Introduction

Vaccines are one of the greatest advances in medicine and an important public health tool, as they not only prevent infection, morbidity and mortality individually, but also reduce and eliminate disease prevalence locally, ultimately leading to eradication of disease globally [1] . Since the development of the smallpox vaccine in 1798 [2] and rabies vaccine in 1885 [3], vaccine technology progressed from the use of inactivated and attenuated pathogens, to the use of subunits that only contain those pathogen components that can trigger an immunologic response (Figure 1). Key milestones include the development of virus-like particle vaccines, recombinant viral-vectored vaccines, and toxoids, polysaccharides or protein-based vaccines, which can be conjugated with different protein carriers to improve immune response.

Vaccines save 6 million lives every year and are one of the major responsible for the increase of human longevity [6]. Their impact on the economic viability of the healthcare system is also very large, since vaccines lower the treatment costs of diseases [7], and reduce the impact and risk of outbreaks [8]. Additionally, by preventing bacterial infection and, subsequently, reducing the need for antibiotic treatment, vaccines can have an impact on antimicrobial resistance [9]. The use of vaccines goes beyond prevention of infectious diseases. Technology advances coupled with progress in target selection and understanding of the immunosuppressive mechanisms have led to the development of therapeutic cancer vaccines [10].

Despite the proven effectiveness of current vaccines, there is still room for improvement in the vaccine technology field. Traditional attenuated and inactivated vaccines are still widely used today (e.g., Bacillus Calmette–Guérin vaccine, BCG and Inactivated Polio vaccine, IPV) owing to their robustness and stability. However, they present safety concerns due to the use of whole pathogens and in many cases, they don´t have a defined composition. In the case of toxoid and subunit vaccines, and despite their safety and stability profile, the use of adjuvants is required for a strong immune response and the protection lifetime is limited (Table 1). The manufacturing of new vaccines is typically a lengthy (6 to 36 months), challenging and expensive process, as no standard process is available [11,12]. To deliver effective, precise, and consistent vaccines it is imperative

to use good manufacturing practice (GMP) compliant equipment, facilities, and procedures. However, this is costly and difficult to implement at a large scale. Vaccines developed on the basis of traditional technology have failed to respond effectively to several diseases, such as malaria, tuberculosis, AIDS or flu. Furthermore, SARS and Ebola epidemic outbreaks and, more recently, the CODVID-19 pandemic, show that many of the current platforms are not well suited for a very fast, efficient, and cost-effective response.

New vaccine technology approaches are thus necessary to improve our response to outbreaks and enable vaccination worldwide. Ideally, a new vaccine should be safe, effective, stable, available to all populations and not susceptible to antigenic variance [13]. The manufacturing must be reliable, efficient, low-cost, and flexible to allow on-demand production. Viral vectors and DNA technology are two cutting-edge platforms that have the flexibility and characteristics to support faster vaccine development and manufacturing [14]. However, the costly and complex manufacturing of viral vectored vaccines and the poor immunogenicity presented by DNA vaccines (Table 1) can make them unattractive for some clinical applications.

The rise of mRNA technology

mRNA vaccines have reached the spotlight during the Covid-19 pandemic, as the forefront technology used for the development of vaccines by many companies. In fact, a mRNA vaccine candidate was the first to reach phase I clinical trials [15]. The potential of mRNA vaccines was first hinted at in 1990, when the *in vivo* expression of a protein was observed after injecting the coding mRNA into mouse skeletal muscle [16]. These early experiments proved that *in vitro* transcribed mRNA (IVT) can induce the production of proteins in live tissues. During the following 10 years, several studies demonstrated that mRNA could induce an immunologic response to the expressed protein in many mammalian cell types both *in vitro* and *in vivo* [17–19]

mRNA technology presents several advantages that makes it an attractive alternative over traditional vaccines or even DNA vaccines. Unlike attenuated or inactivated vaccines, mRNA is precise as it will only express a specific antigen and induce a directed immune response. Additionally, it promotes both humoral and cellular immune response and induces the innate immune system [20]. Compared with DNA-based vaccines, mRNA is

more effective, since expression does not require nuclear entry, and safer, since the probability of random genome integration is virtually zero [21,22]. Additionally, expression of the coded antigens is transient since mRNA is quickly degraded by cellular processes, with no traces found after 2-3 days [23]. The flexible nature of the mRNA vaccine platform is also advantageous for manufacturing since a change in the encoded antigen does not affect the mRNA backbone physical-chemical characteristics [24], and hence allow production to be standardized. Additionally, since production is based on an *in vitro* cell-free transcription reaction, safety concerns regarding the presence of cell-derived impurities and viral contaminants commonly found in other platforms are minimised.

mRNA Vaccine Structure

Construction of mRNA vaccines requires the insertion of the encoded antigen in a DNA template from where the mRNA is transcribed *in vitro*. Unlike DNA, mRNA only needs to reach the cytosol, where it will be transcribed into the antigen *in vivo,* using the cell machinery. This way, any desired sequence can be designed, produced *in vitro,* and delivered to any type of cell [21]. Inside the cells, RNA is recognised by endosomal or cytosolic receptors, which can lead to the activation of the type I interferon (IFN-I) pathway, and to the promotion of the production of chemokines and proinflammatory cytokines. These signal molecules lead to antigen-presenting cell (APC) activation and, subsequently, to a strong adaptive response [25].

The structure of mRNA vaccines is similar to eukaryotic mRNA - a single-stranded molecule with a cap at the 5' end, a poly(A) tail at the 3’ end and an open reading frame (ORF) flanked by untranslated regions (UTR) [20]. The 5’ cap is an important component as it enables the translation initiation by binding to a eukaryotic translation initiation factor (eIF4E) [26]. Different structures are possible for the 5’ cap. The Cap 0 structure, which features a methyl-7 guanine nucleotide linked to the 5’ position through a 5’ triphosphate, is the simplest. The Cap 1 structure is achieved by the methylation of the mRNA first nucleotide at the ribose 2’-O position. Both caps can be added during *in vitro* mRNA transcription using a synthetic cap analogue [27] or the proprietary Cap dinucleotide CleanCap® [28]. Another capping approach uses a post-transcription enzymatic

reaction based on the vaccinia capping system [29]. This modification brings with it a number of advantages as it improves the translation initiation by recruiting translation initiation factors, protects the synthetic mRNA against exonuclease degradation [30], and avoids an innate immunity overactivation response [25]. The addition of a 3' poly(A) tail also improves mRNA stability and translational activities, as it protects mRNA from nuclease degradation by the poly(A)-binding protein (PABP) [31]. This tail can be added to the transcript by inserting a poly(A) sequence in the DNA template or by an enzymatic reaction [27]. Tail size optimization is an important factor for the stabilization and expression of mRNA. Longer poly-A tails can improve mRNA stability and translation. However, this effect is not linear, and the best tail size is dependent on cell type [31]. The untranslated regions (UTRs) are responsible for the transcription regulation and mRNA stability. These regions strongly affect translation efficiency as the sequences used are involved in the translation machinery recognition, recruitment, and mRNA trafficking. Strategies to modulate the innate immune response, such as the introduction of unnatural nucleosides (NTPs), and to improve translation efficiency, by using codon optimisation, are also commonly used in mRNA production [27, 28].

Two forms of mRNA structure are being extensively studied for vaccine applications: conventional or non-replicating mRNA and self-amplifying mRNA. In the conventional mRNA form, the antigen of choice is only flanked by UTR regions, a 3' poly(A) tail and a 5' cap. This form presents several advantages - molecules are simple and small, and the possibility of unwanted immune response is lowered since no other proteins are encoded [32]. However, this mRNA expression is limited to its transient nature, and higher mRNA doses may be necessary to achieve high expression [33]. Efforts have been made to overcome this bottleneck by using sequence optimization and formulation [34]. Self-amplifying mRNA (saRNA) is based on the addition of a viral replicase gene to enable the mRNA to self-replicate. Usually, sequences of single-stranded RNA viruses, such as alphaviruses, flaviviruses, and picornaviruses, are used [35]. Upon cytoplasm delivery, this type of mRNA produces high levels of the antigen of interest. Despite the use of viral genes, no viral infectious particles or virus-like-particles are observed during expression, reducing the safety concerns [21]. Evaluation of an saRNA vaccine for protection of mouse models against H1N1/PR8 infection showed that a 64-fold lower dose was

required to induce an immunologic response when compared with the conventional mRNA vaccine counterpart [36].

Trans-amplifying mRNA (taRNA) is a new structural modality of mRNA vaccines. The taRNA results from the splitting of the self-amplifying mRNA in a system with two templates, one containing the gene of interest and a second containing the replicase system. The amplification is performed *in trans* by the replicase in the cytoplasm. This system presents some advantages over saRNA since it is safer, more versatile and cost-effective to manufacture, as the production of shorter RNAs with high yield and high quality is less challenging. taRNA has already been used to protect mice against influenza with results showing induction of antibodies and protection[37].

mRNA Delivery

mRNA must cross the cell membrane to reach the cytosol. This is challenging due to the negative charge of the molecule, its relatively large size (300-5000 kDa) and degradability, which can hamper its passive pass through the cell membrane [38]. To overcome this, mRNA can be delivered using different strategies including: i) direct injection of naked mRNA; ii) conjugation with lipid-based carriers, polymers, or peptides; iii) via transfection of dendritic cells (DC) [39].

The induction of an immune response by injection of naked mRNA in conventional and self-amplifying forms has been widely reported [40–44]. However, mRNA delivery can be limited by the presence of extracellular exonucleases in the target tissues, inefficient cell uptake or unsuccessful endosomal release [27]. Liposomes or lipid nanoparticles (LNPs) are one of the most promising mRNA delivery tools [45]. For example, LNP-mediated delivery of mRNA vaccines against Zika and influenza has shown encouraging results [46–49]. Although less explored, polymer-based delivery systems can also be used. Polyethylenimine (PEI) systems were successfully implemented as a strategy to deliver mRNA to cells [50], and intranasally [51]. Additionally, PEI-based systems improved the response to sa-mRNA vaccines in skin explants [52] and in mice [36]. Peptide-based delivery is a less explored system, as only protamine has been evaluated in clinical trials [53]. New delivery approaches

include the use of cationic cell-penetrating peptides (CPPs) and anionic peptides. CPPs systems have proved to improve T-Cell immunity response *in vivo* [54], modulate innate immune response and enhance protein expression in both DC and human cancer cells *in vitro* [55,56]. mRNA polyplexes conjugated with an anion peptide, exhibited an increase in cellular uptake without inducing cytotoxicity in DC cells [57].

Despite the efforts to improve mRNA delivery, there are still challenges that must be considered, such as the delivery efficiency, cell targeting, materials safety, route of administration and vaccine thermostability. This topic is extensively revised elsewhere [39].

Applications

Since Wolf *et al.* [16] showed that proteins can be produced from *in vitro* transcribed mRNA in live tissues, mRNA vaccines have been demonstrating efficacy in a number of applications [58]. The first record of a clinical trial using mRNA technology based on RNA-pulsed DC cancer vaccine dates back to 2003 [59]. Today, more than 140 clinical trials can be found that use mRNA to address different conditions such as cancer or infectious disease (Figure 2).

From the first applications, mRNA has emerged as a potential therapy for cancer. *Boczkowski et al* [60] produced one of the first breakthroughs by using mRNA to generate vaccines based on RNA-pulsed dendritic cells (DC) against tumour cells. Using this system, the antigen-presenting immune response was induced, and tumour regression was observed. Since then, mRNA-based DC vaccines have shown their potential in cancer applications in over 70 completed clinical trials. Recently, a phase I study where RNA transduced DCs were evaluated as a post-remission therapy in acute myeloid leukaemia (AML) was published [61]. This treatment induced an immune response with a positive relation between higher survival rate of patients with ≤ 65 years. The use of mRNA has also been explored to engineer T- or Natural Killer (NK) cells to express chimeric antigen receptor (CAR) that are used as a cancer cell therapy [62,63]. In fact, this this system was successfully implemented in a phase I clinical trial designed to evaluate its potential in the treatment of colorectal cancers [64].

146 The direct injection of mRNA is a more cost-effective delivery alternative to DC vaccines. *In vivo* delivery of the
147 naked, complexed, or encapsulated mRNA can be successfully performed by a number of administration
148 routes such as intradermal, intramuscular, intranasal, intratumoral, intranodal or even intravenous [45]. Using
149 this method, a dose consisting of only a few tenths or hundreds of micrograms of mRNA (10-250 μg) is
150 administered to each patient to trigger an immune response [65]. The first clinical trial evaluating direct
151 injection used naked mRNA in patients with melanoma [66]. This approach was feasible and safe but no clinical
152 effectiveness was observed. Self-adjuvanted RNActive® vaccines is a technology developed by CureVac that
153 uses a mixture of protamine-complexed and naked mRNA to improve the immunostimulatory effect of the
154 vaccine [67]. This technology was successfully applied in phase I and I/II clinical trials targeting liver [68],
155 prostate [69], lungs [70] and melanoma [71] cancers. New delivery approaches using lipoplexes and LNPs have
156 been extensively used in clinical trials studies in the last couple of years. Recent results show that both
157 technologies can be successfully applied to treat melanoma [72], lymphoma [73,74], and solid tumours
158 [75,76].
159 Cancer is currently the target of choice for mRNA technology. Over 50% of the clinical trials focus on the
160 treatment of melanomas, prostate and brain cancer (Figure 3), with most of the trials still in the early phases (I
161 and II). The lack of benchmarks for cancer treatment hampers the evaluation of the vaccine's effectiveness
162 beyond the safety profile and the immunological response [21]. However, this is not the case for infectious
163 diseases since many conventional vaccines are available to serve as benchmarks to validate the new mRNA
164 vaccines. mRNA have also shown potential, not only for the treatment of cancer, but also as a therapeutic for
165 protein expression in the treatment a number of other diseases, such a cardiovascular disease [87,88] and type
166 II diabetes [88].
167 Owing to its versatility and flexible manufacture, mRNA is an excellent platform for the development of
168 prophylactic or therapeutic vaccines against infectious diseases (Figure 3). The first studies using mRNA
169 technology for infectious diseases therapeutics targeted HIV. Using DC-based and naked delivery systems,

phase I and II clinical trials presented mixed results despite the vaccine's safe profile [77], as a lack of an efficient immunologic response against HIV was observed [78,79].

Prophylactic vaccines using mRNA technology were also directed to rabies, with the first clinical trial using a self-adjuvanted delivery system [80]. Interestingly, this trial showed that the vaccine effectiveness depended on the route of administration, as only those patients that received the vaccine via needle-free devices produced antibodies above the WHO predefined titre (≥0.5 IU mL^{-1}). A new formulated mRNA vaccine based on LNPs delivery system is currently being evaluated in a phase I clinical trial [25].

mRNA technology is a perfect fit to overcome the bottlenecks faced by the conventional influenza vaccine. Indeed, studies on influenza immunisation provided the first demonstration of the efficacy of mRNA vaccines against infectious diseases in animals models (mice, ferrets and pigs) [81]. An LNP-based vaccine encoding H10N8 and H7N9 is currently being evaluated in phase I clinical trials. The first published results demonstrated that the H10N8 encoding vaccine was safe and triggered a robust prophylactic immunity [48].

mRNA vaccines have also shown promising results against other infectious diseases. For example, experiments with an LNP-based system against Zika have been performed in cells, mice and primates [46,47]. Currently, phase I clinical trials against Zika virus, Chikungunya virus, and a phase II trial against Human Cytomegalovirus using LNPs-bases systems are on-going.

During the current Covid-2019 pandemic, mRNA vaccines took the spotlight as the first vaccines to be approved for the prophylactic treatment. Furthermore, at least nine clinical trials can be found using mRNA technology, two of which are in phase III. Three recently published studies describe encouraging results obtained in phase I clinical trials using LNP-based systems [82–86]. All studies reported a safe profile with mild to moderate reactions, despite the greater reactogenicity observed following the administration of the second dose. Furthermore, an immunologic response was also observed in all studies, thus supporting the advance of this technology to late-stage clinical evaluation. A recent phase III study reported an efficacy of 95% [86].

mRNA Manufacturing: from upstream to downstream

One of the most important advantages of mRNA over conventional vaccines is its relatively simple manufacturing. To produce the mRNA product with specific quality attributes, a series of manufacturing steps must be carried out. Currently, a well-established manufacturing platform is still lacking and a number of combinations of steps is possible. These can be grouped into the upstream processing, which comprises the enzymatic generation of mRNA, and the downstream processing, which includes the unit operations required to purify the mRNA product (Figure 4). These are complemented with LNP formulation and Fill-to-Finish steps [92]. Nonetheless, the choice of the unit operations is still dependent on the purpose. For example, a lab scale production usually consists of a one-step synthesis reaction followed by a nuclease digestion and a precipitation [58]. The exact unit operations used can have an impact on the manufacturing price [92] and on the cost per dose. Ultimately, the cost will be greatly influenced by the quantity of RNA per dose, production titres and production scale used. The purchase price of 5' cap analogue and modified UTP seem to have an impact on the cost [92].

mRNA is produced in a cell-free system and uses no animal derived raw materials. Cell-derived impurities or adventitious contaminations are thus absent, which makes the manufacturing of these molecules safer [58, 65]. The *in vitro* transcription (IVT) enzymatic reaction used to generate mRNA relies on T7, SP6 or T3 RNA polymerases to catalyse the synthesis of the target mRNA from the corresponding DNA template (Figure 4). This template must be produced in advance, usually by linearisation of a purified plasmid or by amplification of the region of interest using PCR. Apart from the linear DNA template, the IVT components must then include an RNA polymerase, nucleotide triphosphates (NTPs) substrates, the polymerase cofactor $MgCl_2$, a pH buffer containing polyamine and antioxidants [33,89]. The reaction only takes a few hours in contrast with the time-consuming processes used to manufacture conventional vaccines. Furthermore, this reduced time lowers the probability for contamination to occur [65]. In general, milligrams of mRNA per millilitre of reaction can be obtained [90]. Additionally, the production process can be standardized as it is not dependent on the antigen encoded in the template.

As for mRNA capping, it can be performed during the IVT reaction by substituting a part of the guanosine triphosphate (GTP) substrate for a cap analog [91]. Alternatively, mRNA can be capped in a second enzymatic reaction using the vaccinia capping enzyme (VCC) and a methyl donor as a substrate (Figure 4). Although the capping efficiency of this method is higher (100% compared to 60-80% obtained with the use of a cap analog), the process with cap analogs is faster as it does not require the set-up of a second enzymatic reaction [25]. However, due to their price, cap analogues can have an impact on production costs [92], especially if large scale manufacturing is considered. Nevertheless, a cost analysis should be performed to compare the costs of the one-step and two-step production options [93]. Alternatively, co-transcriptional capping can be performed using CleanCap® Reagent AG [28]. Although this method does not compete with GTP and delivers a Cap 1 construct, it requires the use of templates with a modified T7 promoter.

Although several commercial kits are available to produce mRNA for preclinical studies at laboratory scale, their costs are high [94]. The generation of mRNA by IVT at large scale and under current good manufacturing practice (cGMP) conditions is also challenging. For example, the specialised components of the IVT reaction must be acquired from certified suppliers that guarantee that all the material is animal component-free and GMP-grade. Furthermore, the availability of large amounts of these materials is limited and purchasing costs are high [58]. This is true, for example, in the case of the enzymes used for translation and capping. Nevertheless, the expedite and simple nature of the production process is expected to lower production and operational costs when compared with the cell-based manufacturing of other biologicals such as proteins, antibodies, plasmid DNA and virus-like particles [94].

Once the mRNA is generated by IVT, it must be isolated and purified from the reaction mixture using multiple purification steps to achieve clinical purity standards (Figure 4). The reaction mixture contains not only the desired product, but also a number of impurities, which includes enzymes, residual NTPs and DNA template, and aberrant mRNAs formed during the IVT. Traditional lab scale purification methods are based on DNA removal by DNAse digestion followed by lithium chloride (LiCl) precipitation [31,58]. However, these methods do not allow the removal of aberrant mRNA species such as dsRNA and truncated RNA fragments. The removal

of these product-related impurities is crucial for mRNA performance, as they lower translation efficiency and modify the immunostimulatory profile. For example, a 10-1000-fold increase in protein production was observed when nucleoside-modified mRNA was purified by reverse phase HPLC prior to delivery to primary DC [95].

Chromatography is a mainstream purification process widely accepted in the pharmaceutical industry. Its high popularity is derived from several attributes such as selectively, versatility, scalability and cost-effectiveness [96]. The first published protocol for large scale purification of synthetically produced RNA oligonucleotides used size exclusion chromatography (SEC) in a gravity-flow mode to separate molecules according to size. [97]. Further studies applying SEC with fast performance liquid chromatography were performed [98,99]. These techniques allowed a preparative scale purification process, achieving high purity and high yields. However, SEC presents limitations, as it is not able to remove similar size impurities, such as dsDNA.

The use of ion pair reverse-phase chromatography (IPC) proved to be an excellent method for mRNA purification [44,95,100,101]. In IPC, the negatively charged sugar-phosphate backbone of the oligonucleotides will pair with quaternary ammonium compounds present in the mobile phase (in this case triethylammonium acetate) to become lipophilic and then interact with the stationary phase of a reverse-phase chromatography column [90]. Elution is then performed with a gradient of an adequate solvent, e.g., acetonitrile. Using this approach, dsRNA impurities are effectively removed while maintaining the process's high yield. However, IPC is challenging and costly to scale, and the use of toxic reagents such as acetonitrile, is not desirable. A new cellulose-based chromatography process for the removal of dsRNA has been described that leverages the ability of dsRNA to bind to cellulose in presence of ethanol [102]. This method reported a mRNA yield of >65% with a dsRNA removal of over 90%. Still, the removal of other impurities was not addressed, and thus the introduction of pre-purification steps is likely to be required.

Ion exchange chromatography (IEC) can also be used to purify mRNA at large scale. This technique explores the charge difference between the target mRNA species and the different impurities. For example, weak anion exchange chromatography has been successfully implemented to separate mRNA from IVT impurities [103].

IEC presents several advantages: it is scalable and cost-effective; it allows the separation of longer RNA transcripts; and it presents higher binding capacities (when compared with IPC) [104]. Nevertheless, this chromatography must be performed under denaturing conditions. This makes the process more complex as it requires a mobile phase heater and a tight control of the temperature during chromatography.

Affinity based separation is another mRNA purification approach. A single-stranded sequence of deoxythymidine (dT) - Oligo dT - is routinely used for the capture of mRNA in laboratory applications. This sequence binds to the poly-A tails present in the mRNA. Chromatographic beads with immobilized oligo dT could thus be used for the process scale purification using affinity chromatography: the poly-A tails of the single stranded mRNA produced during IVT would bind to the stationary phase while impurities are washed out. This way, IVT unconsumed reagents, the DNA template and dsRNA could be efficiently removed [105]. While high purity products can be obtained using affinity chromatography, several drawbacks are present such as low binding capacities and a less cost-effective process.

The removal of small size impurities can also be achieved while concentrating or diafiltrating solutions by tangential flow filtration (TFF) [106,107]. Core bead chromatography can also be used for this purpose [108]. In this case, small impurities are trapped inside the beads, and the product will be in the flowthrough. However, both techniques rely on DNase digestion or denaturing agents to remove high size molecules such as the DNA template or the polymerase. DNA removal can also be achieved using hydroxyapatite chromatography without the use of a DNase [108]. As a polishing step, hydrophobic interaction chromatography (HIC) can be applied using connective interaction media monolith (CIM) containing OH or SO_3 ligands [109].

Large scale adaptations of the traditional laboratory scale mRNA purification methods are also being explored. For example, mRNA precipitation can be combined with TFF technique [106]. During TFF, the membrane captures the precipitated mRNA product while other impurities are removed by diafiltration. The product is then eluted by re-solubilizing the mRNA. Furthermore, DNA template removal can be achieved by performing the digestion with immobilised DNase [110]. Another approach is to use tagged DNA template that can then be removed after IVT using affinity chromatography [110]. Despite being scalable, these methods present a

limited effectiveness since they only focus on the removal of some specific impurities and hence must be coupled with other purification steps.

New perspectives

The current IVT mRNA production methods must be improved to move mRNA technology to commercialisation and to support market demand. As process yields and production scale have an impact on the manufacturing costs and consequentially on the cost per dose [92], we speculate that continuous processing would have a particular advantage to lower costs. Continuous processing is already used in the chemical and pharmaceutical industry to run flexible and cost-effective processes and will ultimately offer on demand production. Additionally, the process integration made possible by continuous manufacturing may also reduce operation time and facilitate automation and process analytical technologies (PAT), which can result in a higher productivity and higher product quality [111,112]. The relative simplicity of mRNA manufacturing makes the process well suited for continuous processing, and in particular at a microfluidic scale (Figure 5). At this scale, reaction rates can be accelerated under specific conditions, the use of expensive reagents can be minimised, and cascade reactions can be compartmentalised easily [113]. Further, *in situ* product removal (ISPR) and substrate feed and product recovery (SFPR) strategies can be implemented in flow to facilitate process control, recirculation, and re-use of compounds [113]. These strategies will allow the separation of molecules, such as enzymes (if free enzymes are used), co-factors or NTPs, that can be recirculated in the process. Different unit operations, such as TFF, aqueous two-phase systems (ATPS) or precipitation, could be used for this purpose. These potentially will lower the burden on the downstream processing as well as the overall processing costs. furthermore, the proposed system could be coupled with a microfluidic formulation step, in which the mRNA is encapsulated into lipid nanoparticles (LNPs) [115]. This would allow the establishment of continuous mRNA processing until the fill-to-finish steps.

Downstream processing, together with fill-to-finish, is still the major bottleneck in the mRNA vaccine production due to the lack of well-established and cost-effective processes. Despite the effort to develop methods that achieve high purity products, most of them are coupled with the traditional precipitation or nuclease digestion techniques [102,108]. Moreover, most methods are not cost-effective which can make the process infeasible for the market needs. Alternative cost-effective techniques, such a single-pass tangential flow filtration (SPTFF) or aqueous two-phase systems (ATPS), that can be applied in a continuous mode, could potentially improve the process time and manufacturing flexibility while reducing cost and maintaining the quality [113]. Additionally, new chromatographic operation modes can overcome the need for having multiple mRNA purification steps (Figure 5). For example, the use of multimodal chromatography is highly promising as the combination of interactions between the molecule and the matrix could result in an integrated and intensified purification process without the need for multiple chromatographic steps [114].

mRNA Safety and Quality

mRNA manufacturing is advantageous when compared to the production of most biologicals since it does not require the use of cell cultures. Owing to its fast reaction time, the risk of contamination is lower than what is observed with other complex vaccine manufacturing processes. Additionally, the non-integrative nature and the transient expression inside the cells favours the mRNA safety profile [58,116].

Regulation guidelines for the evaluation of quality, safety and efficacy of RNA-based prophylactic vaccines for infection diseases are now being considered [117]. The emphasis is now on the establishment of manufacturing processes that can deliver a high quality and consistent product. Specifications for a number of critical process steps and acceptance criteria, intermediates, drug substances (DS) and drug product (DP) must therefore be defined, e.g., in terms of product yields, and analytical technologies that allows for rigorous product quantification and characterisation (product identity, purity and quality). mRNA quality can be assessed using several analytical techniques, such as gel electrophoresis and high-performance liquid

chromatography (HPLC) [116], while the identity can be assured using sequencing techniques, such as reverse transcription polymerase chain reaction (RT-PCR) or next-generation sequencing [117]. The presence of residual amounts of DNA, enzymes and solvents [118], as well as dsRNA and truncated RNA fragments, must be determined. Additionally, as a general quality control, aspects like the presence of endotoxins, overall sterility and mRNA stability, must also be evaluated [117].

Concluding Remarks

mRNA is a rising star in the field of biopharmaceuticals. The interest in this new type of vaccine derives from the flexibility, safety, and precision that these vaccines present when compared to conventional approaches. The growing number of clinical trials for cancer therapies and infectious diseases demonstrates an increased interest from the industry to release these types of vaccines to the market. mRNA vaccines are precise, safe and flexible, which can be easily manufactured on a large scale for clinical grade applications. These vaccines can be an answer to quickly respond to epidemic outbreaks in terms of manufacturing.

However, to achieve this status, the development of sustainable and cost-effective manufacturing processes must be addressed. Although the IVT reaction of mRNA is safer and quicker than most of the established vaccines production, it relies on the use of expensive and limited materials. Downstream processing of the vaccine is still poorly established, and it is dependent on methods that lack scalability and cost-effectiveness. Moving the process to continuous manufacturing can overcome these bottlenecks. We propose a microfluidics approach with the compartmentalisation of enzymatic reactions coupled with *in situ* product removal (ISPR) and substrate feed and product recovery (SDPR) modules and the use of multimodal chromatography to replace the use of multiple chromatographic steps (Figure 5). The use of new production methods that allow the reuse and recirculation of compounds integrated with high-throughput purification and well-defined analytical methods in a continuous manufacturing process can be the answer for a sustainable, flexible and cost-effective vaccine manufacture that can allow an on-demand response.

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Declaration of Competing Interest

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

All authors attest they meet the ICMJE criteria for authorship.

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692 Table 1. Advantages (+) and disadvantages (x) of the currently available types of vaccines.
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Properties	Inactivated	Live attenuated	Toxoid	Subunit (conjugate; protein-based; polysaccharide)	Viral Vectors	DNA
Humoral and cellular immune response	✗	+			+	
Lasting protection		+	✗	✗		
Stability	+		+	+	+	+
Safety	+	✗	+	+	✗	+
Manufacturing	+		+	✗	✗	+
Presence of adjuvants		+	✗	✗	+	✗
Cold chain		✗			✗	+