibodies elicited in mice following vaccine loaded MN patch application	91
	Bacillus Calmette–Guérin (BCG) vaccine loaded dissolving MN patch prepared using sodium alginate and trehalose	An increase in IgG antibodies from 3 g L^{-1} to 5.98 g L^{-1} Leucocytes increased from 2.6×10^9 L^{-1} to 18.45×10^9 L^{-1} Granulocytes increased from 14.4% to 29.15% Lymphocytes increased from 58.75% to 85.3%	73
	Tumor antigen peptide (OVA257–264: SIINFEKL) fused with hepatitis B core protein virus like particles and mesoporous silica nanoparticles	Dendritic cell maturation stimulated Antigen specific anti-tumor immune response stimulated	92
Liquid injector	Hepatitis A vaccine	Virus specific antibody titre elicited in human subjects by a liquid injector was significantly greater than that induced with a conventional hypodermic needle	93
	Hepatitis B plasmid DNA vaccine	In regenerating muscle, the gene transfer was ~10 times more efficient than that in normal mouse muscle A relatively rapid and potent humoral response was recorded in regenerating muscle compared with normal mature muscle	94
Powder injector	Influenza vaccine formulated with trehalose into a powder	In mice, the antibody titre induced by the powder injector approach was ~300% and ~700% higher than that elicited by intramuscular and subcutaneous injection, respectively	95
	Influenza antigen coated gold particles	Histological examination indicated the presence of antigen-coated gold particles in the epidermis Transmission electron microscopy analysis indicated the intracellular localization of particles In mice, antigen-specific cytotoxic T lymphocytes were recorded as well as antibody responses	96

Table 2 Transcutaneous delivery of vaccines using a combination of lipidic/polymeric carriers and skin barrier disruption techniques

Approach	Vaccine formulation	Observations	Ref.
Iontophoresis, liposomes, nanoparticles	Vaccine formulation comprising ovalbumin (as model antigen) containing liposomes and silver nanoparticles	Iontophoresis mediated delivery led to a 92-fold increase in the epidermal permeation of ovalbumin compared to passive delivery In mice, antibody induction and differentiation of immune-competent cells was successfully achieved	111
Iontophoresis, nanoparticles	Hen egg-white lysozyme (model antigen) encapsulated poly lactic-*co*-glycolic acid (PLGA) nanoparticles coated with chitosan hydroxypropyltrimonium chloride	Iontophoresis mediated delivery led to a ~9.6 times higher concentration of lysozyme in mouse skin compared with the amount that permeated in the absence of iontophoresis A 2.1 times higher concentration of lysozyme was recorded in mouse skin following iontophoretic delivery of antigen loaded nanoparticles compared to plain antigen solution Lysozyme specific IgG1 and IgG2a titres were significantly higher following iontophoresis and nanoparticle combination treatment compared with the subcutaneous injection counterpart	112
Solid MNs, liposomes	Ovalbumin (model antigen) loaded CD11c monoclonal antibody immunoliposomes	Tumour growth was inhibited and overall survival was prolonged in mice A potent humoral response (in terms of the antibody titre ratio of IgG1/IgG2a) was achieved following solid MN and liposome combination treatment	113
Hollow MNs, nanoparticles	Ovalbumin, toll-like receptor agonist imiquimod, monophosphoryl lipid A encapsulated in PLGA nanoparticles	Antigen loaded nanoparticles administered using polymeric hollow MN patch induced a significantly higher IgG2a antibody response and number of interferon (IFN)-γ secreting lymphocytes compared with intramuscular delivery of antigen loaded nanoparticles	114
Electroporation, nanoparticles	PLGA nanoparticles containing luciferase encoding plasmid as a reporter and vaccine plasmids encoding antigens from the porcine reproductive and respiratory syndrome virus	Electroporation treatment successfully induced luciferase expression in the skin Local production of IL-1β, IL-8 and IL-17 was achieved The combination of electroporation and nanoparticles promoted the recruitment of various myeloid subsets A relatively potent and broad interferon-γ T-cell response was recorded compared with the conventional hypodermic needle mediated delivery and MN patches	115

ture reports indicate efficient vaccine delivery using a combination of micro- or nano-scaled carriers and skin treatment/stratum corneum breaching approaches (Table 2).

Hair follicles offer an opportunity to circumvent the skin barrier; however, the hair orifices cover only ~0.1% of the skin surface. Nevertheless, the infundibula of hair follicles, which can penetrate up to ~2000 mm into the skin, increases the surface area. Transfollicular delivery allows the uptake of vaccine antigens by the rich pool of perifollicular and skin antigen presenting cells. However, approximately 30% of hair follicles were inactive and closed due to the presence of keratinized materials, lipids, cell debris *etc*. Pretreatment, such as hot waxing, can enhance transfollicular permeation of vaccines. Hot waxing involves the application of a warm and viscous liquid wax to the skin, followed by hair depilation. The enlargement of follicular pores due to hot wax and hair depilation can facilitate vaccine diffusion. Recently, transfollicular vaccine delivery has received increased attention; however, research into this area is still in its initial stages.[99]

4. Types of vaccines

Vaccines are generally categorized based on their ability to multiply within the host or the technology employed in their development. Conventional vaccines typically comprise single or multiple antigens obtained from inactivated/weakened pathogenic organisms, or their constituents, such as protein subunits, toxins *etc*. Currently, most commercially available vaccines are based on this conventional approach. However, this traditional methodology cannot generate efficient vaccines against complex pathogenic microorganisms that display high variability and rapidly evade the immune system.[116] Advanced technologies using recombinant DNA (deoxyribonucleic acid) generate a relatively more potent immune response.[10] A schematic presentation of the induction of immune responses by these vaccine types is shown in Fig. 7. In this section, both conventional and advanced vaccine platforms/types will be discussed in detail.

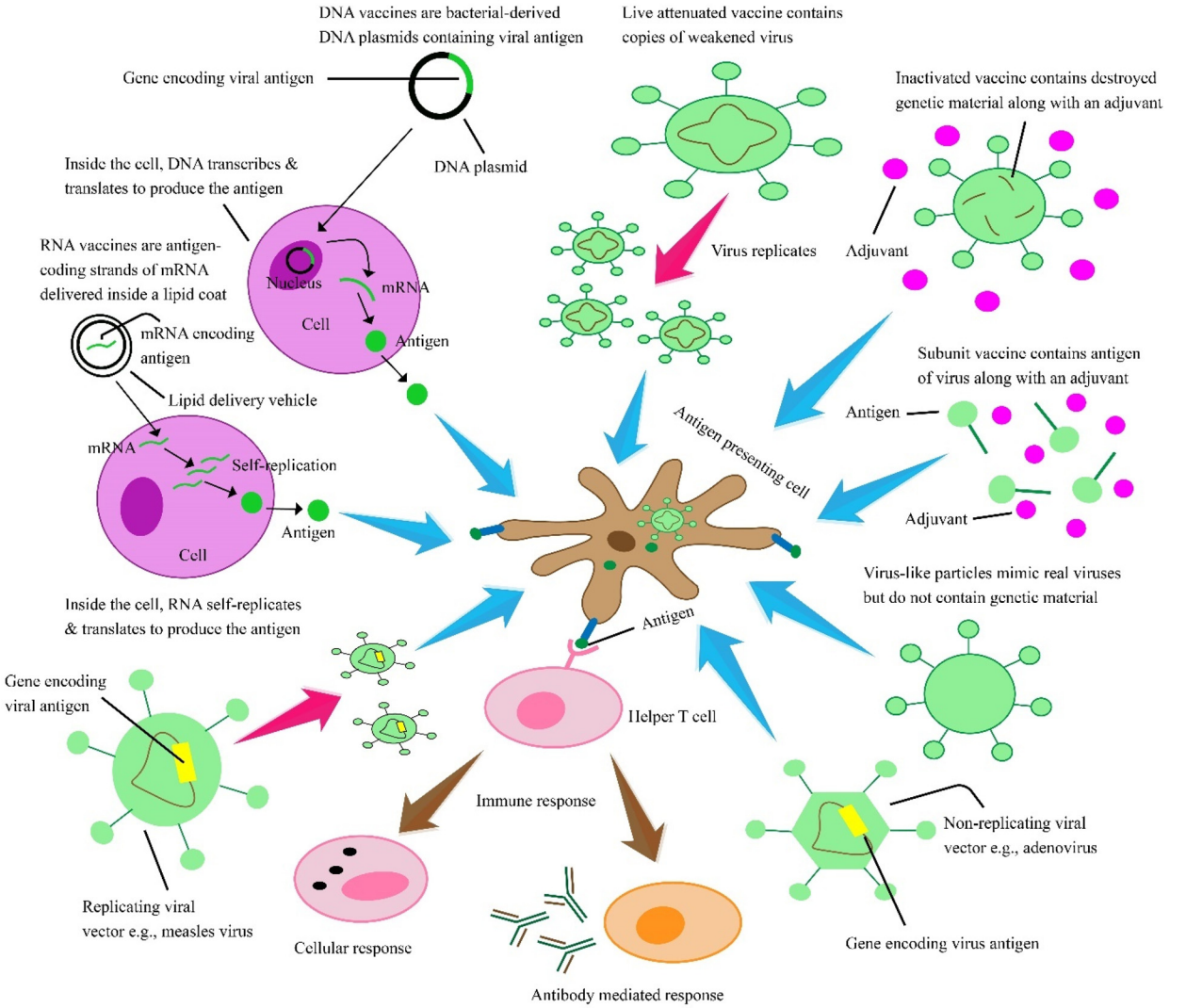

Fig. 7 Schematic of induction of immune responses by different vaccine types.

4.1. Conventional vaccines

Conventional vaccines include live attenuated, inactivated/killed, toxoid, polysaccharide and polysaccharide conjugate, synthetic peptide and virus-like particles.

4.1.1. Live attenuated vaccines. Live attenuated vaccines comprise weakened pathogenic organisms with reduced virulence, meaning that they have a decreased ability to cause infection. Since the attenuated pathogen mimics natural infection, it maintains the microorganism's multiplication ability without causing disease/reversion to virulence, which is one of the most significant considerations for developing this vaccine type.[117] Attenuation can be achieved by serially passaging or exposing the virulent microbe in suboptimal conditions or temperatures that induce a selective pressure, thereby reducing pathogen's infection potential.[18,117,118] An increase in replication fidelity, meaning a reduction in error rate, is an approach which is currently being investigated to minimize the risk of virulence. Vignuzzi *et al.* reported that a reduction in the count of errors generated from the viral replication machinery (RNA (ribonucleic acid) dependent RNA polymerase) prevented the reversion of poliovirus to its virulent wild-type phenotype.[119] Another promising technique to attenuate pathogens is codon de-optimization. This approach involves changing the positions of synonymous codons, whereby the microbial genome is recoded and the number of suboptimal codon pairs is increased. This technique is reported to reduce messenger RNA (mRNA) stability, translation efficiency, protein production and attenuation of the deoptimized virus.[120]

Live attenuated vaccines induce immunity by activating molecular sensors of innate immune cells, sustaining antigen expression and presentation. In addition, this vaccine type activates pattern recognition receptors on dendritic cells, which upregulate costimulatory molecules and the expression

of interferons/cytokines. This is followed by differentiation and activation of T helper type 1 cells.[121,122] These mechanisms result in the induction of potent immune responses.

Most attenuated vaccines confer long-term immunity by a single dose. For example, live attenuated smallpox vaccine provides humoral protection for approximately 75 years.[123,124] However, a major limitation associated with this vaccine type includes risk of infection in normal and immunocompromised individuals. Moreover, the development of live attenuate vaccines requires intensive labor, stringent quality control and trained personnel leading to a high production cost and slow manufacturing, which is particularly unsuitable in pandemics.[117,125–127] Despite these drawbacks, this traditional approach has enabled the development of various FDA approved, commercially available highly effective vaccines such as measles, mumps, rubella, tuberculosis, cholera, influenza, and smallpox.[10]

4.1.2. Inactivated/killed vaccines. Inactivated vaccines are obtained from killed virulent pathogenic organisms and primarily induce antibody-mediated immunity. Several approaches are used to inactivate the virulent pathogens, including chemical, physical and their combination. Some of the chemicals employed to inactivate virulent pathogens include β-propiolactone, formaldehyde, hydrogen peroxide, ethylenimine derivatives, glutaraldehyde and ascorbic acid. The inactivation process is significantly influenced by the chemical concentration, duration of exposure and temperature. Thus, these conditions must be optimized carefully to attain satisfactory inactivation while preserving the physicochemical properties, structure, and immunogenicity.[10,128–133] Formaldehyde or formalin (37% v/v) is the most widely used chemical to inactivate pathogenic organisms. This aldehyde-based crosslinker causes inactivation by several chemical modifications involving methylol groups, Schiff bases and methylene bridges.[19,134] Physical approaches involve the use of heat, pH, ultraviolet light and gamma irradiation for inactivating virulent pathogenic microbes. Heat and pH are the commonly employed physical approaches; however, inactivation by formaldehyde under optimal conditions is preferred over these treatments due to a reduced risk of secondary/tertiary structure degradation, antigen aggregation and increased thermal stability.[135–139]

Whole pathogen-based vaccines, including live attenuated and inactivated/killed, elicit a broad immune response against multiple targets. Inactivation prevents replication of the pathogen within the host; hence, this vaccine type is relatively safer than the live attenuated counterpart. Other advantages associated with inactivated vaccines include relatively cost-effective manufacturing and thermal stability, which permits long-term storage. However, inactivated/killed vaccines are unable to induce potent cellular immunity against intracellular pathogens. Furthermore, high doses and booster injections (which lead to poor compliance and increased production cost) are required to ensure long-lasting protection because of the reduced immunogenicity of these vaccines.[10] A common approach to enhance the immunogenicity of these vaccines is the inclusion of an adjuvant in the formulation. Several inactivated/killed vaccines have been approved by the FDA against polio, hepatitis A, diphtheria, tetanus toxoid, Japanese encephalitis and zika virus.[10,19]

4.1.3. Toxoid vaccines. Toxoid vaccines are obtained by inactivating toxins (hazardous substances/chemicals produced and secreted by bacteria). This vaccine type induces immunity against infection causing agents instead of the pathogenic microbes. Toxins cause numerous infections, including pseudomembranous colitis, diphtheria, tetanus and cholera.[140,141] Toxin inactivation is achieved using the chemicals mentioned earlier (subsection 3.1.2 Inactivated/killed vaccines). These chemicals change specific amino acids and result in conformational modifications in the structure of the toxin, thereby leading to inactivation.[134,142]

Toxoid vaccines elicit antibody-mediated immunity, which prevents the cytopathological effects of toxins on tissues, and decreases the invading potential and invasiveness of pathogenic organisms. The toxoid-specific T-cell response is primarily exhibited by $CD4^+$ cells, which also promotes a robust antigen-specific B cell response. As the anti-toxin response does not target the bacteria, decolonization (the elimination of infection-causing bacteria) takes place, usually mediated by engaging the innate immune cells using antibacterial agents and fostering competition amongst the pathogenic microbe and normal microbiota.[20,143]

Toxoid vaccines are safe, stable and suitable for long-term storage; however, they require repeated dosing and the addition of adjuvants in the formulation to attain a potent immune response. Some examples of FDA approved clinically used toxoid vaccines include diphtheria, tetanus toxoid and acellular pertussis vaccines.[10]

4.1.4. Polysaccharide and polysaccharide conjugate vaccines. The polysaccharide and polysaccharide conjugate vaccines are obtained from carbohydrate-based polymeric materials (*e.g.*, glycoproteins, teichoic-acids, peptidoglycans *etc.*) that form the capsular structure of some pathogenic organisms such as *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Neisseria meningitidis*.[144]

The immune responses generated by this vaccine type are primarily T cell independent because polysaccharides are not processed and displayed on major histocompatibility complex (MHC) molecules. In this case, a specific B cell subtype found in spleen (marginal zone $CD21^+$ B cell) plays a significant role in the detection and binding of naked or complement-coated polysaccharide antigens. This interaction amongst polysaccharide antigens and B cell receptors results in secretion of immunoglobulin M (IgM). The absence of T cell responses prevents the generation of IgG and durable B cell memory.[145,146]

Polysaccharides serve as promising targets for developing vaccines, particularly when inactivation methods are unsuitable. However, this vaccine type is unable to elicit protective responses in individuals <2 years of age. Infant marginal zone $CD21^+$ B cells are underdeveloped and they are unable to recognize polysaccharides and elicit the required immune response.[147] Some approaches used to improve the immuno-

genicity of polysaccharide-based vaccines and stimulate T cell responses are the inclusion of adjuvants and formation of glycoconjugates. The polysaccharides and carrier proteins (*e.g.*, diphtheria, tetanus toxoid *etc*.) are covalently attached to develop conjugates. These conjugates exhibit increased immunogenicity and improved protection in infants/children.[21,148] The action of polysaccharide conjugate vaccine resembles that of the polysaccharide counterpart. However, in case of glycoconjugate vaccine, both the conjugate protein and the polysaccharide are displayed on MHC-II, resulting in recognition by T-cell receptors and stimulation of the T helper cell response. An improved titer, quality of antibodies and B cell memory are achieved by this interaction between T helper and B cells.[140,146,149] Various polysaccharide (*e.g.*, meningococcal and typhoid) as well as polysaccharide conjugate (*e.g.*, pneumococcal, meningococcal and haemophilus B) vaccines have been approved by the FDA.[10]

4.1.5. Synthetic peptide vaccines. Immune responses to pathogenic microorganisms are governed by effector cells that recognize epitopes on antigens. These immunodominant peptide sequences are identified and synthesized, resulting in the development of novel vaccine modalities. Extensive *in vitro* screening and modelling are conducted to identify suitable immunodominant peptides. Peptide vaccines are developed using fragment condensation or solid-phase techniques and are subjected to rigorous purification and evaluation. As peptides are small-sized molecules, these vaccines are co-formulated with adjuvants to ensure uptake by antigen-presenting cells and to elicit a potent immune response. The selection of adjuvants requires careful consideration to avoid denaturation or emulsification of synthetic epitopes in their presence.[23]

Peptide vaccines are relatively safer than live attenuated and inactivated/killed vaccines due to the absence of pathogenic or toxic contaminants. Another advantage is the ability to induce epitope-specific immune responses due to complete control over peptide engineering, synthesis, quality and comprehensive knowledge about the vaccine antigens' constituents. Moreover, peptide sequences can be rapidly modified to elicit strain-specific immune responses.[150,151] However, immune breadth is reduced because the response is restricted to a few epitopes. This limitation can be addressed by using multiple T and B cell epitopes to increase the immune breadth to enhance the overall efficiency of the vaccine.[23,116,152] Another issue is the difficulty in mimicking the conformational B cell epitopes because the choice is limited to linear counterparts. Nevertheless, assembling peptides on appropriate backbones to reconstitute the native epitope conformation or synthesizing peptide nanoparticles can lead to an improved immune response. A drawback associated with the peptide vaccine type is the risk of cross-reaction between antibodies (elicited in response to peptides) and normal tissues, particularly when pathogens mimic the host to evade the immune response. Despite these issues, synthetic peptide vaccines are highly flexible and can serve as promising candidates alone or in combination with other types of vaccines. An example of synthetic peptide formulation approved by the FDA is meningococcal group B vaccine.[10]

4.1.6. Virus-like particles. Virus-like particles mimic the morphological attributes of a native virus, such as the size, shape, and surface epitopes. Virus-like particles are classified based on the presence or absence of a lipid envelope as well as the number of protein layers forming the capsid. These vaccines are produced in bioreactors following the transfection of microbial/mammalian/plant cells, insects or yeasts with single or multiple genetic constructs. At least two structural constituents of the original virus are encoded in the construct and self-assembled into replication-incompetent particles.[153–157] Some approaches adopted to enhance the immunogenicity of virus-like particles include chemical modification of the surface and the addition of immune-dominant peptides or adjuvants.[158,159]

Virus-like particle-based vaccines generally target B cells and stimulate a robust immune response following antigen by displaying on MHC-II and stimulating $CD4^+$ cells. Virus-like particles exhibit multivalent epitopes of specified morphology on their surface, which facilitates their interaction and cross-linking with B cell receptors. These particles bind with multivalent components of the innate immune system, which mediates effective opsonization and uptake by antigen presenting cells. Virus-like particles are internalized by dendritic cells, B-cells and sub-capsular macrophages. Their nano-scale size (~20–200 nm) facilitates their extravasation and drainage into the lymphatic system. This enables cross-presentation of these particles on MHC-I, followed by the activation of $CD8^+$ cells.[160,161]

Virus-like particle-based vaccines exhibit increased potency compared with previously described traditional vaccines due to their multivalent interaction with immune cells leading to their stimulation. For example, human papillomavirus vaccine is an FDA-approved virus-like particle-based formulation.[162–166] Different carry-over agonists (*e.g.*, nucleic acids, lipids *etc*.) are typically packaged during the assembly of virus-like particles, which also leads to enhanced immunogenicity of these vaccines. However, manufacturing challenges, purification, increased production cost and storage issues hinder successful commercial development of these vaccines on a large scale.

4.2. Advanced vaccines

The rapid development of conventional vaccines (particularly during pandemics) faces inherent challenges, such as high production cost, storage requirements and slow manufacturing. Advanced vaccine platforms or technologies can overcome the aforementioned limitations associated with traditional vaccine development approaches. These advanced technologies enable rapid large-scale manufacturing at lower cost. However, these technologies (particularly nucleic acid based-platforms) have limitations, such as their inability to encode non-protein antigens such as polysaccharides. In this scenario, conventional platforms remain the method of choice for developing non-protein antigen-based vaccines.[10] Advanced vaccine technologies that will be discussed in this subsection include viral vectors,[167] bacterial vectors,[25] synthetic DNA[27] and mRNA[168] vaccines.

4.2.1. Viral vector-based vaccines. Viral vector-based vaccines are obtained from viruses that have been modified to carry genes encoding single or multiple antigens. Viral vectors can be engineered to be replication-incompetent, meaning they cannot replicate within host cells, while still retaining the ability to infect cells and express the encoded antigens. The development process of viral vectors involves several steps, including (1) plug-and-play type of genetic engineering techniques, (2) transfection with subsequent expansion of cultured mammalian cells, (3) collection of the produced viral vectors, (4) purification, (5) concentration, (6) diafiltration and (7) formulation of the final vaccine product. Some examples of replication-incompetent vectors include poxvirus,[169] modified vesicular stomatitis virus,[170] Newcastle disease virus,[171] human adenovirus[172] and chimpanzee adenovirus.[26,173–175]

Viral vector-based vaccines mimic natural infections and induce robust humoral and cellular ($CD4^+$ and $CD8^+$) immune responses for several reasons. These include broad tropism, inherent immunogenicity of vectors (due to the presence of pathogenic microbe-associated molecular patterns or carry-over during development), and high transduction efficiency of vectors into the target cells. Finally, these vaccines result in potent long-lasting antigen expression achieved by using strong promoters to drive transcription.[26,176]

Replication-incompetent viral vector-based vaccines are safer and easier to develop compared with replication-competent counterparts, which can cause true infections similar to live-attenuated vaccines. Typically, viral vector-based vaccines are administered either as a single dose or as part of a mixed and matched heterologous vaccination regimen. This vaccine technology has exhibited high immunogenicity in preclinical studies; however, it failed to show similar efficacy in clinical trials. The immunogenicity and efficacy of these vaccines can be enhanced by using adjuvants.[26,176] The major drawbacks associated with these vaccines include pre-existing immunity against the vector and decreased efficacy in the case of subsequent administrations because of anti-vector immunity. These limitations can be circumvented using chimeric vectors, vectors from other species (such as chimpanzees or cattle), and vector serotypes with low seroprevalence among the human population. The seroprevalence can vary across different parts of the world; hence, it should be considered cautiously when developing this vaccine type. Examples of FDA approved viral vector-based vaccines include vesicular stomatitis virus-derived Ebola vaccine and human adenovirus serotype 26-based SARS-CoV-2 vaccine.[2,10]

4.2.2. Bacterial vector-based vaccines. Bacteria enter the human body *via* mucosal membranes and can serve as carriers/vectors for developing mucosal vaccines capable of inducing mucosal immune responses. Live bacterial vectors are categorized into non-pathogenic and attenuated pathogenic bacteria. A major limitation associated with bacterial vectors is the risk of infection, particularly in pediatric-, geriatric- and immunocompromised populations. Non-pathogenic bacteria (*e.g.*, *Lactobacillus* sp.) can act as suitable vaccine vectors. Advanced genetic engineering approaches can help to identify and delete bacterial virulence genes to attenuate pathogenic bacteria.[25,177]

4.2.3. DNA vaccines. DNA vaccines based on DNA plasmid molecules encoding one or several antigens are large, polyanionic and sensitive to nucleases.[100]

Intramuscular delivery of DNA transfects tissue-residing antigen-presenting cells, myocytes and keratinocytes. The internalized DNA undergoes translocation into the nucleus, followed by transcription into mRNA which is then exported for protein translation. The generated antigen displays on MHC-I and II, leading to a robust T cell response. The resident antigen-presenting cells that display the antigen drain to the lymph nodes and subsequently initiate immune responses. Antigen expression on the myocytes results in the induction of an immune response by translation and shedding of antigen into the local microenvironment. As a result, uptake and cross-presentation (MHC-II) by untransfected antigen-presenting cells are promoted. B cells are also capable of recognizing the shed antigen, resulting in their T cell independent stimulation. The soluble antigen itself drains to the lymph nodes and extends antigen presentation locally, leading to re-expansion of lymph node primed T cells ($CD4^+$ and $CD8^+$). MHC-I and other co-stimulatory molecules (*e.g.*, CD80) prime naïve $CD8^+$ T cells and contribute to T cell-based immune responses.[10,178]

DNA vaccines offer several advantages compared to conventional counterparts (*e.g.*, inactivated, live attenuated) such as the ability to induce both humoral and cellular immune responses, ease of manufacture, and rapid and cost-effective development. Over time, advancements in purification approaches, immune-informatics, immunogen design, codon optimization, molecular and structural biology have improved the potency of DNA vaccines.[178–182] A major limitation associated with this vaccine type is the safety concern due to the persistence of DNA in the nucleus, which can increase the risk of integration into the genomic DNA. Nevertheless, experimental data suggests that integration into genomic DNA is a very rare event and below the FDA limit for non-persistence (<100 copies of plasmid per mg of host DNA). DNA vaccines are not currently licensed for human use despite positive clinical data, possibly because this advanced technique displays less efficient passive entry into cells and requires multiple doses to induce a potent B and T cell-base immune response.[10,183,184] Several advanced delivery systems enhance the entry of DNA vaccines into cells, including electroporation and nanoparticles-based approaches.[184–186]

4.2.4. RNA vaccines. *In vitro* transcribed and endogenously produced (extracted from cells) mRNA can be administered for protein expression, leading to the induction of immune responses. mRNA vaccines induce immunity by transfecting the mRNA encoding viral antigens into human cells. When the mRNA reaches the cytosol, the transfected cells translate the genetic information to specific antigens which are then presented on the cell surface and recognized by the immune cells.[187] In the initial phases of development and use, several issues associated with mRNA vaccines (such as physico-

chemical instability, severe inflammation, swelling-mediated inhibition of protein translation, poor transfection, decreased *in vivo* translation because of mRNA's short half-life and high sensitivity to nucleases) limited the therapeutic applications of this vaccine type.[188–190] Several approaches resulted in the production of safe and robust mRNA vaccines, such as the inclusion of modified nucleosides into *in vitro* transcribed mRNA and removal of impurities or contaminants by employing purification chromatography.[191,192] Over time, advancements in sequence engineering and codon optimization, innovations in cap moieties and capping approaches, and the evolution of advanced delivery systems (*e.g.*, nanoparticles) led to the development of efficient mRNA-based vaccines.[168,193–195] For example, improved tolerability and enhanced antigen expression were recorded by modifying nucleoside and removing double-stranded RNA contaminants produced during development. Advanced cap analogs and capping strategies led to a high yield of properly capped mRNA and efficient recognition by cytoplasmic innate immune sensors.[28,188,196,197]

mRNA vaccines are broadly divided into three classes, including conventional mRNA, self-amplifying mRNA and circular RNA.[10] Conventional *in vitro* transcribed mRNAs are developed using a cell-free template-directed enzymatic synthesis. Linear plasmid DNAs containing a promoter sequence, 50 and 30 untranslated regions and the desired gene are employed as templates for mRNA synthesis. The polyadenine tail, responsible for stability and expression of mRNA, is engineered into the plasmid or incorporated enzymatically after synthesis. The stability and expression can be further improved by incorporating the 5′ cap structure either co-transcriptionally or enzymatically. The conventional technology is further classified into nucleoside modified or non-modified mRNA. Nucleoside modified mRNA shows higher protection (~94%) compared with the unmodified counterpart (~47%).[10,198]

Self-amplifying mRNA comprises viral-derived molecular machines (*e.g.*, alphavirus-derived replicases) and conserved sequence elements designed to achieve intracellular amplification of mRNA sequence. The architecture of self-amplifying mRNA is constructed from an expression cassette containing sub-genomic promoter and the desired antigen. This cassette is cloned between alphavirus-derived non-structural proteins 1–4 and a polyadenosine tail encoded sequences. The structural proteins assemble into an RNA-dependent RNA polymerase complex that can recognize the conserved sequence elements incorporated in the construct. The mRNA vaccine is replicated within the cytoplasm, resulting in efficient and long-term antigen expression. The manufacture of self-amplifying mRNA vaccines is relatively more complex and challenging than the conventional mRNA counterparts because of low yield and difficulty in purification. Moreover, self-amplifying mRNA vaccines are prone to autocatalysis and physical degradation. mRNA vaccines are unable to undergo nucleoside modification because of impaired interactions between RNA-dependent RNA polymerase and nucleoside modified sequences. As a result, the amplification of mRNA in target cells is reduced. Nevertheless, a low dose of self-amplifying mRNA vaccines (~100-fold lower compared with conventional mRNA counterparts) induces robust humoral and cellular immune responses. A modified approach of self-amplifying mRNA is *trans*-amplifying mRNA, which is easier to develop and manufacture. Self-amplifying mRNA is split into two different transcripts. One transcript encodes non-structural proteins 1–4 while the second transcript encodes conserved sequence elements, sub-genomic promoter and the antigen. Both the transcripts are co-delivered. The expression of non-structural proteins 1–4 and their subsequent assembly into RNA-dependent RNA polymerase permits in-*trans* (on a different molecule) amplification of antigen encoding transcript resulting in induction of potent immune responses.[198–201]

Circular RNA is a class of non-coding single-stranded RNAs produced by back-splicing in eukaryotic cells. Circular RNA enables antigen expression by incorporating internal ribosomal entry sites or specific nucleoside modifications in the 50 untranslated terminal region. The prolonged transcript half-life (reduced nuclease resistance) in circular RNA technology generates robust and stable translation in eukaryotic cells. Circular RNA generates robust antigen-specific cellular and humoral immunity.[202–204]

Immune responses to mRNA vaccines are significantly influenced by the immunogenicity of the antigen, the longevity and subcellular localization of antigen expression and the delivery system. A high immunogenicity and local cytokine and chemokine production, which initiates cell recruitment (such as neutrophils and monocytes) for generating immunity, are achieved through intramuscular and intradermal delivery of mRNA vaccines. Unlike DNA vaccines, mRNA vaccines directly translate in the cytoplasm and the antigens are displayed on MHC-I and II. Subsequently, they are presented to T helper cells in the draining lymph nodes. mRNA is not required to enter the nucleus; thus, the expression kinetics are rapid.[205–207]

A major challenge for effective application of these vaccines is rapid degradation of naked mRNA by extracellular RNases. Moreover, mRNA alone cannot efficiently penetrate the cell membranes to be transcribed in the cytosol. Intracellular delivery overcomes these limitations by facilitating the cellular uptake of mRNA and protecting it from RNase. Several methods have been developed for delivering mRNA, including *ex vivo* loading of dendritic cells, cationic peptide protamine and lipid nanoparticles.[208]

Lipid nanoparticles comprising of four components including cationic or ionizable lipids, lipid-anchored polyethylene glycol (PEG), cholesterol and phospholipids have emerged as promising candidates for delivering mRNA vaccines.[208–210] Ionizable lipids remain neutral during systemic circulation and become cationic in acidic environments, such as endosomes. These lipids perform two main roles: improving mRNA entrapment and facilitating endosomal membrane disruption, which enables the release of ribonucleic acid into the cytosol. In addition, these cationic lipids play a significant role in endosomal uptake by interacting with negatively charged cell

membrane or by binding to plasma proteins that facilitate cellular uptake. Ionizable lipids may also exert an adjuvant effect by activating toll-like receptors and inducing proinflammatory cytokines as well as co-stimulatory molecules. Examples of ionizable lipids include 1,2-dioleoyl-3-dimethylammonium-propane (DODAP), 1,2-dioleyloxy-3-dimethylaminopropane (DODMA) and 1,2-dilinoleyloxy-*N*,*N*-dimethyl-3-aminopropane (DLinDMA).[209,211,212] Stereochemistry of ionizable lipids significantly influences the delivery efficiency. Stereopure C12-200-S-based lipidic nanoparticles deliver almost 2.8-fold and 6.1-fold more mRNA in mice compared with the racemic and C12-200-R counterparts.[213] PEG is employed to prevent particle clustering, improve particle stability, increase circulation time, and reduce particle size and uptake by untargeted cells. Cholesterol stabilizes lipid nanoparticle complexes.[208–210] Modified cholesterol increases the stability and circulation time of lipid particles, resulting in an increased delivery efficiency of mRNA. For instance, substituting 50% cholesterol with 7α-hydroxycholesterol enhances mRNA delivery efficiency by 2-fold in primary human T cells *ex vivo*.[214] Phospholipids such as phosphatidylcholines, phosphatidylethanolamines and phosphatidylglycerols support the lipid bilayer.[215]

Lipid carriers are commonly prepared using the batch method, which involves slow addition of the lipidic phase into an agitated aqueous solution. Several limitations are associated with this conventional technique, such as poor control over particle size and low encapsulation efficiency. During recent times, microfluidic methods capable of overcoming the drawbacks of the traditional batch approach have emerged as promising alternatives for producing lipid nanoparticles.[216–218] Usually, the ethanol dilution method is used to prepare lipid nanoparticles by a microfluidic device. Briefly, the ethanolic solution of lipid and acetate, citrate or malic acid buffer containing mRNA is introduced into the microfluidic device. The cationic lipids and anionic mRNA form complexes *via* electrostatic interactions. The complexes then self-assemble, leading to the formation of lipidic nanoparticles. Several types of microfluidic devices (*e.g.*, T-shaped, Y-shaped, sheath-flow and chaotic mixer) have been employed for fabricating lipid nanoparticles. In the T- or Y-shaped devices, ethanol dilutes by a diffusion mechanism and the particles form at the liquid–liquid interface. These types of microfluidic devices produce large sized nanoparticles due to slow dilution of ethanol as well as the ethanol concentration gradient at the liquid–liquid interface. The sheath-flow (three inlet)-type microfluidic device permits rapid dilution of ethanol because the sheath flow increases the ethanol–buffer solution interface.[219–221] The chaotic mixer design allows mixing of solutions at relatively low flow rates, which provides efficient control over particle size compared with T-shaped-, Y-shaped- or sheath-flow type devices. Some other microfluidic systems reported for lipid nanoparticle production include three-dimensional (3D) printed micromixers, capillary-based devices and those involving electrohydrodynamics and ultrasound.[219,222] More recently, automated microfluidic systems with an increased productivity and reduced development lead time have been developed.[216–218]

A major advantage associated with mRNA vaccines compared with viral and DNA-based vaccines is the avoidance of the risk of integration into the genome, ease of manufacture and cost-effectiveness. However, mRNA vaccines present some drawbacks including the requirement of ultra-low cold chain transport, a narrow safety window, poor long-term stability at room temperature and high reactogenicity. Despite these challenges, the FDA has approved an mRNA vaccine against SARS-CoV-2.[10]

5. Challenges and strategies for improving vaccine development

Currently, the high cost ($100 million-1 billion) and time (10–15 years) associated with the development of vaccines is a major challenge, particularly during pandemics. Automation and disposable single use technology can provide a simpler, faster, and relatively low-cost route to vaccine production compared with the conventional approach.[223] Vaccine development faces challenges owing to genomic variability, their ability to evade the immune response (*e.g.*, human immunodeficiency virus, *Mycobacterium tuberculosis*, plasmodium) and rapid mutation. Advanced vaccine types offer the advantage of rapid adaptation to new variants of pathogens; hence, they can help resolve this problem to a certain extent. The development of polyvalent vaccines capable of eliciting a robust immune response against multiple serotypes can serve as a potential solution to this issue. However, the development of a multivalent vaccine is challenging due to several factors such as increased production cost, time of manufacture, technical limitations related to the permissible amount of antigens in a formulation, cross-reactivity between antigens, immunogenic variations among antigens and increased impurities because of multiple antigens.[224,225] In addition, global monitoring and surveillance play vital roles in rapidly and effectively controlling pathogenic outbreaks. Advanced genetic engineering approaches and vaccine platforms can help in overcoming antigenic diversity in pathogenic microbes by identifying more conserved sequences. The structure and the immunogenic components of pathogens can be efficiently predicted by machine learning and computational analyses. Artificial intelligence can also predict the evolution pattern of pathogens.[10,74]

Vaccine development and access is challenging, particularly in underdeveloped and developing nations where resources are limited. Global partnerships, increased funding and provision of resources from the WHO are essential to maintain vaccine development, testing and research activities. International efforts are needed to resolve supply chain issues, share intellectual property as well as prepare vaccines to ensure global access. 'Glassification' approaches involving drying vaccines in the presence of sugar (*e.g.*, trehalose) render vaccines resistant to fluctuating temperatures. The development of vaccines using this technique can relieve pressure on the cold chain in developing countries. Moreover, it is desired

to develop a single dose novel and potent adjuvant along with the vaccine that confers long-term immunity. This would ensure broader access to vaccines, especially in developing countries where access to immunization services is inconvenient. The robustness and scalability of vaccine development/manufacturing can be enhanced by process analytical technology (PAT), design of experiment (DoE) and quality-by-design (QbD) approaches.[2,10,74,226]

Another challenge in the developing world is malnutrition, which results in a compromised immune response. Vitamins can be co-administered with vaccines to resolve this issue.[224] Moreover, edible vaccines prepared by genetically modifying commercial crops (*e.g.*, banana, potato, and spinach) can serve as a source of nutrition and elicit a robust immune response.[227] Currently, research is being conducted on developing edible vaccines of several antigens, including rabies (vector: spinach), human immunodeficiency virus (vector: tomato), cancer (vector: rice), type 1 diabetes (vector: potato) and Alzheimer's disease (vector: tomato).[228]

6. Future of vaccines

Future vaccines will need to be tailored for different age and risk groups to ensure optimal efficacy. For example, vaccines for geriatric population may require higher doses and special adjuvants because this risk group usually exhibit weaker immune responses due to immunosenescence. A personalized approach can be adopted to trigger a potent and robust immune response (vaccines can be recommended on the basis of person's age, genetics, underlying medical conditions *etc.*) Disease prevalence driven by multiple strains of the same pathogen can vary across different age groups. Formulation scientists would possibly prefer including serotypes that are prevalent in pediatric populations. These issues would primarily shift focus to younger age groups due to a relatively higher vaccine efficacy and lower social contact. Another major technical challenge is identifying appropriate antigens and generating sufficient immune responses against viruses such as human immunodeficiency and Ebola. Their virus life cycle changes could be addressed using machine learning and other computational studies. A shift from preventive to therapeutic vaccines is expected in the future to manage serious ailments such as cancer, diabetes, dementia, hypertension, and rheumatoid arthritis. Future research would focus on identifying microbial antigens and human body antigens for developing preventive and therapeutic vaccines, respectively. Global attention would possibly shift to gene-based vaccines such as attenuated viruses, recombinant viral vectors or mRNA for preventive and therapeutic purposes.

Currently, the regulatory process and testing of vaccines is a slow process. The regulatory process and standard operating procedures will be more clearly defined and upgraded, especially for advanced vaccine types such as mRNA and DNA vaccines. Needleless intradermal and nasal vaccines can replace immunization approaches involving invasive hypodermic needles to improve mass vaccination campaigns. Strategies to accelerate vaccine development would be developed to overcome the vaccine inequity observed during the COVID-19 pandemic. Some strategies include decentralized vaccine production, an artificial intelligence (AI)-ready, automated, highly integrated continuous manufacturing approach and efficient supply chain management.

7. Conclusions

The rapid spread of infectious diseases and the associated high morbidity and mortality rates highlight the importance of development of efficient vaccines capable of inducing potent and robust immune responses. A rapid and cost-effective development of vaccines can be achieved by advanced vaccine platforms such as viral vectors, bacterial vectors, and DNA and RNA vaccines. Novel and safe adjuvants must be developed to improve the efficiency of vaccines. Advanced delivery methods that promise efficient and safe administration of vaccines such as MN patches, iontophoresis, electroporation, and lipidic and polymeric carriers should be further explored and employed to develop commercial vaccines. Genetic engineering approaches, machine learning and artificial intelligence should be employed to determine structure and conserved sequences of pathogens to ensure the development of efficient vaccines. Intellectual property sharing, funding and provision of resources from the WHO to developing nations would ensure cost-effective development and easy access to vaccines.

Abbreviations

1βEPP	Interleukin-1β-targeted epitope peptide
APCs	Antigen-presenting cells
BCG	Bacillus Calmette–Guérin
CMC	Carboxymethyl cellulose
COVID-19	Coronavirus disease 2019
CRISPR	Clustered regularly interspaced short palindromic repeats
DNA	Deoxyribonucleic acid
FDA	Food and Drug Administration
GSK	GlaxoSmithKline
IgG	Immunoglobulin G
IFN-γ	Interferon gamma
IL	Interleukin
IgM	Immunoglobulin M
MERS	Middle East respiratory syndrome
mRNA	Messenger ribonucleic acid
MHC	Major histocompatibility complex
M cells	Microfold cells
MNs	Microneedles
MERS-CoV	Middle East respiratory syndrome coronavirus
pH	Negative logarithm of H^+ ion concentration
PEG	Polyethylene glycol

PVP	Polyvinyl pyrrolidone
PLGA	Poly lactic-*co*-glycolic acid
RNase	Ribonuclease
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SARS-CoV-1	severe acute respiratory syndrome coronavirus 1
WHO	World Health Organization
3D	Three dimensional

Author contributions

Conceptualization, M. S. A. and Z. A.; writing—original draft preparation, S. Z.; writing—review and editing, A. A., E. S. and E. O.; supervision, M. S. A. and Z. A. All authors have read and agreed to the published version of the manuscript.

Data availability

This is a review article and therefore does not contain any new data. Any figures that are reproduced, adapted or abridged have been clearly cited in the manuscript. All other figures have been prepared by the authors.

Conflicts of interest

The authors declare no conflicts of interest.

References

1 R. T. Kamorudeen, K. A. Adedokun and A. O. Olarinmoye, Ebola outbreak in West Africa, 2014–2016: Epidemic timeline, differential diagnoses, determining factors, and lessons for future response, *J. Infect. Public Health*, 2020, **13**, 956–962.
2 S. Zafar, M. S. Arshad, S. Fatima, A. Ali, A. Zaman, E. Sayed, M. W. Chang and Z. Ahmad, COVID-19: Current developments and further opportunities in drug delivery and therapeutics, *Pharmaceutics*, 2020, **12**, 1–26.
3 Y. Tian, D. Hu, Y. Li and L. Yang, Development of therapeutic vaccines for the treatment of diseases, *Mol. Biomed.*, 2022, **3**, 1–27.
4 I. Delany, R. Rappuoli and E. De Gregorio, Vaccines for the 21st century, *EMBO Mol. Med.*, 2014, **6**, 708–720.
5 F. Zakir, F. Islam, A. Jabeen and S. Sivagurunathan, Vaccine development: A historical perspective, *Biomed. Res.*, 2019, **30**, 3.
6 A. Saleh, S. Qamar, A. Tekin, R. Singh, R. Kashyap, A. Saleh, S. Qamar, A. Tekin, R. Singh and R. Kashyap, Vaccine Development Throughout History, *Cureus*, 2021, **13**(7), e16635.
7 M. E. Janes, A. P. Gottlieb, K. S. Park, Z. Zhao and S. Mitragotri, Cancer vaccines in the clinic, *Bioeng. Transl. Med.*, 2024, **9**, e10588.
8 D. K. Chellappan, R. R. Bhandare, A. B. Shaik, K. Prasad, N. A. A. Suhaimi, W. S. Yap, A. Das, P. Banerjee, N. Ghosh, T. Guith, A. Das, S. Balakrishnan, M. Candasamy, J. Mayuren, K. Palaniveloo, G. Gupta, S. K. Singh and K. Dua, Vaccine for Diabetes—Where Do We Stand?, *Int. J. Mol. Sci.*, 2022, **23**, 9470.
9 H. Nakagami, T. Ishihama, Y. Daikyoji, C. Sasakura, E. Yamada and R. Morishita, Brief report on a phase I/IIa study to assess the safety, tolerability, and immune response of AGMG0201 in patients with essential hypertension, *Hypertens. Res.*, 2021, **45**, 61–65.
10 M. Ghattas, G. Dwivedi, M. Lavertu and M. G. Alameh, Vaccine technologies and platforms for infectious diseases: Current progress, challenges, and opportunities, *Vaccines*, 2021, **9**, 1–31.
11 O. Sharma, A. A. Sultan, H. Ding and C. R. Triggle, A review of the progress and challenges of developing a vaccine for COVID-19, *Front. Immunol.*, 2020, **11**, 585354.
12 D. Singh, A. Mathur, S. Arora, S. Roy and N. Mahindroo, Journey of organ on a chip technology and its role in future healthcare scenario, *Appl. Surf. Sci. Adv.*, 2022, **9**, 100246.
13 B. C. Buckland, The process development challenge for a new vaccine, *Nat. Med.*, 2005, **11**, S16–S19.
14 P. M. Heaton, Challenges of developing novel vaccines with particular global health importance, *Front. Immunol.*, 2020, **11**, 517290.
15 J. L. Excler, M. Saville, L. Privor-Dumm, S. Gilbert, P. J. Hotez, D. Thompson, S. Abdool-Karim and J. H. Kim, Factors, enablers and challenges for COVID-19 vaccine development, *BMJ Global Health*, 2023, **8**, e011879.
16 W. Wang, S. Wang, X. Meng, Y. Zhao, N. Li, T. Wang, N. Feng, F. Yan and X. Xia, A virus-like particle candidate vaccine based on CRISPR/Cas9 gene editing technology elicits broad-spectrum protection against SARS-CoV-2, *Antiviral Res.*, 2024, **225**, 105854.
17 L. J. J. Hansen, R. Daoussi, C. Vervaet, J. P. Remon and T. R. M. De Beer, Freeze-drying of live virus vaccines: A review, *Vaccine*, 2015, **33**, 5507–5519.
18 W. P. Glezen, Cold-adapted, live attenuated influenza vaccine, *Expert Rev. Vaccines*, 2004, **3**, 131–139.
19 B. Sanders, M. Koldijk and H. Schuitemaker, Inactivated viral vaccines, *Vaccine Anal. Strategies Princ. Control*, 2015, 45–80.
20 P. Angsantikul, R. H. Fang and L. Zhang, Toxoid Vaccination against Bacterial Infection Using Cell Membrane-Coated Nanoparticles, *Bioconjugate Chem.*, 2018, **29**, 604–612.
21 M. E. Pichichero, Protein carriers of conjugate vaccines Characteristics, development, and clinical trials, *Hum. Vaccines Immunother.*, 2013, **9**, 2505–2523.
22 M. Allahyari and E. Mohit, Peptide/protein vaccine delivery system based on PLGA particles, *Hum. Vaccines Immunother.*, 2016, **12**, 806–828.
23 W. Li, M. D. Joshi, S. Singhania, K. H. Ramsey and A. K. Murthy, Peptide vaccine: Progress and challenges, *Vaccines*, 2014, **2**, 515–536.

24 Q. Zhao, S. Li, H. Yu, N. Xia and Y. Modis, Virus-like particle-based human vaccines: Quality assessment based on structural and functional properties, *Trends Biotechnol.*, 2013, **31**, 654–663.
25 A. J. da Silva, T. C. Zangirolami, M. T. M. Novo-Mansur, R. de Campos Giordano and E. A. L. Martins, Live bacterial vaccine vectors: An overview, *Braz. J. Microbiol.*, 2014, **45**, 1117–1129.
26 H. C. Ertl, Viral vectors as vaccine carriers, *Curr. Opin. Virol.*, 2016, **21**, 1–8.
27 E. F. Fynan, R. G. Webster, D. H. Fuller, J. R. Haynes, J. C. Santoro and H. L. Robinson, DNA vaccines: Protective immunizations by parenteral, mucosal, and gene-gun inoculations, *Proc. Natl. Acad. Sci. U. S. A.*, 1993, **90**, 11478–11482.
28 G. Hartmann, Nucleic Acid Immunity, *Adv. Immunol*, 2017, **133**, 121–169.
29 J. B. Sandbrink and R. J. Shattock, RNA Vaccines: A Suitable Platform for Tackling Emerging Pandemics?, *Front. Immunol.*, 2020, **11**, 608460.
30 A. Kocourkova, J. Honegr, K. Kuca and J. Danova, Vaccine ingredients: Components that influence vaccine efficacy, *Mini-Rev. Med. Chem.*, 2016, **17**, 451–466.
31 A. Facciolà, G. Visalli, A. Laganà and A. Di Pietro, An overview of vaccine adjuvants: Current evidence and future perspectives, *Vaccines*, 2022, **10**, 1–26.
32 E. J. Ko and S. M. Kang, Immunology and efficacy of MF59-adjuvanted vaccines, *Hum. Vaccines Immunother.*, 2018, **14**, 3041–3045.
33 C. L. Smith, B. Richardson, M. Rubsamen, M. J. Cameron, C. M. Cameron and D. H. Canaday, Adjuvant AS01 activates human monocytes for costimulation and systemic inflammation, *Vaccine*, 2024, **42**, 229–238.
34 Y. Zheng, L. Bian, H. Zhao, Y. Liu, J. Lu, D. Liu, K. Zhang, Y. Song, Y. Luo, C. Jiang, Y. Chen, Y. Zhang and W. Kong, Respiratory syncytial virus F subunit vaccine with AS02 adjuvant elicits balanced, robust humoral and cellular immunity in BALB/c mice, *Front. Immunol.*, 2020, **11**, 526965.
35 I. Leroux-Roels, J. M. Devaster, G. Leroux-Roels, V. Verlant, I. Henckaerts, P. Moris, P. Hermand, P. Van Belle, J. T. Poolman, P. Vandepapelière and Y. Horsmans, Adjuvant system AS02 V enhances humoral and cellular immune responses to pneumococcal protein PhtD vaccine in healthy young and older adults: Randomised, controlled trials, *Vaccine*, 2015, **33**, 577–584.
36 N. Garçon, D. W. Vaughn and A. M. Didierlaurent, Development and evaluation of AS03, an adjuvant system containing α-tocopherol and squalene in an oil-in-water emulsion, *Expert Rev. Vaccines*, 2012, **11**, 349–366.
37 A. M. Didierlaurent, S. Morel, L. Lockman, S. L. Giannini, M. Bisteau, H. Carlsen, A. Kielland, O. Vosters, N. Vanderheyde, F. Schiavetti, D. Larocque, M. Van Mechelen and N. Garçon, AS04, an aluminum salt- and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity, *J. Immunol.*, 2009, **183**, 6186–6197.
38 J. Zhang, H. Zeng, J. Gu, H. Li, L. Zheng and Q. Zou, Progress and Prospects on Vaccine Development against SARS-CoV-2, *Vaccines*, 2020, **8**, 153.
39 S. Awate, L. A. Babiuk and G. Mutwiri, Mechanisms of action of adjuvants, *Front. Immunol.*, 2013, **4**, 50795.
40 P. Wang, Natural and Synthetic Saponins as Vaccine Adjuvants, *Vaccines*, 2021, **9**, 222.
41 I. Dmour and N. Islam, Recent advances on chitosan as an adjuvant for vaccine delivery, *Int. J. Biol. Macromol.*, 2022, **200**, 498–519.
42 G. Wei, H. Tan, S. Ma, G. Sun, Y. Zhang, Y. Wu, S. Cai, Y. Huang and J. Jian, Protective effects of β-glucan as adjuvant combined inactivated Vibrio harveyi vaccine in pearl gentian grouper, *Fish Shellfish Immunol.*, 2020, **106**, 1025–1030.
43 P. M. Chandrashekar and Y. P. Venkatesh, Fructans from aged garlic extract produce a delayed immunoadjuvant response to ovalbumin antigen in BALB/c mice, *Immunopharmacol. Immunotoxicol.*, 2012, **34**, 174–180.
44 M. Liu, E. MacHová, Z. Neščáková, I. Medovarská, K. V. Clemons, M. Martinez, V. Chen, S. Bystrický and D. A. Stevens, Vaccination with mannan protects mice against systemic aspergillosis, *Med. Mycol.*, 2012, **50**, 818–828.
45 M. S. Arshad, S. Hussain, S. Zafar, S. J. Rana, N. Ahmad, N. A. Jalil and Z. Ahmad, Improved Transdermal Delivery of Rabies Vaccine using Iontophoresis Coupled Microneedle Approach, *Pharm. Res.*, 2023, **40**, 2039–2049.
46 A. Dalla Pietà, D. Carpanese, A. Grigoletto, A. Tosi, S. Dalla Santa, G. K. Pedersen, D. Christensen, L. Meléndez-Alafort, V. Barbieri, P. De Benedictis, G. Pasut, I. M. Montagner and A. Rosato, Hyaluronan is a natural and effective immunological adjuvant for protein-based vaccines, *Cell. Mol. Immunol.*, 2021, **18**, 1197–1210.
47 S. Mallakpour, E. Azadi and C. M. Hussain, Chitosan, alginate, hyaluronic acid, gums, and β-glucan as potent adjuvants and vaccine delivery systems for viral threats including SARS-CoV-2: A review, *Int. J. Biol. Macromol.*, 2021, **182**, 1931–1940.
48 V. Mohammadzadeh, N. Rahiman, H. Cabral, S. Quader, M. R. Zirak, M. E. Taghavizadeh Yazdi, M. R. Jaafari and S. H. Alavizadeh, Poly-γ-glutamic acid nanoparticles as adjuvant and antigen carrier system for cancer vaccination, *J. Controlled Release*, 2023, **362**, 278–296.
49 Z. Jin, Y. T. Dong, S. Liu, J. Liu, X. R. Qiu, Y. Zhang, H. Zong, W. T. Hou, S. Y. Guo, Y. F. Sun, S. M. Chen, H. Q. Dong, Y. Y. Li, M. M. An and H. Shen, Potential of Polyethyleneimine as an Adjuvant To Prepare Long-Term and Potent Antifungal Nanovaccine, *Front. Immunol.*, 2022, **13**, 843684.
50 W. Zhang, L. Wang, Y. Liu, X. Chen, J. Li, T. Yang, W. An, X. Ma, R. Pan and G. Ma, Comparison of PLA microparticles and alum as adjuvants for H5N1 influenza split vaccine: Adjuvanticity evaluation and preliminary action mode analysis, *Pharm. Res.*, 2014, **31**, 1015–1031.

51 J. Kreuter, Poly(Methyl Methacrylate) Nanoparticles As Vaccine Adjuvants, *Vaccine Adjuvants*, 2000, 105–119.
52 J. T. van Dissel, S. A. Joosten, S. T. Hoff, D. Soonawala, C. Prins, D. A. Hokey, D. M. O'Dee, A. Graves, B. Thierry-Carstensen, L. V. Andreasen, M. Ruhwald, A. W. de Visser, E. M. Agger, T. H. M. Ottenhoff, I. Kromann and P. Andersen, A novel liposomal adjuvant system, CAF01, promotes long-lived Mycobacterium tuberculosis-specific T-cell responses in human, *Vaccine*, 2014, **32**, 7098–7107.
53 F. Liu, X. Sun, J. Fairman, D. B. Lewis, J. M. Katz, M. Levine, T. M. Tumpey and X. Lu, A cationic liposome–DNA complexes adjuvant (JVRS-100) enhances the immunogenicity and cross-protective efficacy of pre-pandemic influenza A (H5N1) vaccine in ferrets, *Virology*, 2016, **492**, 197–203.
54 D. M. Lidgate, Preparation of the Syntex Adjuvant Formulation (SAF, SAF-m, and SAF-1), *Vaccine Adjuvants*, 2000, 229–237.
55 K. Lövgren Bengtsson, B. Morein and A. D. Osterhaus, ISCOM technology-based Matrix MTM adjuvant: success in future vaccines relies on formulation, *Expert Rev. Vaccines*, 2011, **10**, 401–403.
56 S. G. Reed, S. Bertholet, R. N. Coler and M. Friede, New horizons in adjuvants for vaccine development, *Trends Immunol.*, 2009, **30**, 23–32.
57 T. A. Olafsdottir, K. Lingnau, E. Nagy and I. Jonsdottir, IC31®, a Two-Component Novel Adjuvant Mixed with a Conjugate Vaccine Enhances Protective Immunity against Pneumococcal Disease in Neonatal Mice, *Scand. J. Immunol.*, 2009, **69**, 194–202.
58 E. Ramin, A. G. Cardillo, R. Liebers, J. Schmölder, E. von Lieres, W. Van Molle, B. Niebel, L. Natalis, I. Meln, M. Perea-Vélez, D. Clénet, J. B. Jørgensen, B. Nilsson, D. G. Bracewell and K. V. Gernaey, Accelerating vaccine manufacturing development through model-based approaches: current advances and future opportunities, *Curr. Opin. Chem. Eng.*, 2024, **43**, 100998.
59 S. Mukherjee Id, K. Kalra and A. L. Phelan, Expanding global vaccine manufacturing capacity: Strategic prioritization in small countries, *PLOS Global Public Health*, 2023, **3**, e0002098.
60 J. M. Robinson, in *The Vaccine Book*, ed. B. R. Bloom and P.-H. Lambert, Academic Press, 2nd edn, 2016, pp. 77–96.
61 P. L. Gomez, J. M. Robinson and J. A. Rogalewicz, in *Vaccines*, ed. S. A. Plotkin, W. A. Orenstein and P. A. Offit, W.B. Saunders, 6th edn, 2013, pp. 44–57.
62 D. H. Thornton, *Quality control of vaccines*, 1988, 347–365.
63 B. Metz, G. Den Van Dobbelsteen, C. Van Els, J. Der Van Gun, L. Levels, L. Der Van Pol, N. Rots and G. Kersten, Quality-control issues and approaches in vaccine development, *Expert Rev. Vaccines*, 2009, **8**, 227–238.
64 T. H. Mogensen, Pathogen recognition and inflammatory signaling in innate immune defenses, *Clin. Microbiol. Rev.*, 2009, **22**, 240–273.
65 A. Iwasaki and S. B. Omer, Why and How Vaccines Work, *Cell*, 2020, **183**, 290–295.
66 A. J. Pollard and E. M. Bijker, A guide to vaccinology: from basic principles to new developments, *Nat. Rev. Immunol.*, 2021, **21**, 83–100.
67 M. Embgenbroich and S. Burgdorf, Current concepts of antigen cross-presentation, *Front. Immunol.*, 2018, **9**, 01643.
68 A. Chauveau, G. Pirgova, H. W. Cheng, A. De Martin, F. Y. Zhou, S. Wideman, J. Rittscher, B. Ludewig and T. I. Arnon, Visualization of T Cell Migration in the Spleen Reveals a Network of Perivascular Pathways that Guide Entry into T Zones, *Immunity*, 2020, **52**, 794–807.
69 S. Crotty, T Follicular Helper Cell Biology: A Decade of Discovery and Diseases, *Immunity*, 2019, **50**, 1132–1148.
70 C. G. Vinuesa, M. A. Linterman, D. Yu and I. C. M. Maclennan, Follicular Helper T Cells, *Annu. Rev. Immunol.*, 2016, **34**, 335–368.
71 G. M. Griffiths, C. Berek, M. Kaartinen and C. Milstein, Somatic mutation and the maturation of immune response to 2-phenyl oxazolone, *Nature*, 1984, **312**, 271–275.
72 M. S. Arshad, S. Gulfam, S. Zafar, N. A. Jalil, N. Ahmad, O. Qutachi, M. W. Chang, N. Singh and Z. Ahmad, Engineering of tetanus toxoid-loaded polymeric microneedle patches, *Drug Delivery Transl. Res.*, 2023, **13**, 852–861.
73 M. S. Arshad, S. Fatima, K. Nazari, R. Ali, M. Farhan, S. A. Muhammad, N. Abbas, A. Hussain, I. Kucuk, M. W. Chang, P. Mehta and Z. Ahmad, Engineering and characterisation of BCG-loaded polymeric microneedles, *J. Drug Targeting*, 2020, **28**, 525–532.
74 M. M. Levine and M. B. Sztein, Vaccine development strategies for improving immunization: the role of modern immunology, *Nat. Immunol.*, 2004, **5**, 460–464.
75 M. Skwarczynski and I. Toth, Non-invasive mucosal vaccine delivery: advantages, challenges and the future, *Expert Opin. Drug Delivery*, 2020, **17**, 435–437.
76 K. Khatri, A. K. Goyal, P. N. Gupta, N. Mishra, A. Mehta and S. P. Vyas, Surface modified liposomes for nasal delivery of DNA vaccine, *Vaccine*, 2008, **26**, 2225–2233.
77 A. K. Shakya, M. Y. E. Chowdhury, W. Tao and H. S. Gill, Mucosal vaccine delivery: Current state and a pediatric perspective, *J. Controlled Release*, 2016, **240**, 394–413.
78 M. Huang, M. Zhang, H. Zhu, X. Du and J. Wang, Mucosal vaccine delivery: A focus on the breakthrough of specific barriers, *Acta Pharm. Sin. B*, 2022, **12**, 3456–3474.
79 E. C. Lavelle and R. W. Ward, Mucosal vaccines—fortifying the frontiers, *Nat. Rev. Immunol.*, 2021, **22**, 236–250.
80 J. C. Joyce, H. E. Sella, H. Jost, M. J. Mistilis, E. S. Esser, P. Pradhan, R. Toy, M. L. Collins, P. A. Rota, K. Roy, I. Skountzou, R. W. Compans, M. S. Oberste, W. C. Weldon, J. J. Norman and M. R. Prausnitz, Extended delivery of vaccines to the skin improves immune responses, *J. Controlled Release*, 2019, **304**, 135–145.
81 S. Hansen and C. M. Lehr, Transfollicular delivery takes root: the future for vaccine design?, *Expert Rev. Vaccines*, 2014, **13**, 5–7.

82 V. M. Meidan, M. C. Bonner and B. B. Michniak, Transfollicular drug delivery—Is it a reality?, *Int. J. Pharm.*, 2005, **306**, 1–14.
83 G. M. Glenn, D. N. Taylor, X. Li, S. Frankel, A. Montemarano and C. R. Alving, Transcutaneous immunization: A human vaccine delivery strategy using a patch, *Nat. Med.*, 2000, **6**, 1403–1406.
84 G. M. Glenn, R. T. Kenney, L. R. Ellingsworth, S. A. Frech, S. A. Hammond and J. P. Zoeteweij, Transcutaneous immunization and immunostimulant strategies: capitalizing on the immunocompetence of the skin, *Expert Rev. Vaccines*, 2003, **2**, 253–267.
85 E. L. Giudice and J. D. Campbell, Needle-free vaccine delivery, *Adv. Drug Delivery Rev.*, 2006, **58**, 68–89.
86 E. M. Mucker, J. W. Golden, C. D. Hammerbeck, J. M. Kishimori, M. Royals, M. D. Joselyn, J. Ballantyne, A. Nalca and J. W. Hooper, A Nucleic Acid-Based Orthopoxvirus Vaccine Targeting the Vaccinia Virus L1, A27, B5, and A33 Proteins Protects Rabbits against Lethal Rabbitpox Virus Aerosol Challenge, *J. Virol.*, 2022, **96**(3), e0150421.
87 A. Generotti, R. Contreras, B. Zounes, E. Schade, A. Kemme, Y. Rane, X. Liu, D. Elwood, K. Schultheis, J. Marston, J. McCoy, K. Broderick and P. Fisher, Intradermal DNA vaccine delivery using vacuum-controlled, needle-free electroporation, *Mol. Ther.–Nucleic Acids*, 2023, **34**, 102070.
88 M. Hasan, A. Khatun and K. Kogure, Intradermal Delivery of Naked mRNA Vaccines via Iontophoresis, *Pharmaceutics*, 2023, **15**, 2678.
89 M. Toyoda, S. Hama, Y. Ikeda, Y. Nagasaki and K. Kogure, Anti-cancer vaccination by transdermal delivery of antigen peptide-loaded nanogels via iontophoresis, *Int. J. Pharm.*, 2015, **483**, 110–114.
90 Y. C. Kim, F. S. Quan, R. W. Compans, S. M. Kang and M. R. Prausnitz, Formulation and coating of microneedles with inactivated influenza virus to improve vaccine stability and immunogenicity, *J. Controlled Release*, 2010, **142**, 187–195.
91 E. Kim, G. Erdos, S. Huang, T. W. Kenniston, S. C. Balmert, C. D. Carey, V. S. Raj, M. W. Epperly, W. B. Klimstra, B. L. Haagmans, E. Korkmaz, L. D. Falo and A. Gambotto, Microneedle array delivered recombinant coronavirus vaccines: Immunogenicity and rapid translational development, *EBioMedicine*, 2020, **55**, 102743.
92 Q. Guo, C. Wang, Q. Zhang, K. Cheng, W. Shan, X. Wang, J. Yang, Y. Wang and L. Ren, Enhanced cancer immunotherapy by microneedle patch-assisted delivery of HBc VLPs based cancer vaccine, *Appl. Mater. Today*, 2021, **24**, 101110.
93 J. Williams, L. Fox-Leyva, C. Christensen, D. Fisher, E. Schlicting, M. Snowball, S. Negus, J. Mayers, R. Koller and R. Stout, Hepatitis A vaccine administration: comparison between jet-injector and needle injection, *Vaccine*, 2000, **18**, 1939–1943.
94 H. L. Davis, M. L. Michel, M. Mancini, M. Schleef and R. G. Whalen, Direct gene transfer in skeletal muscle: plasmid DNA-based immunization against the hepatitis B virus surface antigen, *Vaccine*, 1994, **12**, 1503–1509.
95 D. Chen, R. L. Endres, C. A. Erickson, K. F. Weis, M. W. McGregor, Y. Kawaoka and L. G. Payne, Epidermal immunization by a needle-free powder delivery technology: Immunogenicity of influenza vaccine and protection in mice, *Nat. Med.*, 2000, **6**, 1187–1190.
96 D. Chen, K. F. Weis, Q. Chu, C. Erickson, R. Endres, C. R. Lively, J. Osorio and L. G. Payne, Epidermal Powder Immunization Induces both Cytotoxic T-Lymphocyte and Antibody Responses to Protein Antigens of Influenza and Hepatitis B Viruses, *J. Virol.*, 2001, **75**, 11630–11640.
97 A. Alexander, S. Dwivedi, Ajazuddin, T. K. Giri, S. Saraf, S. Saraf and D. K. Tripathi, Approaches for breaking the barriers of drug permeation through transdermal drug delivery, *J. Controlled Release*, 2012, **164**, 26–40.
98 A. Z. Alkilani, M. T. C. McCrudden and R. F. Donnelly, Transdermal drug delivery: Innovative pharmaceutical developments based on disruption of the barrier properties of the stratum corneum, *Pharmaceutics*, 2015, **7**, 438–470.
99 Z. Chen, Y. Lv, J. Qi, Q. Zhu, Y. Lu and W. Wu, Overcoming or circumventing the stratum corneum barrier for efficient transcutaneous immunization, *Drug Discovery Today*, 2018, **23**, 181–186.
100 M. P. Morrow, N. A. Hutnick, T. H. Shin, C. E. Lucke and D. B. Weiner, Clinical applications of DNA vaccines: Current progress, *Clin. Infect. Dis.*, 2011, **53**, 296–302.
101 G. Ahlén, J. Söderholm, T. Tjelle, R. Kjeken, L. Frelin, U. Höglund, P. Blomberg, M. Fons, I. Mathiesen and M. Sällberg, In Vivo Electroporation Enhances the Immunogenicity of Hepatitis C Virus Nonstructural 3/4A DNA by Increased Local DNA Uptake, Protein Expression, Inflammation, and Infiltration of CD3+ T Cells, *J. Immunol.*, 2007, **179**, 4741–4753.
102 M. Gujjar and A. K. Banga, Iontophoretic and microneedle mediated transdermal delivery of glycopyrrolate, *Pharmaceutics*, 2014, **6**, 663–671.
103 M. S. Arshad, S. Zafar, A. T. Zahra, M. H. Zaman, A. Akhtar, I. Kucuk, M. Farhan, M. W. Chang and Z. Ahmad, Fabrication and characterisation of self-applicating heparin sodium microneedle patches, *J. Drug Targeting*, 2020, **29**, 60–68.
104 M. S. Arshad, S. Zafar, S. J. Rana, K. Nazari, M. W. Chang and Z. Ahmad, Fabrication of gentamicin sulphate laden stimulus responsive polymeric microarray patches for the treatment of bacterial biofilms, *J. Drug Delivery Sci. Technol.*, 2023, **84**, 104504.
105 S. Zafar, M. S. Arshad, S. J. Rana, M. Patel, B. Yousef and Z. Ahmad, Engineering of clarithromycin loaded stimulus responsive dissolving microneedle patches for the treatment of biofilms, *Int. J. Pharm.*, 2023, **640**, 123003.
106 T. Waghule, G. Singhvi, S. K. Dubey, M. M. Pandey, G. Gupta, M. Singh and K. Dua, Microneedles: A smart

approach and increasing potential for transdermal drug delivery system, *Biomed. Pharmacother.*, 2019, **109**, 1249–1258.

107 R. Haj-Ahmad, H. Khan, M. S. Arshad, M. Rasekh, A. Hussain, S. Walsh, X. Li, M. W. Chang and Z. Ahmad, Microneedle coating techniques for transdermal drug delivery, *Pharmaceutics*, 2015, **7**, 486–502.

108 E. Larrañeta, R. E. M. Lutton, A. D. Woolfson and R. F. Donnelly, Microneedle arrays as transdermal and intradermal drug delivery systems: Materials science, manufacture and commercial development, *Materials Science and Engineering: R: Reports*, 2016, **104**, 1–32.

109 D. Psimadas, P. Georgoulias, V. Valotassiou and G. Loudos, Molecular Nanomedicine Towards Cancer, *J. Pharm. Sci.*, 2012, **101**, 2271–2280.

110 M. Trovato, Novel antigen delivery systems, *World J. Virol.*, 2015, **4**, 156.

111 D. S. Bernardi, C. Bitencourt, D. S. C. da Silveira, E. L. C. M. da Cruz, M. A. Pereira-da-Silva, L. H. Faccioli and R. F. V. Lopez, Effective transcutaneous immunization using a combination of iontophoresis and nanoparticles, *Nanomedicine*, 2016, **12**, 2439–2448.

112 I. Takeuchi, T. Suzuki and K. Makino, Iontophoretic transdermal delivery using chitosan-coated PLGA nanoparticles for transcutaneous immunization, *Colloids Surf., A*, 2021, **608**, 125607.

113 Y. Yu, H. Wang, B. Guo, B. Wang, Z. Wan, Y. Zhang, L. Sun and F. Yang, Microneedle-based two-step transdermal delivery of Langerhans cell-targeting immunoliposomes induces a Th1-biased immune response, *Eur. J. Pharm. Biopharm.*, 2022, **177**, 68–80.

114 L. Niu, L. Y. Chu, S. A. Burton, K. J. Hansen and J. Panyam, Intradermal delivery of vaccine nanoparticles using hollow microneedle array generates enhanced and balanced immune response, *J. Controlled Release*, 2019, **294**, 268–278.

115 C. Bernelin-Cottet, C. Urien, J. McCaffrey, D. Collins, A. Donadei, D. McDaid, V. Jakob, C. Barnier-Quer, N. Collin, E. Bouguyon, E. Bordet, C. Barc, O. Boulesteix, J. J. Leplat, F. Blanc, V. Contreras, N. Bertho, A. C. Moore and I. Schwartz-Cornil, Electroporation of a nanoparticle-associated DNA vaccine induces higher inflammation and immunity compared to its delivery with microneedle patches in pigs, *J. Controlled Release*, 2019, **308**, 14–28.

116 W. C. Koff, D. R. Burton, P. R. Johnson, B. D. Walker, C. R. King, G. J. Nabel, R. Ahmed, M. K. Bhan and S. A. Plotkin, Accelerating next-generation vaccine development for global disease prevention, *Science*, 2013, **340**(6136), 1232910.

117 T. Mak and M. Saunders, in *The Immune Response*, ed. S. Tw, Burlington, NJ, USA, Academic Press, 2006, pp. 695–749.

118 R. L. Ward and D. I. Bernstein, Rotarix: A rotavirus vaccine for the world, *Clin. Infect. Dis.*, 2009, **48**, 222–228.

119 M. Vignuzzi, E. Wendt and R. Andino, Engineering attenuated virus vaccines by controlling replication fidelity, *Nat. Med.*, 2008, **14**, 154–161.

120 N. Groenke, J. Trimpert, S. Merz, A. M. Conradie, E. Wyler, H. Zhang, O. G. Hazapis, S. Rausch, M. Landthaler, N. Osterrieder and D. Kunec, Mechanism of Virus Attenuation by Codon Pair Deoptimization, *Cell Rep.*, 2020, **31**(4), 107586.

121 T. B. H. Geijtenbeek and S. I. Gringhuis, Signalling through C-type lectin receptors: Shaping immune responses, *Nat. Rev. Immunol.*, 2009, **9**, 465–479.

122 B. Pulendran and R. Ahmed, Immunological mechanisms of vaccination, *Nat. Immunol.*, 2011, **12**, 509–517.

123 R. LeBlanc, Y. Vasquez, D. Hannaman and N. Kumar, Markedly enhanced immunogenicity of a Pfs25 DNA-based malaria transmission-blocking vaccine by in vivo electroporation, *Vaccine*, 2008, **26**, 185–192.

124 E. Hammarlund, M. W. Lewis, S. G. Hansen, L. I. Strelow, J. A. Nelson, G. J. Sexton, J. M. Hanifin and M. K. Slifka, Duration of antiviral immunity after smallpox vaccination, *Nat. Med.*, 2003, **9**, 1131–1137.

125 H. Shimizu, B. Thorley, F. J. Paladin, K. A. Brussen, V. Stambos, L. Yuen, A. Utama, Y. Tano, M. Arita, H. Yoshida, T. Yoneyama, A. Benegas, S. Roesel, M. Pallansch, O. Kew and T. Miyamura, Circulation of Type 1 Vaccine-Derived Poliovirus in the Philippines in 2001, *J. Virol.*, 2004, **78**, 13512–13521.

126 T. Müller, H. J. Bätza, A. Beckert, C. Bunzenthal, J. H. Cox, C. M. Freuling, A. R. Fooks, J. Frost, L. Geue, A. Hoeflechner, D. Marston, A. Neubert, L. Neubert, S. Revilla-Fernández, E. Vanek, A. Vos, E. Wodak, K. Zimmer and T. C. Mettenleiter, Analysis of vaccine-virus-associated rabies cases in red foxes (Vulpes vulpes) after oral rabies vaccination campaigns in Germany and Austria, *Arch. Virol.*, 2009, **154**, 1081–1091.

127 C. Fehlner-Gardiner, S. Nadin-Davis, J. Armstrong, F. Muldoon, P. Bachmann and A. Wandeler, ERA vaccine-derived cases of rabies in wildlife and domestic animals in Ontario, Canada, 1989–2004, *J. Wildl. Dis.*, 2008, **44**, 71–85.

128 F. M. Richards and J. R. Knowles, Glutaraldehyde as a protein cross-linking reagent, *J. Mol. Biol.*, 1968, **37**, 231–233.

129 P. V. McGucken and W. Woodside, Studies on the Mode of Action of Glutaraldehyde on Escherichia coli, *J. Appl. Bacteriol.*, 1973, **36**, 419–426.

130 S. N. Madhusudana, R. Shamsundar and S. Seetharaman, In vitro inactivation of the rabies virus by ascorbic acid, *Int. J. Infect. Dis.*, 2004, **8**, 21–25.

131 J. L. Dembinski, O. Hungnes, A. G. Hauge, A. C. Kristoffersen, B. Haneberg and S. Mjaaland, Hydrogen peroxide inactivation of influenza virus preserves antigenic structure and immunogenicity, *J. Virol. Methods*, 2014, **207**, 232–237.

132 J. P. Uittenbogaard, B. Zomer, P. Hoogerhout and B. Metz, Reactions of β-propiolactone with nucleobase analogues, nucleosides, and peptides: Implications for the inactivation of viruses, *J. Biol. Chem.*, 2011, **286**, 36198–36214.

133 P. Wu, Y. Y. Rodríguez, B. J. Hershey, Y. Tadassa, K. A. Dodd and W. Jia, Validation of a binary ethylenimine (BEI) inactivation procedure for biosafety treatment of foot-and-mouth disease viruses (FMDV), vesicular stomatitis viruses (VSV), and swine vesicular disease virus (SVDV), *Vet. Microbiol.*, 2021, **252**, 108928.

134 R. Paliwal and E. London, Comparison of the conformation, hydrophobicity, and model membrane interactions of diphtheria toxin to those of formaldehyde-treated toxin (diphtheria toxoid): Formaldehyde stabilization of the native conformation inhibits changes that allow membrane insertion, *Biochemistry*, 1996, **35**, 2374–2379.

135 D. E. Swayne and J. R. Beck, Heat inactivation of avian influenza and Newcastle disease viruses in egg products, *Avian Pathol.*, 2004, **33**, 512–518.

136 L. Durno and O. Tounekti, Viral Inactivation: Low pH and Detergent, *PDA J. Pharm. Sci. Technol.*, 2015, **69**, 163–172.

137 A. J. Hume, J. Ames, L. J. Rennick, W. P. Duprex, A. Marzi, J. Tonkiss and E. Mühlberger, Inactivation of RNA viruses by gamma irradiation: A study on mitigating factors, *Viruses*, 2016, **8**(7), v8070204.

138 F. Stauffer, T. El-Bacha and A. Da Poian, Advances in the Development of Inactivated Virus Vaccines, *Recent Pat. Anti-Infect. Drug Discovery*, 2008, **1**, 291–296.

139 M. Nishide, K. Tsujimoto, M. Uozaki, K. Ikeda, H. Yamasaki, A. H. Koyama and T. Arakawa, Effects of electrolytes on virus inactivation by acidic solutions, *Int. J. Mol. Med.*, 2011, **27**, 803–809.

140 D. Baxter, Active and passive immunity, vaccine types, excipients and licensing, *Occup. Med.*, 2007, **57**, 552–556.

141 B. Bizzini, J. Blass, A. Turpin and M. Raynaud, Chemical Characterization of Tetanus Toxin and Toxoid: Amino Acid Composition, Number of SH and S–S Groups and N–Terminal Amino Acid, *Eur. J. Biochem.*, 1970, **17**, 100–105.

142 M. S. Salnikova, S. B. Joshi, J. H. Rytting, M. Warny and C. R. Middaugh, Physical characterization of Clostridium difficile toxins and toxoids: effect of the formaldehyde crosslinking on thermal stability, *J. Pharm. Sci.*, 2008, **97**, 3735–3752.

143 S. Mayer, M. Laumer, A. Mackensen, R. Andreesen and S. W. Krause, Analysis of the immune response against tetanus toxoid: Enumeration of specific T helper cells by the Elispot assay, *Immunobiology*, 2002, **205**, 282–289.

144 T. Kaijalainen, S. M. Kharit, A. S. Kvetnaya, K. Sirkiä, E. Herva, O. V. Parkov and H. Nohynek, Invasive infections caused by Neisseria meningitidis, Haemophilus influenzae and Streptococcus pneumoniae among children in St Petersburg, Russia, *Clin. Microbiol. Infect.*, 2008, **14**, 507–510.

145 G. B. Lesinski and M. A. J. Westerink, Novel vaccine strategies to T-independent antigens, *J. Microbiol. Methods*, 2001, **47**, 135–149.

146 D. F. Kelly, E. R. Moxon and A. J. Pollard, Haemophilus influenzae type b conjugate vaccines, *Immunology*, 2004, **113**, 163–174.

147 S. Kruetzmann, M. M. Rosado, H. Weber, U. Germing, O. Tournilhac, H. H. Peter, R. Berner, A. Peters, T. Boehm, A. Plebani, I. Quinti and R. Carsetti, Human immunoglobulin M memory B cells controlling Streptococcus pneumoniae infections are generated in the spleen, *J. Exp. Med.*, 2003, **197**, 939–945.

148 R. Rappuoli, Glycoconjugate vaccines: Principles and mechanisms, *Sci. Transl. Med.*, 2018, **10**(456), eaat4615.

149 R. Rappuoli, E. De Gregorio and P. Costantino, On the mechanisms of conjugate vaccines, *Proc. Natl. Acad. Sci. U. S. A.*, 2019, **116**, 14–16.

150 R. J. Malonis, J. R. Lai and O. Vergnolle, Peptide-Based Vaccines: Current Progress and Future Challenges, *Chem. Rev.*, 2020, **120**, 3210–3229.

151 K. El Bissati, Y. Zhou, S. M. Paulillo, S. K. Raman, C. P. Karch, S. Reed, A. Estes, A. Estes, J. Lykins, P. Burkhard and R. McLeod, Engineering and characterization of a novel Self Assembling Protein for Toxoplasma peptide vaccine in HLA-A*11:01, HLA-A*02:01 and HLA-B*07:02 transgenic mice, *Sci. Rep.*, 2020, **10**(1), 16984.

152 A. Singh, M. Thakur, L. K. Sharma and K. Chandra, Designing a multi-epitope peptide based vaccine against SARS-CoV-2, *Sci. Rep.*, 2020, **10**, 16219.

153 S. Nooraei, H. Bahrulolum, Z. S. Hoseini, C. Katalani, A. Hajizade, A. J. Easton and G. Ahmadian, Virus-like particles: Preparation, immunogenicity and their roles as nanovaccines and drug nanocarriers, *J. Nanobiotechnol.*, 2021, **19**, 1–27.

154 X. Ding, D. Liu, G. Booth, W. Gao and Y. Lu, Virus-Like Particle Engineering: From Rational Design to Versatile Applications, *Biotechnol. J.*, 2018, **13**(5), e1700324.

155 L. H. L. Lua, N. K. Connors, F. Sainsbury, Y. P. Chuan, N. Wibowo and A. P. J. Middelberg, Bioengineering virus-like particles as vaccines, *Biotechnol. Bioeng.*, 2014, **111**, 425–440.

156 A. Roldão, M. C. M. Mellado, L. R. Castilho, M. J. T. Carrondo and P. M. Alves, Virus-like particles in vaccine development, *Expert Rev. Vaccines*, 2010, **9**, 1149–1176.

157 B. V. Syomin and Y. V. Ilyin, Virus-Like Particles as an Instrument of Vaccine Production, *Mol. Biol.*, 2019, **53**, 323–334.

158 M. O. Mohsen, L. Zha, G. Cabral-Miranda and M. F. Bachmann, Major findings and recent advances in virus–like particle (VLP)-based vaccines, *Semin. Immunol.*, 2017, **34**, 123–132.

159 V. Cimica and J. M. Galarza, Adjuvant formulations for virus-like particle (VLP) based vaccines, *Clin. Immunol.*, 2017, **183**, 99–108.

160 P. M. Folegatti, K. J. Ewer, P. K. Aley, B. Angus, S. Becker, S. Belij-Rammerstorfer, D. Bellamy, S. Bibi, M. Bittaye, E. A. Clutterbuck, C. Dold, S. N. Faust, A. Finn, A. L. Flaxman, B. Hallis, P. Heath, D. Jenkin, R. Lazarus, R. Makinson, A. M. Minassian, K. M. Pollock, M. Ramasamy, H. Robinson, M. Snape, R. Tarrant,

M. Voysey, C. Green, A. D. Douglas, A. V. S. Hill, T. Lambe, S. C. Gilbert, A. J. Pollard, J. Aboagye, K. Adams, A. Ali, E. Allen, J. L. Allison, R. Anslow, E. H. Arbe-Barnes, G. Babbage, K. Baillie, M. Baker, N. Baker, P. Baker, I. Baleanu, J. Ballaminut, E. Barnes, J. Barrett, L. Bates, A. Batten, K. Beadon, R. Beckley, E. Berrie, L. Berry, A. Beveridge, K. R. Bewley, E. M. Bijker, T. Bingham, L. Blackwell, C. L. Blundell, E. Bolam, E. Boland, N. Borthwick, T. Bower, A. Boyd, T. Brenner, P. D. Bright, C. Brown-O'Sullivan, E. Brunt, J. Burbage, S. Burge, K. R. Buttigieg, N. Byard, I. Cabera Puig, A. Calvert, S. Camara, M. Cao, F. Cappuccini, M. Carr, M. W. Carroll, V. Carter, K. Cathie, R. J. Challis, S. Charlton, I. Chelysheva, J. S. Cho, P. Cicconi, L. Cifuentes, H. Clark, E. Clark, T. Cole, R. Colin-Jones, C. P. Conlon, A. Cook, N. S. Coombes, R. Cooper, C. A. Cosgrove, K. Coy, W. E. M. Crocker, C. J. Cunningham, B. E. Damratoski, L. Dando, M. S. Datoo, H. Davies, H. De Graaf, T. Demissie, C. Di Maso, I. Dietrich, T. Dong, F. R. Donnellan, N. Douglas, C. Downing, J. Drake, R. Drake-Brockman, R. E. Drury, S. J. Dunachie, N. J. Edwards, F. D. L. Edwards, C. J. Edwards, S. C. Elias, M. J. Elmore, K. R. W. Emary, M. R. English, S. Fagerbrink, S. Felle, S. Feng, S. Field, C. Fixmer, C. Fletcher, K. J. Ford, J. Fowler, P. Fox, E. Francis, J. Frater, J. Furze, M. Fuskova, E. Galiza, D. Gbesemete, C. Gilbride, K. Godwin, G. Gorini, L. Goulston, C. Grabau, L. Gracie, Z. Gray, L. B. Guthrie, M. Hackett, S. Halwe, E. Hamilton, J. Hamlyn, B. Hanumunthadu, I. Harding, S. A. Harris, A. Harris, D. Harrison, C. Harrison, T. C. Hart, L. Haskell, S. Hawkins, I. Head, J. A. Henry, J. Hill, S. H. C. Hodgson, M. M. Hou, E. Howe, N. Howell, C. Hutlin, S. Ikram, C. Isitt, P. Iveson, S. Jackson, F. Jackson, S. W. James, M. Jenkins, E. Jones, K. Jones, C. E. Jones, B. Jones, R. Kailath, K. Karampatsas, J. Keen, S. Kelly, D. Kelly, D. Kerr, S. Kerridge, L. Khan, U. Khan, A. Killen, J. Kinch, T. B. King, L. King, J. King, L. Kingham-Page, P. Klenerman, F. Knapper, J. C. Knight, D. Knott, S. Koleva, A. Kupke, C. W. Larkworthy, J. P. J. Larwood, A. Laskey, A. M. Lawrie, A. Lee, K. Y. Ngan Lee, E. A. Lees, H. Legge, A. Lelliott, N. M. Lemm, A. M. Lias, A. Linder, S. Lipworth, X. Liu, S. Liu, R. Lopez Ramon, M. Lwin, F. Mabesa, M. Madhavan, G. Mallett, K. Mansatta, I. Marcal, S. Marinou, E. Marlow, J. L. Marshall, J. Martin, J. McEwan, L. McInroy, G. Meddaugh, A. J. Mentzer, N. Mirtorabi, M. Moore, E. Moran, E. Morey, V. Morgan, S. J. Morris, H. Morrison, G. Morshead, R. Morter, Y. F. Mujadidi, J. Muller, T. Munera-Huertas, C. Munro, A. Munro, S. Murphy, V. J. Munster, P. Mweu, A. Noé, F. L. Nugent, E. Nuthall, K. O'Brien, D. O'Connor, B. Oguti, J. L. Oliver, C. Oliveira, P. J. O'Reilly, M. Osborn, P. Osborne, C. Owen, D. Owens, N. Owino, M. Pacurar, K. Parker, H. Parracho, M. Patrick-Smith, V. Payne, J. Pearce, Y. Peng, M. P. Peralta Alvarez, J. Perring, K. Pfafferott, D. Pipini, E. Plested, H. Pluess-Hall, K. Pollock, I. Poulton, L. Presland, S. Provstgaard-Morys, D. Pulido, K. Radia, F. Ramos Lopez, J. Rand, H. Ratcliffe, T. Rawlinson, S. Rhead, A. Riddell, A. J. Ritchie, H. Roberts, J. Robson, S. Roche, C. Rohde, C. S. Rollier, R. Romani, I. Rudiansyah, S. Saich, S. Sajjad, S. Salvador, L. Sanchez Riera, H. Sanders, K. Sanders, S. Sapaun, C. Sayce, E. Schofield, G. Screaton, B. Selby, C. Semple, H. R. Sharpe, I. Shaik, A. Shea, H. Shelton, S. Silk, L. Silva-Reyes, D. T. Skelly, H. Smee, C. C. Smith, D. J. Smith, R. Song, A. J. Spencer, E. Stafford, A. Steele, E. Stefanova, L. Stockdale, A. Szigeti, A. Tahiri-Alaoui, M. Tait, H. Talbot, R. Tanner, I. J. Taylor, V. Taylor, R. Te Water Naude, N. Thakur, Y. Themistocleous, A. Themistocleous, M. Thomas, T. M. Thomas, A. Thompson, S. Thomson-Hill, J. Tomlins, S. Tonks, J. Towner, N. Tran, J. A. Tree, A. Truby, K. Turkentine, C. Turner, N. Turner, S. Turner, T. Tuthill, M. Ulaszewska, R. Varughese, N. Van Doremalen, K. Veighey, M. K. Verheul, I. Vichos, E. Vitale, L. Walker, M. E. E. Watson, B. Welham, J. Wheat, C. White, R. White, A. T. Worth, D. Wright, S. Wright, X. L. Yao and Y. Yau, Safety and immunogenicity of the ChAdOx1 nCoV-19 vaccine against SARS-CoV-2: a preliminary report of a phase 1/2, single-blind, randomised controlled trial, *Lancet*, 2020, **396**, 467–478.

161 M. Kovacsovics-Bankowski, K. Clark, B. Benacerraf and K. L. Rock, Efficient major histocompatibility complex class I presentation of exogenous antigen upon phagocytosis by macrophages, *Proc. Natl. Acad. Sci. U. S. A.*, 1993, **90**, 4942–4946.

162 R. Xu, M. Shi, J. Li, P. Song and N. Li, Construction of SARS-CoV-2 Virus-Like Particles by Mammalian Expression System, *Front. Bioeng. Biotechnol.*, 2020, **30**(8), 00862.

163 H. Swann, A. Sharma, B. Preece, A. Peterson, C. Eldridge, D. M. Belnap, M. Vershinin and S. Saffarian, Minimal system for assembly of SARS-CoV-2 virus like particles, *Sci. Rep.*, 2020, **10**(1), 21877.

164 H. Garg, T. Mehmetoglu-Gurbuz and A. Joshi, Virus Like Particles (VLP) as multivalent vaccine candidate against Chikungunya, Japanese Encephalitis, Yellow Fever and Zika Virus, *Sci. Rep.*, 2020, **10**, 4017.

165 S. Dai, H. Wang and F. Deng, Advances and challenges in enveloped virus-like particle (VLP)-based vaccines, *J. Immunol. Sci.*, 2018, **2**, 36–41.

166 E. V. L. Grgacic and D. A. Anderson, Virus-like particles: Passport to immune recognition, *Methods*, 2006, **40**, 60–65.

167 Y. Choi and J. Chang, Viral vectors for vaccine applications, *Clin. Exp. Vaccine Res.*, 2013, **2**, 97.

168 N. Chaudhary, D. Weissman and K. A. Whitehead, mRNA vaccines for infectious diseases: principles, delivery and clinical translation, *Nat. Rev. Drug Discovery*, 2021, **20**, 817–838.

169 M. J. Mastrangelo, L. C. Eisenlohr, L. Gomella and E. C. Lattime, Poxvirus vectors: orphaned and underappreciated, *J. Clin. Invest.*, 2000, **105**, 1031–1034.

170 A. Fathi, C. Dahlke and M. M. Addo, Recombinant vesicular stomatitis virus vector vaccines for WHO blueprint priority pathogens, *Hum. Vaccines Immunother.*, 2019, **15**, 2269–2285.

171 M. F. Díaz, K. Calderón, A. Rojas-Neyra, V. N. Vakharia, R. Choque-Guevara, A. Montalvan-Avalos, A. Poma-Acevedo, D. Rios-Matos, A. Agurto-Arteaga, M. D. G. Cauti-Mendoza, N. Perez-Martinez, G. Isasi-Rivas, L. Tataje-Lavanda, Y. Sernaque-Aguilar, F. Ygnacio, M. Criollo-Orozco, E. Huaccachi-Gonzalez, E. Delgado-Ccancce, D. Villanueva-Pérez, R. Montesinos-Millán, K. Gutiérrez-Manchay, K. Pauyac-Antezana, I. Ramirez-Ortiz, S. Quiñones-Garcia, Y. Cauna-Orocollo, K. Vallejos-Sánchez, A. Rios-Angulo, D. Núñez-Fernández, M. I. Salguedo-Bohorquez, J. Ticona, M. Fernández-Sánchez, E. Icochea, L. A. Guevara-Sarmiento, M. Zimic, A. Agurto-Arteaga, R. Antiparra, M. Ardiles-Reyes, K. Calderón, Y. Cauna-Orocollo, M. D. G. Cauti-Mendoza, N. Chipana-Flores, R. Choque-Guevara, X. Chunga-Girón, M. Criollo-Orozco, L. De La Cruz, E. Delgado-Ccancce, N. E. Delgado-Pease, C. Elugo-Guevara, M. Fernández-Díaz, M. F. Sánchez, L. A. Guevara-Sarmiento, K. Gutiérrez-Manchay, O. Heredia-Almeyda, E. H. Gonzalez, P. Huerta-Roque, E. Icochea, G. Isasi-Rivas, G. Jiménez-Avalos, R. A. Juscamaita-Bartra, A. Licla-Inca, A. Montalvan-Avalos, D. Núñez-Fernández, A. Ochoa-Ortiz, G. E. Olivos-Ramirez, E. Páucar-Montoro, K. Pauyac-Antezana, J. L. Perez-Martinez, N. Perez-Martinez, A. Poma-Acevedo, S. Quiñones-Garcia, I. Ramirez-Ortiz, D. Ramos-Sono, A. Rios-Angulo, D. Rios-Matos, Y. K. Romero, M. I. Salguedo-Bohorquez, Y. Sernaque-Aguilar, P. Sheen, L. F. Soto, L. Tataje-Lavanda, J. Ticona, K. Vallejos-Sánchez, A. P. Vargas-Ruiz, D. Villanueva-Pérez, R. G. Villena and M. Zimic, Intranasal vaccination of hamsters with a Newcastle disease virus vector expressing the S1 subunit protects animals against SARS-CoV-2 disease, *Sci. Rep.*, 2022, **12**, 1–18.

172 F. Sakurai, M. Tachibana and H. Mizuguchi, Adenovirus vector-based vaccine for infectious diseases, *Drug Metab. Pharmacokinet.*, 2022, **42**, 100432.

173 I. R. Humphreys and S. Sebastian, Novel viral vectors in infectious diseases, *Immunology*, 2018, **153**, 1–9.

174 M. Olbert, A. Römer-Oberdörfer, C. Herden, S. Malberg, S. Runge, P. Staeheli and D. Rubbenstroth, Viral vector vaccines expressing nucleoprotein and phosphoprotein genes of avian bornaviruses ameliorate homologous challenge infections in cockatiels and common canaries, *Sci. Rep.*, 2016, **10**(6), 36840.

175 N. Tatsis and H. C. J. Ertl, Adenoviruses as vaccine vectors, *Mol. Ther.*, 2004, **10**, 616–629.

176 A. Milicic, C. S. Rollier, C. K. Tang, R. Longley, A. V. S. Hill and A. Reyes-Sandoval, Adjuvanting a viral vectored vaccine against pre-erythrocytic malaria, *Sci. Rep.*, 2017, **4**(7), 7284.

177 V. Yurina, Live Bacterial Vectors-A Promising DNA Vaccine Delivery System, *Med. Sci.*, 2018, **6**(2), 1–12.

178 I. Tombácz, D. Weissman and N. Pardi, Vaccination with Messenger RNA: A Promising Alternative to DNA Vaccination, *Methods Mol. Biol.*, 2021, **2197**, 13–31.

179 R. L. Sheets, J. Stein, T. S. Manetz, C. Andrews, R. Bailer, J. Rathmann and P. L. Gomez, Toxicological Safety Evaluation of DNA Plasmid Vaccines against HIV-1, Ebola, Severe Acute Respiratory Syndrome, or West Nile Virus Is Similar Despite Differing Plasmid Backbones or Gene-Inserts, *Toxicol. Sci.*, 2006, **91**, 620–630.

180 D. Baumjohann, S. Preite, A. Reboldi, F. Ronchi, K. M. Ansel, A. Lanzavecchia and F. Sallusto, Persistent Antigen and Germinal Center B Cells Sustain T Follicular Helper Cell Responses and Phenotype, *Immunity*, 2013, **38**, 596–605.

181 H. H. Tam, M. B. Melo, M. Kang, J. M. Pelet, V. M. Ruda, M. H. Foley, J. K. Hu, S. Kumari, J. Crampton, A. D. Baldeon, R. W. Sanders, J. P. Moore, S. Crotty, R. Langer, D. G. Anderson, A. K. Chakraborty and D. J. Irvine, Sustained antigen availability during germinal center initiation enhances antibody responses to vaccination, *Proc. Natl. Acad. Sci. U. S. A.*, 2016, **113**, E6639–E6648.

182 J. Yan, H. Yoon, S. Kumar, M. P. Ramanathan, N. Corbitt, M. Kutzler, A. Dai, J. D. Boyer and D. B. Weiner, Enhanced Cellular Immune Responses Elicited by an Engineered HIV-1 Subtype B Consensus-based Envelope DNA Vaccine, *Mol. Ther.*, 2007, **15**, 411–421.

183 P. J. Bergman, M. A. Camps-Palau, J. A. McKnight, N. F. Leibman, D. M. Craft, C. Leung, J. Liao, I. Riviere, M. Sadelain, A. E. Hohenhaus, P. Gregor, A. N. Houghton, M. A. Perales and J. D. Wolchok, Development of a xenogeneic DNA vaccine program for canine malignant melanoma at the Animal Medical Center, *Vaccine*, 2006, **24**, 4582–4585.

184 Z. Wang, P. J. Troilo, X. Wang, T. G. Griffiths, S. J. Pacchione, A. B. Barnum, L. B. Harper, C. J. Pauley, Z. Niu, L. Denisova, T. T. Follmer, G. Rizzuto, G. Ciliberto, E. Fattori, N. L. Monica, S. Manam and B. J. Ledwith, Detection of integration of plasmid DNA into host genomic DNA following intramuscular injection and electroporation, *Gene Ther.*, 2004, **11**, 711–721.

185 G. Anderluzzi, G. Lou, S. Woods, S. T. Schmidt, S. Gallorini, M. Brazzoli, R. Johnson, C. W. Roberts, D. T. O'Hagan, B. C. Baudner and Y. Perrie, The role of nanoparticle format and route of administration on self-amplifying mRNA vaccine potency, *J. Controlled Release*, 2022, **342**, 388–399.

186 X. L. Peng, J. S. Y. Cheng, H. L. Gong, M. Di Yuan, X. H. Zhao, Z. Li and D. X. Wei, Advances in the design and development of SARS-CoV-2 vaccines, *Mil. Med. Res.*, 2021, **8**, 67.

187 T. Schlake, A. Thess, M. Fotin-Mleczek and K. J. Kallen, Developing mRNA-vaccine technologies, *RNA Biol.*, 2012, **9**, 1319–1330.

188 K. Karikó, H. Muramatsu, F. A. Welsh, J. Ludwig, H. Kato, S. Akira and D. Weissman, Incorporation of Pseudouridine

Into mRNA Yields Superior Nonimmunogenic Vector With Increased Translational Capacity and Biological Stability, *Mol. Ther.*, 2008, **16**, 1833–1840.

189 K. Karikó, M. Buckstein, H. Ni and D. Weissman, Suppression of RNA Recognition by Toll-like Receptors: The Impact of Nucleoside Modification and the Evolutionary Origin of RNA, *Immunity*, 2005, **23**, 165–175.

190 O. Andries, S. Mc Cafferty, S. C. De Smedt, R. Weiss, N. N. Sanders and T. Kitada, N1-methylpseudouridine-incorporated mRNA outperforms pseudouridine-incorporated mRNA by providing enhanced protein expression and reduced immunogenicity in mammalian cell lines and mice, *J. Controlled Release*, 2015, **217**, 337–344.

191 M. Baiersdörfer, G. Boros, H. Muramatsu, A. Mahiny, I. Vlatkovic, U. Sahin and K. Karikó, A Facile Method for the Removal of dsRNA Contaminant from In Vitro-Transcribed mRNA, *Mol. Ther.–Nucleic Acids*, 2019, **15**, 26–35.

192 K. Karikó, H. Muramatsu, J. Ludwig and D. Weissman, Generating the optimal mRNA for therapy: HPLC purification eliminates immune activation and improves translation of nucleoside-modified, protein-encoding mRNA, *Nucleic Acids Res.*, 2011, **39**, e142–e142.

193 K. H. Asrani, J. D. Farelli, M. R. Stahley, R. L. Miller, C. J. Cheng, R. R. Subramanian and J. M. Brown, Optimization of mRNA untranslated regions for improved expression of therapeutic mRNA, *RNA Biol.*, 2018, **15**, 756–762.

194 K. Ali, G. Berman, H. Zhou, W. Deng, V. Faughnan, M. Coronado-Voges, B. Ding, J. Dooley, B. Girard, W. Hillebrand, R. Pajon, J. M. Miller, B. Leav and R. McPhee, Evaluation of mRNA-1273 SARS-CoV-2 Vaccine in Adolescents, *N. Engl. J. Med.*, 2021, **385**, 2241–2251.

195 J. B. Trotman and D. R. Schoenberg, A recap of RNA recapping, *Wiley Interdiscip. Rev.: RNA*, 2019, **10**, e1504.

196 D. Weissman, N. Pardi, H. Muramatsu and K. Karikó, HPLC Purification of In Vitro Transcribed Long RNA, *Methods Mol. Biol.*, 2013, **969**, 43–54.

197 A. Ramanathan, G. B. Robb and S. H. Chan, mRNA capping: biological functions and applications, *Nucleic Acids Res.*, 2016, **44**, 7511–7526.

198 D. Magini, C. Giovani, S. Mangiavacchi, S. MacCari, R. Cecchi, J. B. Ulmer, E. De Gregorio, A. J. Geall, M. Brazzoli and S. Bertholet, Self-Amplifying mRNA Vaccines Expressing Multiple Conserved Influenza Antigens Confer Protection against Homologous and Heterosubtypic Viral Challenge, *PLoS One*, 2016, **11**, e0161193.

199 G. Maruggi, C. Zhang, J. Li, J. B. Ulmer and D. Yu, mRNA as a Transformative Technology for Vaccine Development to Control Infectious Diseases, *Mol. Ther.*, 2019, **27**, 757–772.

200 P. F. McKay, K. Hu, A. K. Blakney, K. Samnuan, J. C. Brown, R. Penn, J. Zhou, C. R. Bouton, P. Rogers, K. Polra, P. J. C. Lin, C. Barbosa, Y. K. Tam, W. S. Barclay and R. J. Shattock, Self-amplifying RNA SARS-CoV-2 lipid nanoparticle vaccine candidate induces high neutralizing antibody titers in mice, *Nat. Commun.*, 2020, **11**, 1–7.

201 M. D. Buschmann, M. J. Carrasco, S. Alishetty, M. Paige, M. G. Alameh and D. Weissman, Nanomaterial Delivery Systems for mRNA Vaccines, *Vaccines*, 2021, **9**, 65.

202 S. Qu, X. Yang, X. Li, J. Wang, Y. Gao, R. Shang, W. Sun, K. Dou and H. Li, Circular RNA: A new star of noncoding RNAs, *Cancer Lett.*, 2015, **365**, 141–148.

203 W. Y. Zhou, Z. R. Cai, J. Liu, D. S. Wang, H. Q. Ju and R. H. Xu, Circular RNA: metabolism, functions and interactions with proteins, *Mol. Cancer*, 2020, **19**, 1–19.

204 L. S. Kristensen, M. S. Andersen, L. V. W. Stagsted, K. K. Ebbesen, T. B. Hansen and J. Kjems, The biogenesis, biology and characterization of circular RNAs, *Nat. Rev. Genet.*, 2019, **20**, 675–691.

205 M. G. Alameh, I. Tombácz, E. Bettini, K. Lederer, C. Sittplangkoon, J. R. Wilmore, B. T. Gaudette, O. Y. Soliman, M. Pine, P. Hicks, T. B. Manzoni, J. J. Knox, J. L. Johnson, D. Laczkó, H. Muramatsu, B. Davis, W. Meng, A. M. Rosenfeld, S. Strohmeier, P. J. C. Lin, B. L. Mui, Y. K. Tam, K. Karikó, A. Jacquet, F. Krammer, P. Bates, M. P. Cancro, D. Weissman, E. T. Luning Prak, D. Allman, M. Locci and N. Pardi, Lipid nanoparticles enhance the efficacy of mRNA and protein subunit vaccines by inducing robust T follicular helper cell and humoral responses, *Immunity*, 2021, **54**, 2877–2892.

206 S. Ndeupen, Z. Qin, S. Jacobsen, A. Bouteau, H. Estanbouli and B. Z. Igyártó, The mRNA-LNP platform's lipid nanoparticle component used in preclinical vaccine studies is highly inflammatory, *iScience*, 2021, **24**, 103479.

207 D. Huang, D. Zhao, X. Wang, C. Li, T. Yang, L. Du, Z. Wei, Q. Cheng, H. Cao, Z. Liang, Y. Huang and Z. Li, Efficient delivery of nucleic acid molecules into skin by combined use of microneedle roller and flexible interdigitated electroporation array, *Theranostics*, 2018, **8**, 2361–2376.

208 Y. Cao and G. F. Gao, mRNA vaccines: A matter of delivery, *eClinicalMedicine*, 2021, **3**(32), 100746.

209 C. Hald Albertsen, J. A. Kulkarni, D. Witzigmann, M. Lind, K. Petersson and J. B. Simonsen, The role of lipid components in lipid nanoparticles for vaccines and gene therapy, *Adv. Drug Delivery Rev.*, 2022, **188**, 114416.

210 X. Hou, T. Zaks, R. Langer and Y. Dong, Lipid nanoparticles for mRNA delivery, *Nat. Rev. Mater.*, 2021, **6**, 1078–1094.

211 J. Wang, Y. Ding, K. Chong, M. Cui, Z. Cao, C. Tang, Z. Tian, Y. Hu, Y. Zhao and S. Jiang, Recent Advances in Lipid Nanoparticles and Their Safety Concerns for mRNA Delivery, *Vaccines*, 2024, **12**, 1148.

212 D. S. Tretiakova and E. L. Vodovozova, Liposomes as Adjuvants and Vaccine Delivery Systems, *Biochem., Suppl. Ser. A: Membr. Cell Biol.*, 2022, **16**, 1–20.

213 A. J. Da Silva Sanchez, K. Zhao, S. G. Huayamares, M. Z. C. Hatit, M. P. Lokugamage, D. Loughrey, C. Dobrowolski, S. Wang, H. Kim, K. Paunovska, Y. Kuzminich and J. E. Dahlman, Substituting racemic

ionizable lipids with stereopure ionizable lipids can increase mRNA delivery, *J. Controlled Release*, 2023, **353**, 270–277.

214 S. K. Patel, M. M. Billingsley, C. Frazee, X. Han, K. L. Swingle, J. Qin, M. G. Alameh, K. Wang, D. Weissman and M. J. Mitchell, Hydroxycholesterol substitution in ionizable lipid nanoparticles for mRNA delivery to T cells, *J. Controlled Release*, 2022, **347**, 521–532.

215 R. Tenchov, R. Bird, A. E. Curtze and Q. Zhou, Lipid Nanoparticles from Liposomes to mRNA Vaccine Delivery, a Landscape of Research Diversity and Advancement, *ACS Nano*, 2021, **15**, 16982–17015.

216 K. J. Hassett, K. E. Benenato, E. Jacquinet, A. Lee, A. Woods, O. Yuzhakov, S. Himansu, J. Deterling, B. M. Geilich, T. Ketova, C. Mihai, A. Lynn, I. McFadyen, M. J. Moore, J. J. Senn, M. G. Stanton, Ö. Almarsson, G. Ciaramella and L. A. Brito, Optimization of Lipid Nanoparticles for Intramuscular Administration of mRNA Vaccines, *Mol. Ther.–Nucleic Acids*, 2019, **15**, 1–11.

217 N. Pardi, M. J. Hogan, F. W. Porter and D. Weissman, mRNA vaccines—a new era in vaccinology, *Nat. Rev. Drug Discovery*, 2018, **17**, 261–279.

218 D. Carugo, E. Bottaro, J. Owen, E. Stride and C. Nastruzzi, Liposome production by microfluidics: potential and limiting factors, *Sci. Rep.*, 2016, **6**, 1–15.

219 M. Maeki, S. Uno, A. Niwa, Y. Okada and M. Tokeshi, Microfluidic technologies and devices for lipid nanoparticle-based RNA delivery, *J. Controlled Release*, 2022, **344**, 80–96.

220 A. Jahn, S. M. Stavis, J. S. Hong, W. N. Vreeland, D. L. Devoe and M. Gaitan, Microfluidic mixing and the formation of nanoscale lipid vesicles, *ACS Nano*, 2010, **4**, 2077–2087.

221 M. Michelon, D. R. B. Oliveira, G. de Figueiredo Furtado, L. Gaziola de la Torre and R. L. Cunha, High-throughput continuous production of liposomes using hydrodynamic flow-focusing microfluidic devices, *Colloids Surf., B*, 2017, **156**, 349–357.

222 D. Chen, K. T. Love, Y. Chen, A. A. Eltoukhy, C. Kastrup, G. Sahay, A. Jeon, Y. Dong, K. A. Whitehead and D. G. Anderson, Rapid discovery of potent siRNA-containing lipid nanoparticles enabled by controlled microfluidic formulation, *J. Am. Chem. Soc.*, 2012, **134**, 6948–6951.

223 D. J. Pollard and A. Pralong, in *Biopharmaceutical Processing: Development, Design, and Implementation of Manufacturing Processes*, ed. G. Jagschies, E. Lindskog, K. Łącki and P. Galliher, Elsevier, 1st edn, 2018, pp. 721–740.

224 R. B. Kennedy, I. G. Ovsyannikova, P. Palese and G. A. Poland, Current Challenges in Vaccinology, *Front. Immunol.*, 2020, **11**, 541543.

225 B. Schlingmann, K. R. Castiglia, C. C. Stobart and M. L. Moore, Polyvalent vaccines: High-maintenance heroes, *PLoS Pathog.*, 2018, **14**, e1006904.

226 M. S. Arshad, S. Zafar, B. Yousef, Y. Alyassin, R. Ali, A. AlAsiri, M. W. Chang, Z. Ahmad, A. Ali Elkordy, A. Faheem and K. Pitt, A review of emerging technologies enabling improved solid oral dosage form manufacturing and processing, *Adv. Drug Delivery Rev.*, 2021, **178**, 113840.

227 P. Naik, Edible vaccines: Current scenario and future prospects, in *Futur. Foods Glob. Trends, Oppor. Sustain. Challenges*, 2022, pp. 305–313.

228 J. Saxena and S. Rawat, Edible vaccines, *Adv. Biotechnol.*, 2014, 207–226.