Integrating plant molecular farming and materials research for next-generation vaccines

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Abstract | Biologics — medications derived from a biological source — are increasingly used as pharmaceuticals, for example, as vaccines. Biologics are usually produced in bacterial, mammalian or insect cells. Alternatively, plant molecular farming, that is, the manufacture of biologics in plant cells, transgenic plants and algae, offers a cheaper and easily adaptable strategy for the production of biologics, in particular, in low-resource settings. In this Review, we discuss current vaccination challenges, such as cold chain requirements, and highlight how plant molecular farming in combination with advanced materials can be applied to address these challenges. The production of plant viruses and virus-based nanotechnologies in plants enables low-cost and regional fabrication of thermostable vaccines. We also highlight key new vaccine delivery technologies, including microneedle patches and material platforms for intranasal and oral delivery. Finally, we provide an outlook of future possibilities for plant molecular farming of next-generation vaccines and biologics.

A human analogue of insulin (Humulin) was the first biologic introduced into the market in 1983. Since then, biologics have been increasingly used as pharmaceutical agents, outpacing the market of small-molecule drugs[1] with an estimated market size of around US$400 billion by 2025 (REF.[2]). Biologics are a class of medications that are derived from a biological source and typically fall into one of four major categories: monoclonal antibodies (mAbs), receptor modulators, enzyme modulators and vaccines[3]. Vaccines have been crucial in eliminating infectious diseases, such as smallpox[4] and rinderpest in 2011 (REF.[5]), and in eradicating once-deadly diseases, such as diphtheria, measles, polio and rubella, by 99%, with many other diseases, such as mumps, pertussis and tetanus, nearing that stage. In the USA alone, it is estimated that in just one generation of children, 13 different vaccines have prevented up to 20 million diseases and 40,000 deaths, with economic savings of up to US$69 billion[4]. On a global scale, these numbers are much higher; one estimate shows that ten vaccines deployed in 94 low- and middle-income countries led to a US$586 billion reduction in illness-related costs, and a reduction of up to US$1.53 trillion when considering additional economic benefits[6].

Vaccines are usually manufactured using cell-based expression systems, such as mammalian and insect cell lines, bacterial or yeast cultures. Plant molecular farming, that is, using plant cells or plants as expression platforms, is a rapidly emerging alternative, which was originally introduced in 1986 for the production of human growth hormone (HGH) in transgenic tobacco and sunflowers[7]. Plant molecular farming is currently used for the production of seasonal influenza vaccines[8] and for Elelyso for Gaucher disease in the USA[9]. Although plant molecular farming may not replace industrial expression systems, it may have a unique role in the generation of vaccines in low-resource areas, for targeting niche and orphan vaccines, and for the production of virus nanoparticles (VNPs) for vaccine applications[10]. Furthermore, the rise of the coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has focused research attention on novel approaches to vaccines. Traditional vaccines are either live attenuated (that is, less virulent forms of the original pathogen), inactivated (that is, inactivated pathogen without disease-producing capacity), or recombinant proteins and viral vectors. In addition, the two vaccines that were first granted emergency authorization in the USA for COVID-19 are messenger RNA (mRNA) vaccines delivered in lipid nanoparticles[11].

In this Review, we discuss challenges in current vaccine development and distribution, such as cold chain

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disruptions and lack of health-care professionals, which are particularly problematic in low-resource areas. Cold chain requirements pose a general logistical challenge for vaccines, as highlighted by the current rollout of the mRNA-based COVID-19 vaccines[12], and traditional administration techniques, such as injections, require trained health-care professionals. Here, we investigate how molecular farming and nanotechnology-based strategies could provide an alternative strategy to traditional vaccines, enabling rapid development, effective deployment and safe administration of vaccines.

Vaccine technology

A brief history of vaccines

The history of modern vaccination began with Edward Jenner's 'live attenuated' smallpox vaccine in the late eighteenth century[13]. Nearly a century later, in 1885, Louis Pasteur developed a rabies vaccine by drying the brains of infected rabbits; however, this vaccine was rather unpredictable and often caused serious side effects[14]. Shortly thereafter it was found that heat or chemical treatment could be used to inactivate bacteria, and vaccines for typhoid (*Salmonella typhi*), plague (*Yersinia pestis*) and cholera (*Vibrio cholerae*) were developed[13]. Vaccine production was greatly improved in the 1950s, when the development of cell culture techniques enabled the in vitro production of non-virulent viruses instead of requiring to be isolated from infected animals[15]. Bacterially expressed hepatitis B surface antigen then became the first vaccine produced by recombinant DNA technology[16], which laid the foundation for the four major categories of modern vaccines: live attenuated, killed/inactivated whole organisms, subunit and toxoid vaccines (TABLE 1). Since then, new vaccine types have emerged, such as viral vectors and nucleic acid vaccines[17]. Today, 85 human vaccines and vaccine combinations are on the market.

Challenges and innovations

Materials design can greatly improve vaccination approaches by enabling stabilization and controlled release, and/or by providing delivery devices for self-administration, which is particularly important for vaccines against viruses with a great degree of variability, such as the human immunodeficiency viruses (HIV-1 and HIV-2). HIV-1 shows worldwide variability as well as direct infection and destruction of immune cells. In addition, there is no validated animal model available for HIV-1 research, and the high rate of mutation allows the virus to escape antibody responses to targeted antigens[18]. High variability may be addressed by universal vaccines, which are effective against all virus subtypes and are impervious to future mutations by the pathogen. For example, the Gardasil-9 vaccine provides immunity against nine human papillomavirus (HPV) types, which should prevent >90% of HPV infections and subsequent cervical cancer[19]; however, expanding immunity protection to all known HPVs would enable HPV eradication. A universal vaccine against influenza would also greatly decrease infection rates, health-care costs and associated mortality, while improving patient compliance[20]. Nanotechnology-based approaches, such as HPV-encapsidating microneedles[21] and slow-release implant technologies[22], could further improve immunization against these difficult pathogens.

Vaccines are also being developed for non-infectious pathophysiological conditions, such as autoimmune diseases and cancers[23]. These vaccines are difficult to produce, because the molecular causes and antigens are often specific to the individual patient. Therefore, unlike for infectious disease vaccines, here it may be more advantageous to produce patient-specific vaccines — a considerable challenge for conventional production methods. Improvements in biotechnology methods, such as whole-exome or RNA sequencing[24], may allow the isolation of patient-specific antigens for the creation of specialized vaccines.

Nanotechnology-based approaches have also played a key part in the development of COVID-19 mRNA vaccines, although it remains to be seen whether mRNA vaccines will provide long- or short-lasting immunity against SARS-CoV-2. Furthermore, the lack of thermostability of lipid nanoparticle formulations requiring storage and distribution in ultralow-temperature freezers presents a great challenge for vaccine distribution to rural and low-resource regions because these vaccines are temperature-stable only when frozen at –20 to –80 °C. Viral vector vaccines may be better suited for vaccination in remote areas, because they can be stored at higher temperatures of up to 4 °C. However, early reports suggest that viral vector vaccines do not reduce symptomatic infection as well as mRNA vaccines[25], although the differing trial protocols and demographics make it difficult to compare the trials equally.

Traditional biologics expression

Humulin, the first biologic used in humans[1], is expressed in *Escherichia coli*[26]. Advances in DNA technology have further enabled vaccine production in yeast, for example, for the production of subunit vaccines against the hepatitis B virus (HBV)[27]. In addition, mammalian cells, insect cells and chicken eggs can be applied as expression systems for vaccine production.

Bacteria are the most widely used expression system, benefiting from low cost, rapid growth and ease of use; in ideal conditions, *E. coli* can double its biomass within 20 minutes[28]. An estimated 30% of all biopharmaceuticals are currently engineered in bacterial systems, with *E. coli* being the most widely used strain[29]. Bacterial systems are also applied to generate DNA plasmids, which are subsequently used to produce the final biologic.

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Table 1 | **Vaccine types used in the clinic**

Vaccine type	Formulation	Advantages	Disadvantages	Example (Brand name; Developer)	Refs
Live attenuated	Weakened live pathogen, usually produced by serial culture	Long-lasting humoral and cell-mediated immunity Lower doses needed than inactivated vaccines	Not given to immunocompromised individuals Cold chain requirement Possible reversion to a virulent form	Measles, mumps, rubella (M-M-RII; Merck)	163
Inactivated	Pathogen killed by heat or chemical treatment	Cold chain not required Cannot reverse to virulence	Usually, no cell-mediated immunity Requires boosters Usually, more side effects than live attenuated vaccines	Hepatitis A (Havrix; GlaxoSmithKline (GSK))	164
Subunit (protein, polysaccharide)	Comprised of only the immunostimulatory parts of the pathogen	Lower risk of side effects than live-attenuated and inactivated vaccines Long-lasting immunity	Requires multiple doses Requires adjuvant to boost immunogenicity	Hepatitis B (Recombivax; Merck)	165
Virus-like particles (VLPs)	Subunit vaccine that self-assembles into non-infectious and non-replicating VLPs; or subunit vaccines presented on a non-infectious VLP	Lower risk of side effects than live attenuated vaccines Long-lasting immunity	More expensive to produce than traditional (less complex) subunit vaccines Requires adjuvant to boost immunogenicity	Human papillomavirus (Gardasil 9; Merck) or SARS-CoV-2 (KBP-201; Kentucky Bioprocessing)	166,167
Nucleic acid	mRNA or DNA, coding for the pathogenic antigen	Induces both humoral and cell-mediated immunity Rapid development and production Relatively inexpensive compared with traditional vaccines	Limited to protein vaccines Requires carrier May require adjuvant	Cervical lesions (VGX-3100; Inovio)	168
Viral vector	Incorporation of the DNA of an antigen within an attenuated or replication-incompetent virus	Induces both humoral and cell-mediated immunity Wide tissue tropism	Pre-existing immunity Possible reversion to virulence, although highly unlikely if replication-incompetent	Ebola (Ervebo; Merck)	169
Toxoid	Toxins secreted by bacteria; purified and deactivated with formaldehyde	Safe Low rate of side effects Stable	Not strongly immunogenic; requires adjuvants Requires larger doses and boosters compared with live-attenuated vaccines	Tetanus, diphtheria (TDVAX; MassBiologics)	NA

NA, not applicable.

The main disadvantage of bacterial systems is the lack of eukaryotic post-translational modifications. Without proper post-translational modification, purified proteins may behave differently in vivo[26] compared with their non-recombinant counterparts, which can lead to diminished or total loss of activity, reduced half-life and decreased stability and/or immunogenicity[30]. Moreover, the presence of rare codons in eukaryotic genes can be problematic, because they can cause early termination during bacterial protein production, necessitating the redesign of genes[31].

To overcome the problems associated with bacterial protein production, mammalian cell lines can be used. From 2016 to 2018, 84% of pharmaceutical proteins were made in mammalian cell lines, predominantly in Chinese hamster ovary (CHO) cells[32]. CHO cells achieve high protein yield (up to 10 g l^{-1} for some proteins)[33], they can grow in suspension[34] and they can withstand changes in external factors, such as temperature and pH[35]. However, although post-translational modifications in CHO cells more closely resemble those of human cells, they are not identical and can induce immune or other adverse reactions in patients[36].

Alternatively, insect cells replicate faster than mammalian cells, enabling faster protein expression, but are more costly than bacteria owing to the requirement of specialized culture media[28]. A main disadvantage of insect cells is also the difference in post-translational modifications, as compared with human cells, which can make the biologics immunogenic[37]. Furthermore, some insects carry pathogenic viruses (for example, arboviruses) and, thus, insect cell lines must be closely examined before regulatory approval[38]. Furthermore, insect cells can produce proteases in response to viral transfection that can digest the protein of interest[37].

Finally, chicken eggs are widely used for vaccine production, for example, for influenza vaccines[39]. Although well established, antigenic drift in chicken eggs can decrease vaccine efficacy, compared with vaccines produced in cell-based systems[40]. In addition, vaccine production in chicken eggs can take up to 6 months, whereas insect cell systems can produce vaccines in

6–8 weeks[39]. Possible contamination with human pathogens can also be a problem, which can be avoided by molecular farming[41].

Molecular farming

Since the production of HGH in transgenic tobacco[7], many proof-of-concept and efficacy tests have been performed of plant-made therapeutics and vaccines for humans and animals[42] (TABLE 2). A tobacco-produced mAb used in the production of a hepatitis B subunit vaccine[43] and a Newcastle disease subunit vaccine for poultry made in cultured tobacco cells were the first plant-made recombinant proteins to receive regulatory approval in 2006 (REF.[44]) (FIG. 1). However, only one human therapeutic (Elelyso, a mitochondrial enzyme deficit therapy for Gaucher disease) produced by molecular farming has thus far been licensed by the US Food and Drug Administration (FDA)[9]. In addition, the first phase III human clinical trial for a plant-produced vaccine has just been successfully concluded. This virus like particle (VLP)-based quadrivalent seasonal influenza virus vaccine is currently undergoing final consideration for licensure in Canada[8]. The fact that only a few plant-produced therapeutic products have

Table 2 | **Vaccines and biologics produced by molecular farming**

Pathogen or condition	Antigenic epitope or biologic	Plant	Transformation method	Company	Ref.
Vaccines					
Influenza virus	Influenza VLP	*Nicotiana benthamiana*	*Agrobacterium tumefaciens*	Medicago, Inc.	[170]
Hepatitis B virus	HBsAg	Tomato	Agrobacterium	–	[171]
Escherichia coli	LT-B	Carrot	Agrobacterium	–	[172]
Rotavirus	Rotavirus VP7	Potato	Agrobacterium	–	[173]
Ebola virus	Ebola glycoprotein (GP1)	*Nicotiana benthamiana*	Agroinfiltration	–	[174]
Foot-and-mouth disease virus	VP1	Tobacco	Biolistic method	–	[175]
Plasmodium falciparum	Pfs25-CP VLP	*Nicotiana benthamiana*	Agrobacterium	–	[176]
Norwalk virus	Norwalk virus VLP	*Nicotiana benthamiana*	*Agrobacterium tumefaciens*	–	[177]
Dengue virus	Dengue virus type 2 E glycoprotein (EIII)	*Nicotiana tabacum* cv. MD609	*Agrobacterium tumefaciens*	–	[178]
SARS-CoV-2	VLP	*Nicotiana benthamiana*	–	Medicago, Inc.	[179]
SARS-CoV-2	RBD of SARS-CoV-2	*Nicotiana benthamiana*	–	Kentucky BioProcessing, Inc.	[167]
SARS-CoV-2	VLP	Tobacco	–	iBio, Inc.	iBio
SARS-CoV-2	Spike protein fused with patented LicKM	Tobacco	–	iBio, Inc.	iBio
Avian H5N1 influenza	Haemagglutinin protein of H5N1	*Nicotiana benthamiana*	Agrobacterium	–	[180]
Biologics					
Skin rejuvenation	Basic fibroblast growth factor (bFGF)	*Nicotiana benthamiana*	*Agrobacterium tumefaciens*	Baiya	–
Skin rejuvenation	Epidermal growth factor (EGF)	*Nicotiana benthamiana*	*Agrobacterium tumefaciens*	Baiya	–
Ebola	ZMapp	*Nicotiana benthamiana*	magnICON	Kentucky BioProcessing	[181]
Diabetes	Insulin	Safflower	*Agrobacterium tumefaciens*	SemBioSys	[182]
Neurotoxic agents	Acetylcholinesterase	Tobacco	PEGylated	Protalix BioTherapeutics	[183]
Inflammatory bowel disease	Lactoferrin	Rice (*Oryza sativa* L.)	ExpressTec	Ventria	[184]
ETEC	Lysozyme	Rice (*Oryza sativa* L.)	ExpressTec	Ventria	[184]
SARS-CoV-2	Neutralizing MAb B38 and H4	*Nicotiana benthamiana*	*Agrobacterium tumefaciens*	Baiya	[185]
SARS-CoV-2	Spike glycoprotein S1 antibody CR3022	*Nicotiana benthamiana*	*Agrobacterium tumefaciens*	Baiya	[186]
Fabry disease	Pegunigalsidase alfa	Carrot cells	*Agrobacterium tumefaciens*	Protalix BioTherapeutics	[187]
Rabies virus	mAb E559	Tobacco and maize	Agrobacterium	–	[188]

ETEC, enterotoxigenic *Escherichia coli*; mAb, monoclonal antibody; PEG, polyethylene glycol; RBD, receptor-binding domain; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; VLP, virus-like particle[189–195].

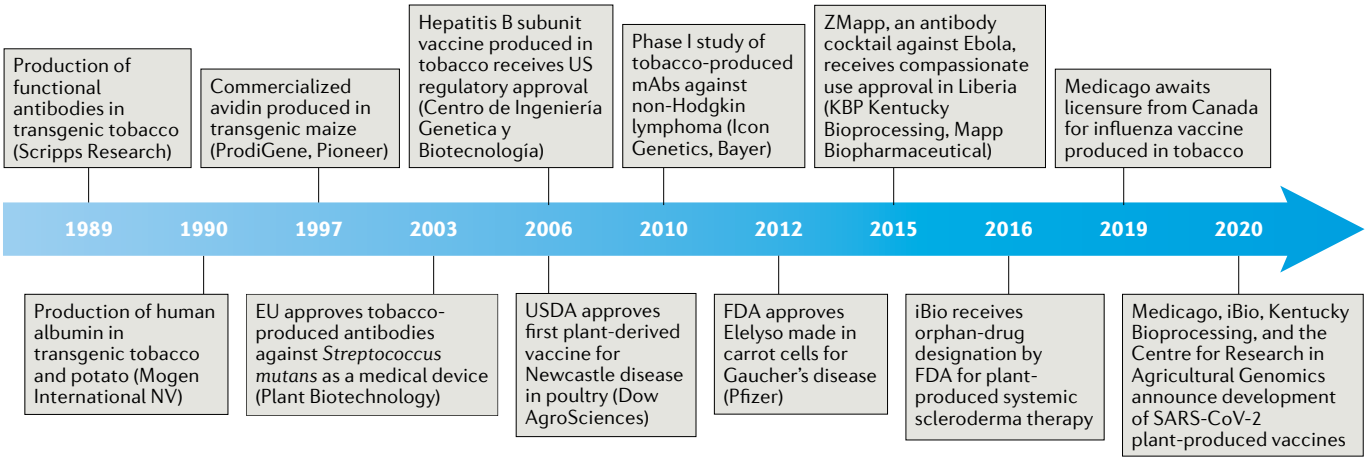


Fig. 1 | **Timeline of the development of plant molecular farming.** FDA, US Food and Drug Administration; mAbs, monoclonal antibodies; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; USDA, US Department of Agriculture.

been clinically translated thus far may be more related to industrial and regulatory inertia than to product inadequacy, especially given the plentiful evidence of functional equivalency.

Transgenic plant and plant cell production of proteins laid the foundation of plant molecular farming; however, it was the advent of transient expression technologies that unleashed its true potential, including plant virus-derived vectors, somatic transformation of cells in normal plants (usually by infiltration of *Nicotiana benthamiana* with *Agrobacterium* suspensions), and hybrid methods involving delivery of replicating vectors by *Agrobacterium*[42] (BOX 1). These transient technologies enable protein expression within days of gene cloning, instead of relying on the generation of stable transgenic plants, which can take years. Moreover, yields are usually higher and more consistent, than transgenic plant systems, because these techniques do not depend on genome insertion sites and expression is not downregulated by methylation or other gene modifications, enabling much faster experimentation and optimization of expression. In addition, production scale-up is more efficient than transgenic plants and conventional cell-based technologies. Only a small volume of recombinant *Agrobacterium* suspension has to be grown for the biologic of interest, which can then be combined by agroinfiltration with as many plants — grown cheaply in nearby facilities — as required. It is striking that Medicago Inc. received funding from the US Defense Advanced Research Projects Agency (DARPA) Blue Angel initiative to produce ten million doses of current good manufacturing practice (cGMP)-level influenza vaccines 1 month after receiving the sequence of the virus[45]. Medicago Inc. has further announced preliminary success in investigating a VLP-based COVID-19 vaccine candidate, which was produced in just over 20 days after receiving the spike protein gene sequence[46]. Phase I clinical trial results showed ten times more production of neutralizing antibodies than in convalescent sera[47]. This vaccine candidate has recently entered phase II/III clinical trials and is administered along with GlaxoSmithKline's AS03 adjuvant[48].

Algal cell expression

The microalga *Chlamydomonas reinhardtii* is the most commonly used algal system for molecular farming[49] and has been applied to express mAbs (against glycoprotein D of the herpes simplex virus and human fibronectin type III), subunit vaccines (viral protein (VP) 1 of foot-and-mouth disease), allergens (peanut allergens), growth factors (vascular endothelial growth factor), and immunotoxins (chimeric antibody to CD22 and exotoxin A)[50]. Although the genomes of the mitochondria, nucleus and chloroplast of *C. reinhardtii* have been sequenced[51], expression has been mainly applied in chloroplasts thus far, because the chloroplast genome is small and simple[50], and biologics can be sequestered in the chloroplast without harming the host cells or being degraded[51]. However, similar to bacterial expression, post-translational modifications are lacking in this system. Alternatively, *Lemna* and moss systems have been explored for biologic expression[52,53].

Plants and plant cells

Using plants for vaccine production has the advantage that plants are cheaper than mammalian systems in terms of biomass production, and they have similar protein folding, assembly and glycosylation[54]. Therefore, plants can be applied to efficiently produce a range of pharmaceuticals at low cost. It is estimated that the final cost of producing biologics in plants is 68% of that in conventional production systems (site and batch costs are lower in plants, whereas downstream processing costs are identical)[55]. Furthermore, post-translational modifications, such as glycosylation, can be manipulated in plants. Thus, plant-produced products have appropriate glycan structures, which can improve half-lives of products and ease downstream processing[56]. For example, Elelyso is considered a 'biobetter' (biologics that improve upon existing biologics), compared with its CHO-produced counterpart, because the end product contains terminal mannose residues that aid in macrophage receptor binding[57]. In CHO cell production, mannose residues are attached in vitro, adding an extra step and increasing the costs[58]. Influenza virus

Box 1 | **Transient expression in plants**

Recombinant proteins can be produced in stable transgenic plants[196], through expression in plant cell cultures[197], and by transient expression in plants using engineered plant viruses or *Agrobacterium tumefaciens*.

Transient expression in plants can be broken down into four main steps.

(1) Choosing the plant host. As with any expression system, it is important to choose a host with desirable characteristics. However, owing to regulatory pressures arising from concerns of possible human and wildlife exposure to modified edible plants, molecular farming is now almost exclusively done in non-edible tobacco plants, such as *Nicotiana benthamiana* and *N. tabacum*.

(2) Choosing the vector. Two different vector types are currently available, that is, *A. tumefaciens*, which is a plant-tumour-causing bacterium with high capacity for transferring DNA into plants, and plant virus-based vectors, which are often delivered by *A. tumefaciens*.

(3) Transfection of plants. Plants can be transfected by mechanical inoculation, agroinfiltration or vacuum infiltration. In mechanical inoculation, the surface of the plant tissue is inoculated with *A. tumefaciens* or with linearized plasmids through gentle disturbance of the cell wall. Agroinfiltration can be achieved by infiltrating the vector into cells on the underside of the plant leaves by making a small nick in the epidermis with a syringe. In vacuum infiltration, a vacuum is applied to allow the vectors to penetrate air spaces throughout the entirety of the plant. In addition, agrospray[198] and agrodip[199] approaches have been investigated.

(4) Purification of the target protein. Protein purification is achieved by recovery, followed by purification. First, plant tissue and cell walls are broken down to release the protein of interest. The proteins are then separated from the plant homogenates using solid–liquid separation methods, such as membrane filtration or centrifugation. The solution is then conditioned for the purification process, which usually comprises a series of multistep chromatography methods. The order of chromatography depends on the biologic (for example, monoclonal antibodies are purified on protein A columns), but often ion exchange chromatography is followed by hydrophobic interaction chromatography. Alternatively, affinity chromatography can be applied: a substance that can bind the protein of interest is attached to a solid matrix (for example, antibodies or enzyme substrates) to pull the protein out of solution. Additional tags, which are recognized and bound, can be added to the N- or C-terminus of the protein to aid purification. These tags may, however, interfere with the biological activity of the protein. The tag can subsequently be removed by adding a protease cleavage site between the tag and the protein (for example, a thrombin cleavage site), although this requires an additional round of purification.

haemagglutinin proteins made in plants were shown not to induce problematic adverse effects in clinical trials despite non-mammalian glycosylation[59]. In proteins that require human glycosylation, such as the HIV-1 Env gp140 protein, the yield can be substantially increased and the glycosylation pattern of HIV-1 Env gp140 can be altered through coexpression of human chaperonins in *N. benthamiana* plants, engineered to not produce xylose or fucose transferases[60].

Plants and plant cells are also inherently safer than bacterial and mammalian systems owing to the low possibility of harmful contamination. In contrast to these systems, plants do not produce endotoxins and cannot be infected by pathogens harmful to humans[61]. Therefore, plants could potentially be used in edible vaccines[62], although concerns of cross-contamination and accidental vaccination of wildlife have stalled development in this field.

Various vaccine candidates have already been produced by molecular farming, including HIV-1 Env[60], ten different HPV L1 proteins[63], West Nile virus envelope protein[64] and dengue virus VLPs[65]. Notably, this list includes three different envelope glycoproteins or their derivatives. In addition, a recombinant double-stranded DNA (dsDNA) molecule has been encapsidated in HPV-16 pseudovirions, which were made in plants[66], proving that DNA vaccines and their delivery systems can be succesfully produced in plants. Similarly, mRNA vaccines can be produced in plants; for example, recombinant mRNA molecules can be expressed in plants from DNA vectors and specifically encapsidated by adding an assembly signal in tobacco mosaic virus (TMV) coat proteins. Synthetic mRNAs made in vitro can also be encapsidated with purified TMV coat proteins, which was first demonstrated in 1956 with TMV RNA[10]. Encapsulation of target and self-replicating mRNA can be achieved in plants through co-expression of the desired mRNA and the TMV coat proteins[67], making this an attractive and scalable approach towards plant-produced mRNA vaccines.

The limited commercialization of plant cell culture-based expression systems may be related to the lower protein yield (usually 0.01–10 mg l^{-1}, although up to 247 mg l^{-1} have been reported)[68], compared with bacterial and mammalian cell culture, which can achieve up to 10 g l^{-1} (REF.[33]). Additionally, controlling the expression levels of certain proteins remains difficult, especially in transgenic plants. Expression can vary between different generations of plants, is highly dependent on the plant type, and can even fluctuate within the same plant's tissues and organs[42]. Plants may also suffer from variability of environmental conditions, such as droughts and extreme heat — although this is less problematic in growth houses.

Cookie technique. The cookie technique, that is, the transient transformation of plant cells to produce high-value proteins, may revolutionize the small-scale production of recombinant proteins and other biologics in cultured plant cells[69]. Here, cell packs or 'cookies' are made on porous supports by filtration of suspended cells, followed by incubation with *Agrobacterium* suspensions without resuspension. The bacteria are then washed out with growth medium, leaving behind a mass with many air spaces. The treatment results in transformation rates of up to 100%, compared with around 1% from co-incubation of cells with *Agrobacterium* in suspension[70]. Cell packs can be incubated for days in situ and can produce up to 47 mg kg^{-1} of recombinant protein. The technique is scalable from microlitre and millilitre volumes up to large-volume preparative columns used for filtration or chromatography, and thus can be used for high-throughput and low-volume screening and for subsequent production of trial batches of proteins.

Clinical applications of molecular farming

Patient-specific antigens. Although molecular farming may not displace fermentation and large-volume cell culture for the production of blockbuster vaccines and therapeutics, it may be advantageous in certain applications; for example, for patient-specific or individualized production of antigens for therapeutic vaccines, which are required for certain diseases, such as non-Hodgkin lymphoma[71]. mAbs can be produced with relative ease in plants and, thus, non-Hodgkin lymphoma was

an early target for molecular farming[72]. Here, the mAbs are produced by introducing recombinant TMV (magnICON) vectors into *N. benthamiana* plants using *Agrobacterium*[73]. Grams of purified protein, enough for a lifetime supply for a patient, were produced at a cost of only about US$15,000 (2015); for comparison, production by conventional expression systems is estimated to cost around ten times more.

Niche and orphan diseases. Molecular farming also has potential for the production of niche or orphan vaccines as well as therapies, for which the market is perceived as too small to invest in a potential vaccine (for example, Lassa fever in West Africa), and/or the target market cannot afford the costs (for example, Rift Valley fever in East Africa). Importantly, such niche or orphan diseases have a role in the One Health Initiative, which aims to produce reagents for a disease that can infect wildlife, livestock or humans and to repurpose these into vaccines for animals or humans[74]. For example, the nucleoproteins of both Crimean–Congo haemorrhagic fever[75] and Rift Valley fever[76] bunyaviruses have been produced in plants with high yield and used in validated serological assays. In addition, SARS-CoV-2 spike glycoproteins have been made in plants in various forms for laboratories and detection kit manufacturers[77].

Virus nanoparticles and virus-like particles. Plant virus-derived VNPs are best produced in their natural hosts, that is, plants, by infection or recombinant expression, yielding milligrams of VNPs or VLPs per gram of leaf tissue[10]. VLPs are the non-infectious version of VNPs and do not contain the viral genome. Plant VNPs are proteinaceous nanomaterials that self-assemble into precise geometries at the nanometre scale. Many of their coat proteins have been mapped to near-atomic resolution, allowing functionalization with spatial control; molecular payloads can be incorporated into the interior cavity, integrated at interfaces, or displayed at the surface using an array of chemical biology approaches[78] (FIG. 2a–c). Thus, VNPs can be used as nanocarriers with a variety of payloads, including small-molecule drugs and prodrugs, nucleic acids, therapeutic proteins, contrast agents and photosensitizers for drug delivery[79], vaccines[80], diagnosis[81], theranostics[82], catalysis[83], live imaging[84] and agricultural applications[85]. Plant-made VNPs confer additional safety benefits compared with their mammalian viral vector counterparts because plant viruses are non-infectious to mammals[86]. Therefore, this technology is ideal for human vaccines and immunotherapies.

Although non-infectious to mammals, the repetitive, multivalent coat protein assemblies are pathogen-associated molecular patterns (PAMPs) that act as danger signals. VNPs administered via various routes (including subcutaneously and intramascular) drain efficiently to lymph nodes and activate immune cells upon recognition by pattern recognition receptors. VNP-based vaccines also facilitate antigen cross-presentation, which is crucial for major histocompatibility complex class I (MHC-I) presentation of extracellular antigens to trigger a robust cytotoxic T cell response. For example, plant VNPs, including papaya mosaic virus, TMV and potato virus X, can generate robust cellular responses against fused epitopes[87]. The potency of the immunostimulatory properties of VNPs has been demonstrated in an in situ cowpea mosaic virus (CPMV) vaccine, showing remarkable efficacy in animal models of melanoma, glioma, breast, colon and ovarian cancer[88–92]. Here, the VNPs are administered directly into the tumour to stimulate innate immune cells within the tumour microenvironment, priming tumour cell killing and antigen processing, which results in systemic antitumour immunity. In contrast to oncolytic viral tumour therapy, pre-existing immunity does not decrease the effectiveness of the immune response induced by the VNPs[89]. Instead, antibody recognition increases opsonization of CPMV, thereby improving the recognition of the virus by innate immune cells, which are its natural targets. The CPMV cancer immunotherapy has also shown efficacy in companion animals with spontaneous tumours[93]. Therefore, VNPs are excellent epitope delivery platforms for antigens (FIG. 2d) and adjuvants for vaccine and immunotherapy applications[94]. The current preclinical development pipeline for VNP-based vaccines includes infectious, cardiovascular and autoimmune diseases as well as substance abuse[95]. Plant VNP vaccine platform technologies also hold promise for pandemic or epidemic vaccines[96] — owing to their high thermal stability they would not be subject to cold chain distribution and/or could be produced in the region for the region through molecular farming.

Distribution challenges of vaccines

The successful vaccination of entire populations in low-resource areas is an arduous task, which will require addressing distribution challenges in relation to cold chain failures and the lack of trained health-care providers with vaccine experience[97].

The cold chain

The majority of traditional (live and inactivated) vaccines approved for use are advised to be distributed through the cold chain at 2–8 °C for optimal activity. The cold chain is a temperature-controlled supply chain of the people, equipment and protocols used in the transportation, storage and handling of vaccines from manufacturer to patient (FIG. 3). When disrupted, vaccine denaturation can lead to less effective vaccines with greater side effects. The 'last mile' vaccine distribution, describing the allocation of vaccines from national distribution centres to regional facilities and patients, is especially challenging, as evidenced in the SARS-CoV-2 vaccine rollout. The World Health Organization (WHO) reported that in 2011, 2.8 million vaccine doses were lost owing to cold chain disruptions in five countries surveyed. Therefore, in 2012, the WHO expressed a preference for vaccines that are heat- and freeze-stable for extended periods above 8 °C, as a part of the controlled temperature chain[98].

Cold chain disruptions can occur from a variety of sources. Vaccines can be exposed to extended periods of heating owing to electricity and power outages, equipment failure, limited ice supplies, and transportation and delivery delays[99]. Vaccine function can also be

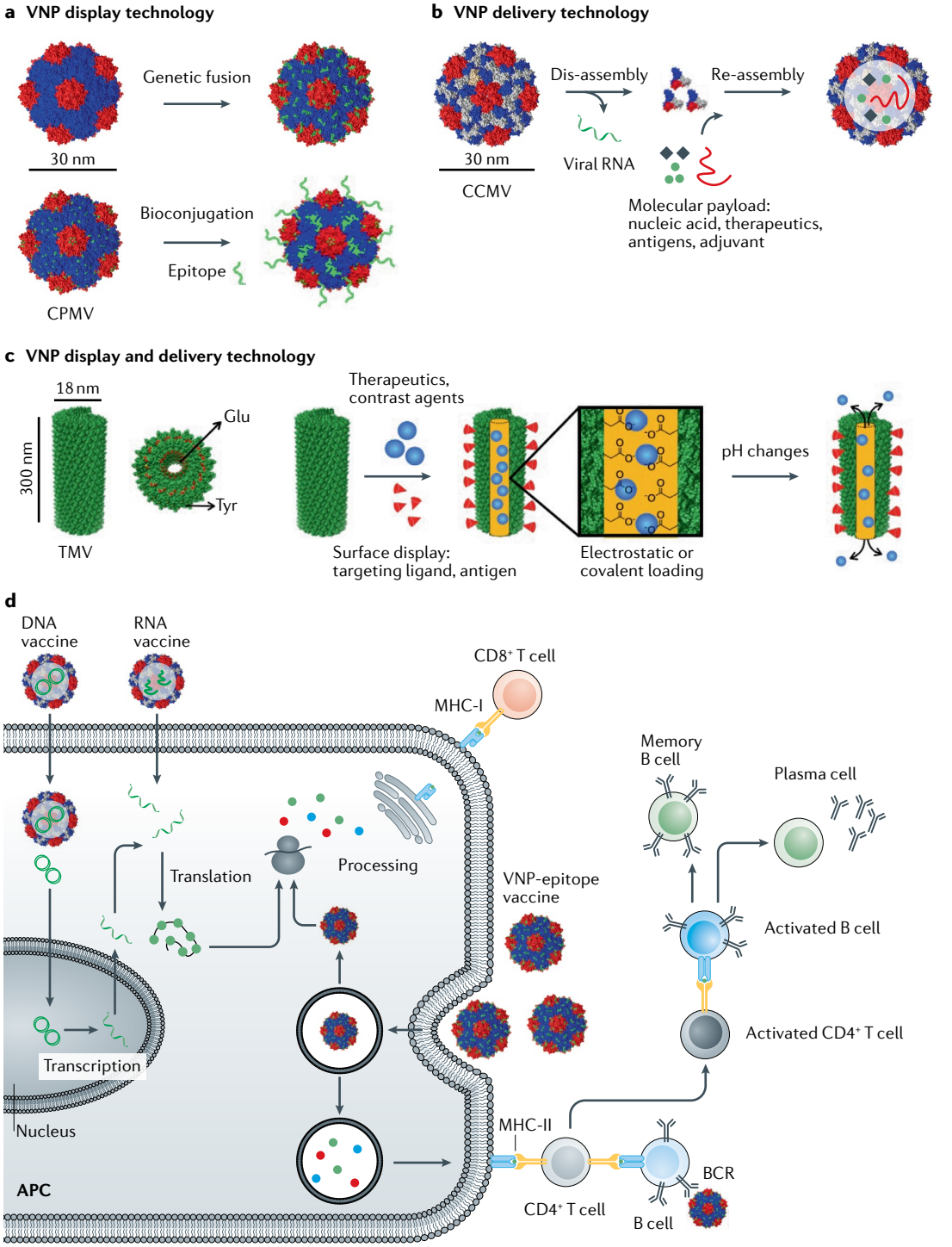


Fig. 2 | **Structure and engineering design space of plant virus nanotechnologies and virus-like particle vaccines.** **a** | Display of epitopes or peptides on cowpea mosaic virus (CPMV) through genetic fusion or bioconjugation. **b** | Encapsidation of molecular payloads within the cowpea chlorotic mosaic virus (CCMV) through dis-assembly and re-assembly of coat proteins following changes in the solvent conditions. **c** | Delivery of molecular payloads with tobacco mosaic virus (TMV) using internal glutamate (Glu) or external tyrosine (Tyr) residues. Therapeutic payloads can be loaded into the interior of the TMV or conjugated to the exterior surface of TMV. **d** | Epitope delivery using plant viral nanoparticles (VNPs). Vaccination with VNPs leads to intracellular processing of the antigen or genomic material encoding the antigen. The antigen is then displayed on the cell surface, leading to the activation of $CD4^+$ and $CD8^+$ T cells. $CD4^+$ T cells go on to activate memory B cells, leading to immune memory against future infections. APC, antigen-presenting cell; BCR, B cell receptor; MHC, major histocompatibility complex; VLP, virus-like particle. The plant VNPs were drawn using UCSF Chimera (CPMV PDB ID: 1NY7; CCMV PDB ID: 1CWP; TMV PDB ID: 2TMV), and parts of FIG. 3d were made using https://smart.servier.com/.

Fig. 3 | **The cold chain of vaccines.** The cold chain is a temperature-controlled supply chain of the people, equipment and protocols used in the transportation, storage and handling of vaccines from the manufacturer to the patient.

compromised by vaccine freezing owing to ice build-up in refrigerators or improper ice conditioning[98].

Immunization challenges

Overcoming immunization challenges requires collaboration between multiple parties involved in the vaccine supply chain. First, a better understanding of the impact of heat or freeze exposure on vaccine stability is needed. The WHO further demands that all vaccines purchased by the United Nations Children's Fund must have vaccine vial monitors[100] that measure cumulative heat exposure and signal whether the vaccine should be discarded in case of cold chain disruptions. Vaccine vial monitors are cheap and indispensable; however, they can fail; for example, an oral polio vaccine with a 48-hour controlled temperature chain (indicated by the manufacturer) maintained viable potency for up to 86.9 hours above 8 °C, even though the vaccine vial monitor indicated it had reached its end point[101]. In addition, it is important that health-care professionals are trained to read and interpret vaccine vial monitor data and in handling procedures, such as preventing sunlight exposure, which can lead to false positives and premature vaccine discards[102]. Similarly, freeze indicators and shake-tests for freeze-induced protein aggregation can measure potency, in cases when freezing temperatures are a concern[98].

Thermostable liquid and dry vaccine formulations can further address cold chain-related challenges. Liquid vaccines can benefit from high-throughput optimization of formulation properties, such as buffer type, pH and ionic strength, to improve stability. Dry vaccines can be more thermostable but must be reconstituted before use. Reconstitution is limited by large product volumes, logistical concerns, and potential sources of error and contaminants. Dry vaccines need simple-to-use reconstitution systems or strategies to bypass the need for reconstitution, for example, by applying aerosols, dry powder jet injection, microneedle patches or biodegradable implants[103]. Moreover, the ability to withstand temporary storage or transport without refrigeration or ice would help mitigate cold chain disruptions, particularly in areas where health workers travel by foot[104] or where there is a lack of cold boxes[102].

Health personnel

Health-care workers connect every step of the vaccine supply chain and, thus, require adequate training for each vaccine, including reconstitution methods, handling of the cold chain and controlled-temperature chain, and reading of vaccine vial monitors. Negligent handling may result in wasted vaccines or the delivery of ineffective vaccines[103]. Therefore, easy-to-use vaccines that require less training may reduce human error, for example, single-use or self-administered systems. UniJect, which is a pre-filled single-use system[105], is easy to use, activated by simply pushing the needle through a membrane and into the drug reservoir, and its valve ensures that the vaccine can only be used once by preventing the needle from retracting. The UniJect system has also been reported to be less painful and anxiety-inducing for patients[105].

Cost per dose

The definition of successful vaccine implementation could further be redefined from cost per dose to cost per dose delivered, to reflect the true cost of introducing a new vaccine[104]. A higher cost per dose would probably be accepted for a more stable vaccine, because of reduced wastage, lower storage and handling costs, better efficacy and protection, and easier management, as compared with less-stable vaccines, making it more cost-effective overall. For example, single-use systems, such as UniJect, add $US0.15–0.30 to the cost per dose, compared with traditional single-use syringes; however, the 20% wastage rate for multi-dose vials and the fact that UniJect can be administered at home lead to a decrease in cost per dose delivered, from $US7.19 to $US6.57 in the case of tetanus[106]. Therefore, dose-sparing technologies in vaccine manufacturing, deployment and administration can have a substantial impact on immunization success and costs.

Plant molecular farming to overcome vaccination challenges

Plants can be used to produce vaccines at small scale and low cost in regions where vaccines are most needed, particularly in areas where it may be difficult to set up cGMP-level bacterial and mammalian cell culture systems. In addition, contaminants from plant molecular farming are not as harmful as endotoxins produced in bacterial expression[61]. Moving vaccine manufacturing on-site would also reduce cold chain requirements, because long-distance shipping would not be needed; this has been especially highlighted during the SARS-CoV-2 vaccine rollout. In 67 low-income countries, an estimated 90% of the population may not gain access to any SARS-CoV-2 vaccine in 2021 (REF.[107]). Therefore, plant molecular farming could reduce vaccine inequity and immunization costs[108]. Furthermore, production of vaccines by plant molecular farming does not require extensive space; for example, it was calculated that Italy could reach a 60% vaccination rate with 12,500 square metres of greenhouses[45]. Moreover, molecular farming is easily scalable, compared with fermentation systems, which must be adapted and optimized for scale-up, and which require costly bioreactors.

By contrast, each plant is a bioreactor, and scale-up is simply achieved by growing more plants.

Plant molecular farming is particularly useful for producing plant viruses as nanocarriers for vaccines. Plant VNPs are very stable and can withstand temperatures outside cold chain requirements; for example, CPMV withstands temperatures of 37 °C for 46 days[109] and 60 °C for up to 1 hour[110]. Thermostability is especially important in areas in which resources for adequate cold chain distribution of vaccines may be limited. Furthermore, plant viruses, owing to their intrinsic stability, can withstand the processing and manufacturing steps of vaccine packaging into new administration technologies, such as microneedles[96]. VNPs do not only act as carriers, but also as adjuvants, reducing the number of vaccine components. Moreover, the simplicity of VNP-based vaccines can improve health-care worker compliance and reduce the need for training in vaccine administration.

Vaccine administration technologies

Administration of vaccines with hypodermic needles, which is currently the most frequently used administration technology, has several drawbacks. For example, blood-borne pathogens can easily be spread by needle reuse or mishandling[111]. Moreover, patients may be apprehensive about needles[112]. Vaccines delivered by hypodermic needles also rely on liquid formulations for injections, which require a cold chain[113].

To overcome these obstacles, vaccines can be encapsulated in polymeric materials. Proteins in the solid state embedded within a polymer matrix further improve the thermal stability of the vaccine, thereby decreasing strict adherence to the cold chain[114]. Polymeric materials could be used in new vaccine delivery platforms that could supplant hypodermic needles, improving patient compliance and reducing the unintended spread of blood-borne pathogens. Next-generation polymeric delivery platforms that are safe, effective and self-administered could thus greatly strengthen concerted vaccination efforts.

Polymeric materials

Considerable research has been devoted to the development of biocompatible and biodegradable polymeric materials for vaccines[115] (TABLE 3). These materials can be of synthetic or natural origin, and processing and manufacturing requirements can differ for each polymer, which may restrict the use of biological components. For example, inactivated or live attenuated vaccines can denature at high temperatures, which restricts the processing conditions (some processes require temperatures above 70 °C) required to encapsulate these vaccines in polymeric materials[113]. By contrast, VLPs and VNPs are chemo- and thermostable, enabling a broader array of device manufacturing conditions[116]. Fabricated by plant molecular farming, VLPs and VNPs can be produced at large scales for material integration at lower cost, as compared with conventional inactivated or live attenuated vaccines[108].

Polymeric materials, developed for next-generation vaccine administration, generally act as delivery vehicles, allowing the controlled release of antigen and adjuvant over weeks to years, depending on the type of material. The antigen release rate can be controlled by tuning the material properties. The presence of degradable bonds in the polymer's backbone and its hydrophilicity, molecular weight and crystallinity influence the degradation rate of both synthetic and natural polymers. For example, to enable instantaneous burst release, antigens can be encapsulated in rapidly dissolving water-soluble polymers, such as polyvinylpyrrolidone (PVP), which can be processed into microneedles for intradermal vaccine delivery[117]. A slow and sustained release can be achieved by choosing slowly degrading polymers, such as poly(lactic-co-glycolic acid) (PLGA)[118]. Increasing the window of antigen delivery can further improve the efficacy of vaccines[119,120]. By combining burst and slow-release strategies, single-dose regimens can be designed that mimic the multi-dose prime–boost regimen of conventional vaccines[121]; for example, a vaccine could be encapsulated in both a burst-release polymer and a slow-release polymer, which can then be simultaneously administered.

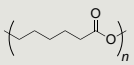
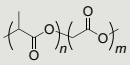
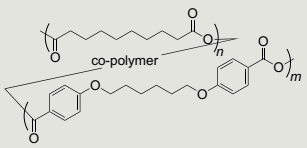
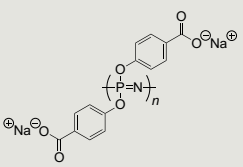
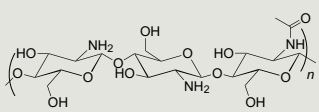
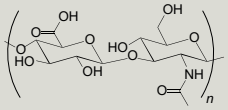
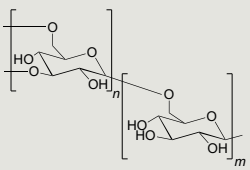
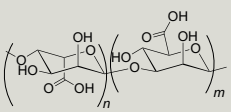
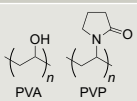
Delivery of adjuvants is key for vaccines. Some subunit vaccines, such as VLPs or recombinant VLPs and VNPs, which deliver subunit antigens, may not require additional adjuvants because the VLP or VNP itself acts as the adjuvant through recognition and activation of pattern recognition receptors[122]. However, for moderately or poorly immunogenic antigens, adjuvants need to be added to the formulation. The most frequently used adjuvants are aluminium-based salts (alum)[113], which serve both as a delivery vehicle by adsorbing antigen to the surface and as an immunostimulator by inducing pro-inflammatory signalling cascades[123]. Similarly, polymers such as alginate, hyaluronic acid and PLGA can act as both delivery vehicle and adjuvant owing to their natural inflammatory properties[124]. Further activation of innate immunity can be achieved through co-delivery of Toll-like receptor (TLR) ligands, such as CpG-oligodeoxynucleotide or monophosphoryl lipid A. Here, materials design is crucial, because a growing body of data suggests that co-delivery of adjuvant and antigen to the same antigen-presenting cell is important to enhance vaccine efficacy and to increase safety by lowering the likelihood of an uncontrolled and unspecific systemic immune response[125].

Vaccine delivery devices and production

As an alternative to subcutaneous injection with hypodermic needles, other routes of administration have been explored, using different device designs and fabrication, which have a considerable effect on immunostimulation. Here, we discuss two delivery constructs for vaccine self-administration: nano- and microparticle formulations, and microneedle patches. Using these devices, vaccines can be self-administered by intradermal, intranasal or oral administration (FIG. 4).

Nano- and microparticles. Vaccines can be encapsulated in polymeric nano- and microparticles. For example, polyester-based microparticles, such as PLGA microparticles, can be fabricated starting with an initial water-in-oil dispersion of polymer and antigen, followed by an

Table 3 | **Biocompatible polymers for vaccine delivery**

Polymer	Structure	Bio-degradation	Properties	Vaccine delivery method	Ref.
Polyesters					
Poly(ε-caprolactone) (PCL)		2–4 years	Does not generate acidic environment upon degradation Adjuvant properties Slow degradation only suitable for long-term delivery applications	NP, MP	189
Poly(lactic acid) (PLA)		3–8 months	Tunable degradation depending on chirality of monomers Adjuvant properties	NP, MP	190
Poly(lactic-co-glycolic acid) (PLGA)		1–6 months	Tunable degradation depending on monomer ratios in copolymer Degradation produces acidic environment Protein encapsulation can cause denaturation	NP, MP, MN	118
Polyanhydrides					
Poly[1,6-bis(p-carboxyphenoxy) hexane-co-sebacic acid] (PCPH-SA)		2–10 weeks	Tunable degradation and material properties owing to synthetic flexibility Adjuvant properties Freezing and anhydrous conditions required for storage	NP, MP	127
Polyphosphazene					
Poly[di(carboxy-latophenoxy) phosphazene] (PCPP)		1–24 months	Tunable degradation and material properties owing to synthetic flexibility Buffering capacity of degradation products Adjuvant properties	NP, MP, coated MN	191
Polysaccharides					
Chitosan		14–60 days	Encapsulation of antigens in aqueous media Mucoadhesive properties Adjuvant properties High variability in quality from commercial sources can affect degradation and immunogenicity	NP, MP, MN, coated MN	144
Hyaluronic acid (HA)		<24 hours	Rapid dissolution in the skin Adjuvant properties Not suitable for long-term delivery applications	MN	192
Dextran		1–42 hours	Rapid dissolution in the skin Microparticle formulations require chemical modification of dextran	MP, MN	193
Alginate		NA	Encapsulation of antigens in aqueous media Mucoadhesive properties Adjuvant properties Stable at low pH, facilitating oral immunization High variability in quality from commercial sources can affect degradation and immunogenicity Limited in vivo degradation unless chemically modified	MP, MN	194
Non-biodegradable polymers					
Polyvinylalcohol (PVA) or polyvinylpyrrolidone (PVP)		NA	Rapid dissolution in the skin Non-biodegradable	MN	195

MN, microneedle; MP, microparticle; NA, not applicable; NP, nanoparticle.

additional emulsion and solvent removal step, polymer phase separation or spray drying[126]. By contrast, polyanhydrides require completely anhydrous conditions (solid–oil–oil dispersions) to prevent premature polymer hydrolysis[127]. Antigens can also be encapsulated in ionic polymers, such as polyphosphazenes and polysaccharides, by crosslinking of the polymers through the addition of oppositely charged multivalent ions in aqueous and ambient conditions. However, these fabrication techniques have several drawbacks, which can limit their use for certain biologics. For example, water-in-oil dispersion has low encapsulation efficiencies, which may limit its use for expensive and difficult-to-produce biologics[128]. Importantly, the fabrication process can affect antigen stability[129]; for example, antigens can degrade or unfold owing to interactions at the aqueous–organic interface or to high shear forces during emulsification[130].

Nano- and microparticles are often used in self-administered intranasal vaccines. Here, antigens are encapsulated within nano- or microparticles, usually made of poly(lactic acid) or PLGA, and administered as a spray or aerosol, ideally targeting immune cells that reside in the nasal-associated lymphoid tissue of the upper respiratory tract[131]. However, following intranasal administration, the mucociliary clearance mechanism may clear the deposited vaccine particles, with a half-life of around 20 minutes[132]. To prevent rapid clearance, the vaccines can be formulated with mucoadhesive polymers, such as chitosan, which can translocate across the nasal mucosal barrier and prolong the residence time to hours or days to improve the immune response[133].

Microneedles. Microneedles are microscale projections, usually 50 to 900 μm in height, which can pierce the upper layers of the skin and facilitate transdermal delivery of vaccines[134]. Microneedles do not puncture deep enough to activate the nociceptors within the skin and are therefore considered painless. Microneedles can achieve greater immunological responses, compared with traditional routes of administration, such as intramuscular or subcutaneous injection, owing to the abundant immune cell population in the upper layers of the skin, including the Langerhans cells in the epidermis and the dendritic cells in the dermis[135,136]. They can also be fabricated to deliver a range of biological vaccines,

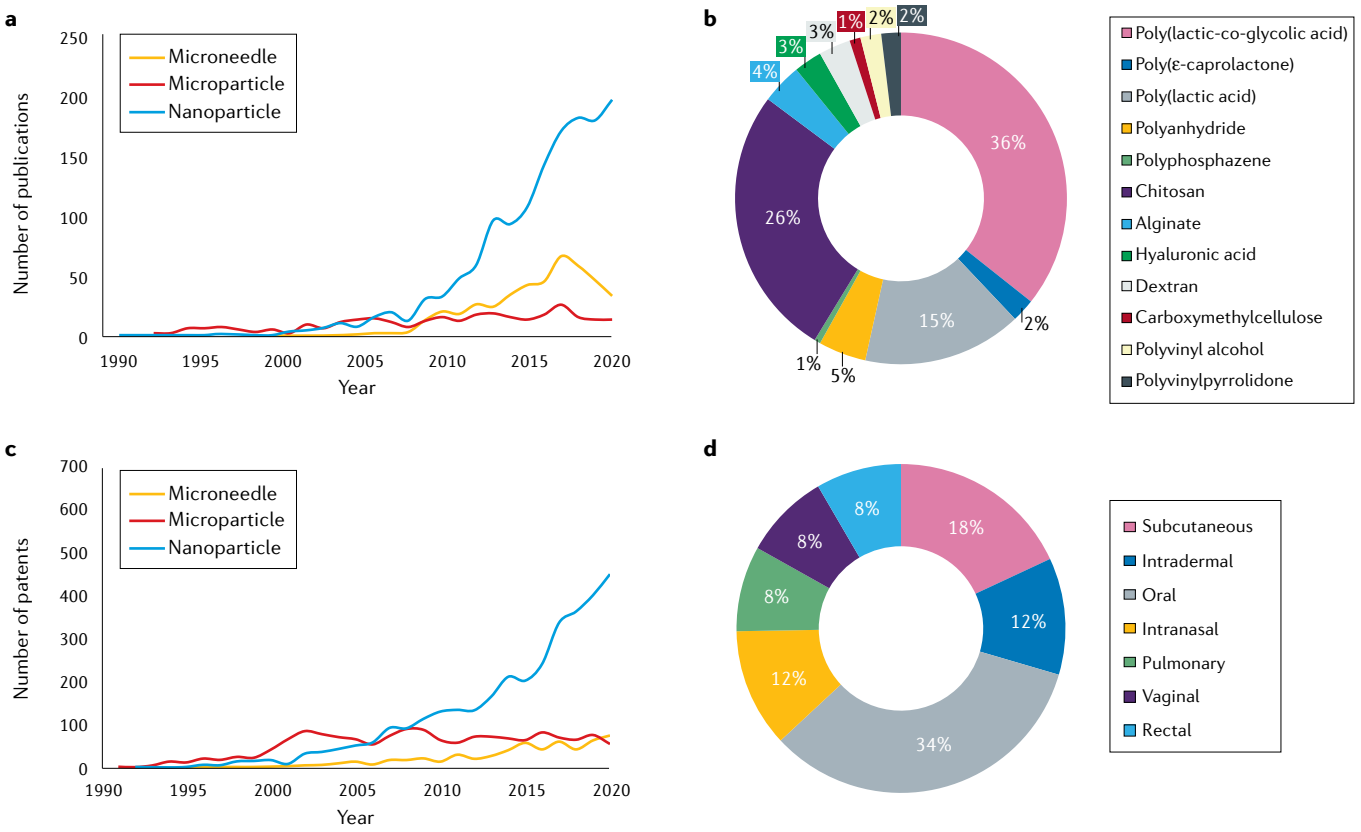


Fig. 4 | **Market analysis and development pipeline of vaccine delivery technologies. a** | Number of research publications for microneedle, microparticle and nanoparticle vaccine delivery technologies. The terms 'microneedle', 'microparticle' and 'nanoparticle' were searched on PubMed, and the total number of publications for each search term were graphed with respect to publication date. **b** | Percentage distribution of biocompatible synthetic and natural polymers frequently used in research publications for vaccine delivery. The number of research publications on PubMed mentioning the respective polymers were counted for each polymer listed in the figure and totalled. The respective percentage of each polymer was then calculated from the total and graphed. **c** | Number of patent applications for microneedle, microparticle and nanoparticle vaccine delivery technologies. The terms 'microneedle', 'microparticle' and 'nanoparticle' were searched on www.uspto.gov, and the total number of publications for each search term was graphed with respect to publication date. **d** | Routes of administration for vaccine delivery proposed by patent applications. The number of patents mentioning the respective route of administration as well as the term "vaccine" were counted on www.uspto.gov and totalled. The respective percentage of each route of administration was then calculated from the total and graphed.

including inactivated and live attenuated viruses, VLPs and VNPs, protein subunits and DNA[137]. Importantly, encapsulation in microneeedle patches increases the thermostability of these biologics, which is particularly important for vaccination in regions without proper cold chain infrastructure. Therefore, microneedles are perhaps the most promising device for widespread vaccine distribution and self-administration.

Coated and dissolving microneedles have also been developed. Coated microneedles are designed by coating the surface of microneedles with antigen by dry-coating[138] or layer-by-layer electrostatic deposition[139]. Upon skin puncture, the coated antigen is immediately released to elicit an immunological response. For dry-coating, a stainless steel or silicon microneedle is dip-coated or dry-sprayed with a mixed solution of antigen and carboxymethylcellulose[140], polysaccharides[141] or synthetic polymers[142]. A disadvantage of this method is its limited loading capacity[134]. However, loading can be increased by layer-by-layer electrostatic deposition; here, layers of oppositely charged polymers and proteins or nucleic acids are deposited in an alternating fashion[139].

For the fabrication of dissolving microneedles, antigen is encapsulated within the microneedle tips, either in the bulk material or in microparticles. Upon insertion into the upper layers of the skin, the microneedles dissolve at rates dependent on the materials used[143]. Each material has advantages and disadvantages (TABLE 3); for example, PVP[117] rapidly dissolves upon contact with the interstitial fluid, which decreases the application time of the microneedles, but requires high doses owing to fast clearance rates. Alternatively, chitosan biodegrades slowly, thereby acting as a sustained-release depot. Chitosan microneedles have been shown to induce a more potent and long-lasting immune response compared with conventional intramuscular injection or injection with alum[121,144]. Dissolving microneedles are usually manufactured by micromoulding[145]. Here, a mould is first filled with a polymer solution containing the antigen and then placed under vacuum or centrifuged to draw the solution into microcavities. The device is then allowed to dry and harden to form robust microneedle arrays. Water-soluble polymers, such as carboxymethyl cellulose and PVA, manufactured into microneedles under mild conditions, can protect encapsulated antigens from degradation[146,147]. Alternatively, dissolving microneedles can be fabricated by ultraviolet photo-polymerization of PVP within the mould[117] or by high-temperature moulding[148]; however, these processes can cause antigen degradation, because they require harsher conditions than solvent-based moulding.

Microneedles with plant-produced antigens have already been shown to be effective. For example, a recombinant influenza VLP vaccine (along with a glucopyranosyl lipid adjuvant) administered by the NanoPass MicroJet microneedle, produced equal levels of haemagglutinin inhibition titres and seroprotection in humans, compared with intramuscular injection[149]. In ferrets, the microneedle formulation led to 100% survival against a H5N1 challenge. Microneedle delivery also enables in situ vaccination; for example, microneedle-delivered CPMV showed greater potency in a mouse model of melanoma compared with intratumoral injection of CPMV, resulting in slower tumour growth and improved overall survival[150].

Outlook

The use of biologics in pharmaceutical applications will continue to grow as expression and purification technologies develop and mature. Plant molecular farming, in particular, is poised to make an impact on an array of applications, and new methodologies, such as the cookie technique, will continue to drive forward its use. Plant molecular farming is currently applied mainly in niche applications; however, recent developments and milestones, such as the production of millions of influenza vaccine doses in record time, has put a spotlight on this manufacturing platform[45]. Following such successes, it is likely that molecular farming will gain in market share. We have just witnessed the worldwide efforts by laboratories and industry in pivoting their particular platforms and tools towards making vaccines, therapeutics or reagents for the ongoing COVID-19 pandemic or the Zika and Ebola epidemics[11,151]. Plant molecular farming requires less sophisticated infrastructure than does conventional vaccine production, and thus could be locally deployed in each region, therefore addressing vaccine rollout challenges. Moreover, plant molecular farming could enable the production of vaccines, therapeutics or reagents in space, either on a space station, or during months-long missions to Mars or other extraterrestrial destinations[152]. The first grants supporting this technology have already been issued, for example, the NASA-funded use of transgenic lettuce and potato leaves to produce growth factors and hormones for astronauts to combat osteoporosis and other spaceflight-related health issues[152].

However, expression levels in plant molecular farming must improve to levels similar to those achieved in mammalian or bacterial systems. Expression in bacterial systems was first achieved in the 1970s, ten years before expression in plants[153]. This gap means that plant molecular farming lags behind other expression systems in terms of expression efficiency, which will be addressed by more efficient plant systems that can produce proteins at larger yields. In addition, downstream processing and purification of biologics need to improve, which remains challenging owing to the complexity of plant cells and tissues (plants produce 30% more solid debris than other expression systems and contain many contaminants, such as phenolics, which interfere with purification). The FDA Critical Path Report states that downstream processes were one of the key reasons that plant-made proteins routinely failed to transition into the clinic or industry[154]. However, new methods, such as using aqueous two-phase partitioning systems, enzymatic hydrolysis, ultrasound, microwaves, pulsed electric fields, high-voltage electric discharge, ohmic heating, vacuum, subcritical and supercritical fluids, and hydrotropic and deep eutectic solvents are being developed to improve downstream processing of plant material[155,156].

Manufacturing of biologics by plant molecular farming is currently performed mainly at small scales

by academic research laboratories and mid-sized pharmaceutical companies, at a lower cost than traditional fermentation-based expression systems (up to ten times and a hundred times lower than the production costs in *E. coli* and CHO cells, respectively)[157]. Once plant molecular farming reaches agricultural crop scales, the margins are expected to widen even further. The lower costs will help to offset the expensive costs of everyday laboratory research. Thus, molecular farming has the potential to reduce the price of recombinant therapeutics to levels similar to those of everyday over-the-counter medications. Molecular farming could also play a key part in producing biosimilars (biologics that are not identical to the original product, but are equally efficacious) to fabricate cheap pharmaceuticals or biological test reagents for all populations. In addition, decreasing the price of medications would also greatly reduce financial strains on the health-care system. For example, the monoclonal antibodies used in the alleviation of rheumatoid arthritis are currently unaffordable outside health-care systems in developed regions, although the majority of sufferers remain in underdeveloped regions.

Future vaccines should be engineered to be free of cold chain restrictions, for example, by producing orally dosed vaccines and therapeutics in plants. Although the 1990s dream of simply 'eating your vaccines' is unattainable (mainly owing to low yield of active ingredient and dose control), transgenic plants consumed by humans, such as potatoes, tomatoes and corn, can be used to not only deliver partially purified and quantified antigen, but also to protect the antigen within a heat-stable environment[158]. The rigid plant cell wall can protect antigens from degradation within the harsh environment of the gastrointestinal system, while allowing antigen delivery[159]. The use of orally dosed vaccines can help reduce cold chain requirements and obviate the need for antigen purification and downstream processing[62]. The amount of land required to vaccinate entire countries by orally dosed vaccines would also be minimal (for example, all of China could be vaccinated with only 40 hectares of land)[62].

Cold chain restrictions could also be mitigated by using new delivery agents, such as plant-made VNPs, that are intrinsically thermostable over a range of temperatures and pH levels, allowing their use in microneedle patches (which require high-temperature processing methods[110]), and in oral vaccines (avoiding denaturation by the acidic gastrointestinal environment[160]). Finally, new formulation chemistries and next-generation materials are anticipated to enhance formulation stability and improve delivery techniques. For example, the biomineralization of viral vaccines generates a mineral exterior, which improves the thermostability of the viral antigen[161]. Furthermore, new combinations of excipients can further extend the half-life of vaccines[162]. Finally, the formulation of biologics in microneedle patches and/or slow-release formulations has tremendous potential for the self-administration of medication.

Thus, platform technologies to produce biologics from living systems continue to develop as new technologies become available, and next-generation nanotechnology and materials science have opened up opportunities in formulation chemistry and the administration of pharmaceuticals.

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Author contributions
Y.C. wrote the first drafts of the manuscript, researched data for the article and prepared figures; D.C. wrote the sections focusing on vaccine administration technology and prepared figures; E.C.K. wrote the sections focusing on biologics and background on vaccines and prepared figures; E.O. wrote the sections focusing on the cold chain and vaccine distribution and prepared figures; S.S. wrote the sections focusing on molecular farming for plant viral nanoparticles and prepared figures. E.P.R. and N.F.S. wrote the section on plant molecular farming. N.F.S. and Y.C. prepared the final version of the manuscript. N.F.S. and J.K.P. conceived the manuscript and were in charge of overall direction and planning. All authors reviewed and edited the final version of the manuscript.

Competing interests
N.F.S. and J.K.P. are co-founders of and have a financial interest with Mosaic IE Inc. E.P.R. has a financial interest in Cape Bio Pharms Ltd in Cape Town. All other authors declare no competing interests.

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