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Review

Quality by Design for enabling RNA platform production processes

Simon Daniel [1], Zoltán Kis [1,2,*], Cleo Kontoravdi [1], and Nilay Shah [1,*]

RNA-based products have emerged as one of the most promising and strategic technologies for global vaccination, infectious disease control, and future therapy development. The assessment of critical quality attributes (CQAs), product–process interactions, relevant process analytical technologies, and process modeling capabilities can feed into a robust Quality by Design (QbD) framework for future development, design, and control of manufacturing processes. QbD implementation will help the RNA technology reach its full potential and will be central to the development, pre-qualification, and regulatory approval of rapid response, disease-agnostic RNA platform production processes.

Highlights

Pfizer-BioNTech's (BNT162b2) and Moderna's (mRNA-1713) vaccines against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) are the first RNA-based biologics to be approved for human use and mass produced.

RNA technology holds great promise beyond infectious disease prophylaxis, from cancer and gene therapy to treatments against cardiovascular and autoimmune diseases.

Current production processes have been developed and scaled up at an unprecedented speed, mostly under a Quality by Testing paradigm.

Recent research has highlighted a strong link between product and process development.

The application of advanced analytical and modeling technologies could rapidly reshape and digitize manufacturing processes.

Promises and challenges of RNA manufacturing

The coronavirus disease 2019 (COVID-19) pandemic and emergence of safe and efficient RNA vaccines have brought RNA technology to the forefront of medical innovations [1,2]. The rapid development and production timelines, in combination with recent genotyping methods, make RNA technology suitable to respond to emerging infectious threats and variants [3]. While most conventional vaccines and biopharmaceuticals require the use of inherently variable cell cultures, RNA manufacturing is based on a relatively simple, scalable, and affordable cell-free production system [4]. Given its mechanism of action, the therapeutic scope of RNA technology is wide, and production processes are versatile (Box 1) [5]. The potential clinical applications encompass infectious disease prophylaxis; rare disease treatment; and gene, cancer, and protein replacement therapy [6–8]. Different products could be manufactured using the same raw materials (excluding DNA template), consumables, equipment, unit operations, and analytical methods. However, a multiproduct platform technology still requires proof of scientific and industrial mastery to be approved and truly disruptive.

In addition, the rapid production of safe and efficient vaccines was only possible thanks to a high-risk financing strategy, government support, and strong incentives for industrial adaptation [20]. In the long run, this approach is neither sustainable nor desirable. International technology transfer and distributed manufacturing are urgently needed. Additionally, despite the high level of safety and efficacy observed in these approved vaccines, product quality remains a critical issue [21]. An example is the occurrence of rare severe adverse events, such as myocarditis and anaphylactic reactions, which could hamper vaccination campaigns and strengthen public mistrust in this new technology [22]. Their clinical success should not overshadow the current need for booster doses and the failures of other mRNA vaccine candidates [23]. The two components, the RNA active substance and the **lipid nanoparticle (LNP)** (see Glossary) (Box 1), are both unstable and prone to degradation [13]. For instance, RNA integrity in the BNT162b2 vaccine is estimated to be approximately 70% at the end of production, with further degradation expected during distribution [24]. This is partially reflected in the low temperature requirements for product storage, which further complicates the vaccine supply chain [25]. Finally, RNA technology is still under intense development and should prove its therapeutic versatility in clinical trials while new manufacturing

[1]Centre for Process Systems Engineering, Department of Chemical Engineering, Faculty of Engineering, Imperial College London, London, UK
[2]Department of Chemical and Biological Engineering, The University of Sheffield, Sheffield, UK

*Correspondence:
z.kis@sheffield.ac.uk (Z. Kis) and
n.shah@imperial.ac.uk (N. Shah).

Box 1. Current RNA-based drug product

RNA-based vaccines and therapeutics are composed of two key elements: the RNA active substance encoding a protein of interest and the LNP structure as the delivery vehicle. Typically, the protein can be a viral antigen, such as the SARS-CoV-2 spike protein, a cancer marker, or a missing protein. Regardless of the route of administration, the RNA enters the cytosol through a receptor-mediated mechanism. Then, the mRNA active substance uses the host cell translation machinery, while saRNA also encodes its own replication machinery [9]. This is one of the major differences between these two classes of RNA-based products. Currently, approved vaccines and most of the clinically advanced candidates are based on nonreplicating mRNA systems. However, saRNA could be advantageous because lower doses of RNA are potentially sufficient for enhanced and prolonged protein expression, thereby also reducing production costs and the occurrence of some adverse reactions [4]. The saRNA nucleotide sequence is also longer (e.g., approximately 10 kb compared with 4.5 kb for mRNA COVID-19 vaccines), implying potential manufacturing differences [10]. In this review, the mRNA and saRNA systems are collectively referred to as 'RNA technology'.

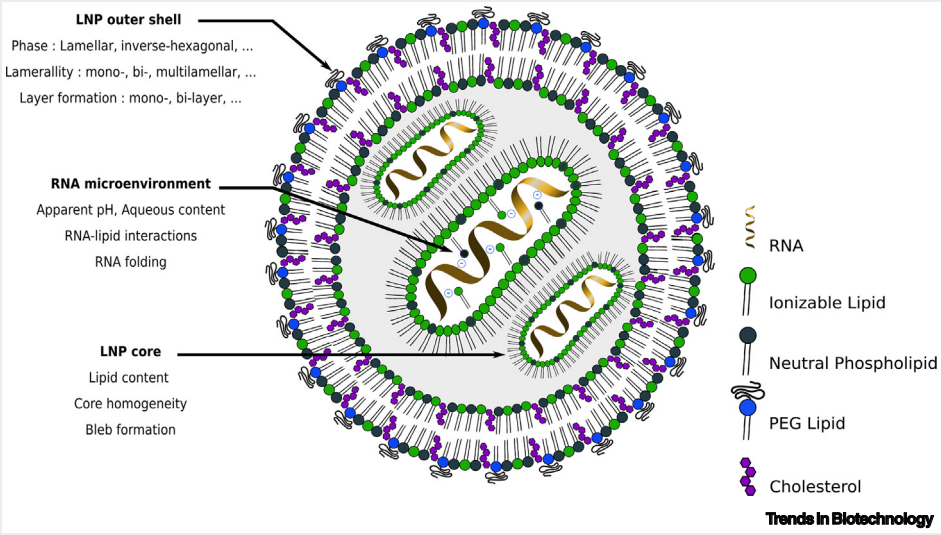

Figure I. Hypothetical structure of an RNA-LNP system. The illustrated LNP is a core dense, unilamellar nanoparticle, with a bilayer outer membrane. Multiple structural models are possible and adequate depending on the lipid type and associated manufacturing process. The RNA microenvironments, LNP morphology, lamerallity, outer shell, and core structures can vary for the different LNP formulations [11–14]. Currently, LNPs represent the most suitable delivery system and are used by three of the most advanced companies for their COVID-19 mRNA vaccines: Moderna, BioNTech, and CureVac [1,2,15]. Recent research further recommends it over polymer-based delivery systems for saRNA active substance [16]. State-of-the-art LNPs are composed of four different lipids: a neutral phospholipid, a PEG lipid, cholesterol, and an ionizable lipid (Figure I) [17]. PEG lipids play a key role in the LNP architecture and are steric barriers to prevent LNP aggregation. Ionizable lipids contain amine groups, which are positively charged at acidic pH, thereby interacting with the RNA cargo and easing its encapsulation during mixing. These lipids are also fundamental in determining LNP fusogenicity and circulation properties [10]. Finally, the phospholipids and cholesterol are at the basis of LNP structuration. Additionally, the LNP patent landscape is complex and encompasses numerous patent licensing and legal actions [18]. This is one of the reasons why LNPs remain diverse in terms of lipid composition and manufacturing processes [19].

requirements are anticipated [26]. Therefore, a deeper understanding of both product and process appears necessary to face these multiple challenges. The absence of rigid RNA-specific regulatory guidelines leaves further room for continuous innovation [27,28]. In particular, the application of a **QbD** approach to this new class of drugs could be a paradigm shift and could unlock the potential of RNA manufacturing technology (Figure 1). This review discusses how these QbD principles can be applied and tuned for RNA-based products and how this new technology can specifically benefit from them. The emerging literature in this rapidly evolving field is analyzed herein and is interpreted through the prism of a multiproduct and patient-centric manufacturing approach. This knowledge assessment offers the first risk-based review of product quality attributes, process

Glossary

Computational fluid dynamics: mathematical modeling of physical phenomena involving fluid flow that are solved by numerical methods.
Critical process parameters (CPPs): process state or input operating condition whose variability within a defined range has an impact on a critical quality attribute.
Critical quality attributes (CQAs): physical, chemical, or biological property that should be within a defined limit, range, or distribution to ensure the desired product quality, safety, and efficacy.
Current Good Manufacturing Practice (cGMP): regulations and guidance provided by regulatory agencies that guide the design, monitoring, and life-cycle management of drug manufacturing.
Design of Experiment (DoE): statistical methods used to optimally plan experiments and collect data from experiments by maximizing the information content when investigating the impact of independent explanatory variables on dependent response variables.
Design space: process operating region leading to the desired product quality.
Lipid nanoparticle (LNP): nucleic acid delivery technology of 50–500-nm size composed of a mixture of lipids.
Mechanistic modeling: mathematical approach describing the underlying physical, chemical, or biological phenomenon in a system such as a production process.
Molecular dynamics: computer simulation methods for analyzing the movements of atoms and molecules in biophysical systems.
Process analytical technology (PAT): set of tools and systems that aim to provide reliable, real-time, and in-process measurements of CQAs, CPPs, and other process indicators in lieu of offline end-product testing.
Process control strategy: set of systems and controls, ideally derived from process and product understanding, ensuring consistent product quality.
Quality by Design (QbD): systematic approach that begins with predefined objectives and emphasizes process control and product and process understanding based on sound science.

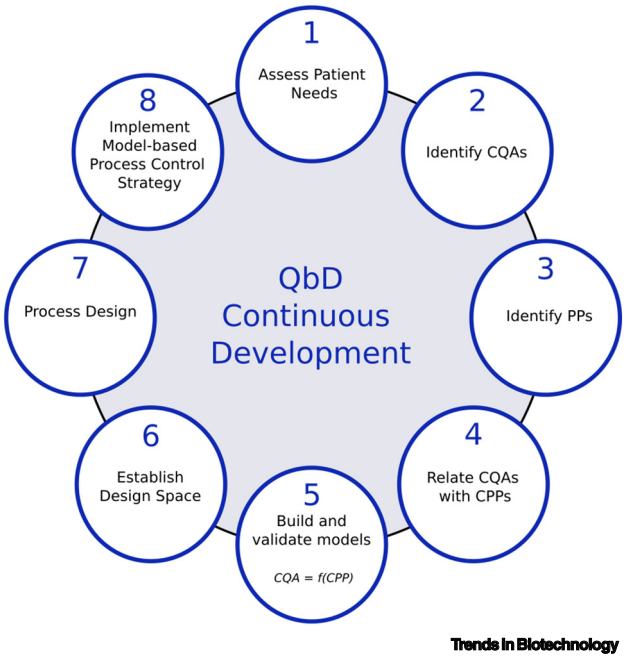

Figure 1. Description of Quality by Digital Design iterative cycle development. Quality by Design (QbD) starts with the assertion that increased testing is not sufficient to improve product quality. It emphasizes instead that quality should be built into the product through deep understanding of the product and process [29]. More precisely, product quality needs to be linked to clinical and nonclinical studies to ensure pharmaceutical safety and efficacy. The production process should be designed to reduce product variability and increase process capability. In addition, enhanced knowledge should enable efficient scale-up, postapproval changes, and root cause analysis [30,31]. In practice, the QbD development begins with (1) the identification of patient needs and the establishment of a Quality Target Product profile (QTPP). (2) Based on this, a list of the critical quality attributes (CQAs) and associated acceptance criteria is established. (3,4) Prior knowledge is then used to identify the process parameters (PPs) and critical process parameters (CPPs) for each process step. (5) From this product–process knowledge, efforts should be made to build reliable and accurate mathematical models relating CQAs to CPPs. (6) These models can be used to identify the process design space and to support simultaneous product and process development. (7) QbD development culminates when the gained knowledge is used to design the process control strategy. A first approach is simply to reduce the need of extensive product testing by using a reliable design space and by identifying all the sources of process variability that impact CQAs. Process parameters are less tightly constrained, and product attributes are more consistent. Process control is also typically shifted upstream. (8) A second, more ambitious QbD approach is to use mathematical modeling for advanced predictive control and real-time process optimization. Once the approach is tested and validated, its implementation can increase quality assurance level and enable real-time release testing, process automation, and digitization. The QbD framework development follows an iterative cycle, as mathematical modeling, analysis, and real-time measurements can be used to reassess parameter criticality and develop an appropriate life-cycle management strategy. QbD is particularly powerful when the gained knowledge is transferred from one product/process to another and, in that sense, is highly promising in a platform technology.

parameters, and their potential interactions. From this, the potential avenues for the development of characterization and modeling tools to underpin an enhanced QbD approach can be identified. Eventually, a theoretical and holistic manufacturing framework encompassing and integrating **CQAs**, **critical process parameters (CPPs)**, and the requirements of current and future RNA-based products can be drawn. At this early stage of development, this analysis further brings new perspectives and a roadmap for the rapid deployment of a versatile, distributed, and affordable RNA platform technology.

Identification of CQAs under a QbD framework

In QbD, CQAs are at the basis of production process development, design, monitoring**,** control, and life-cycle management. The first comprehensive identification of potential CQAs for RNA-based biologicals is presented in Table 1. It is established on the basis of prior knowledge, current structure–function understanding, strategic nonclinical studies, and relevant real-world experience. Furthermore, this list is in accordance with nascent and existing regulatory guidelines and encompasses the specification of mRNA-1713 and BNT162b2 products[i,ii] [27,28]. The sources, risk assessment methodology, and detailed rationale for each attribute are displayed in Tables S1–S3 in the supplemental information online.

Table 1. Identification of quality attributes of RNA-based product[a,b]

Criticality level	AS attributes	AS-related impurities	DP attributes	DP-related impurities	Additional attributes and compendial testing
CQAs	RNA content	RNA purity	RNA content	Lipid–RNA species impurity	Immunogenicity
	RNA sequence identity	dsRNA species	Lipid content		Potency/*in vitro* expression
	RNA sequence integrity	Shorter RNA species	LNP size		Endotoxins
	5′ capping efficiency		LNP polydispersity		Bioburdens
	Poly(A) tail length		LNP surface charge		Sterility
	Poly(A) tail level		Lipid identity		pH
			RNA encapsulation		Osmolality
					Particulate matter
pCQAs	RNA structural integrity	RNA precipitates		Individual lipid impurities	LNP morphology
		Residual enzymes		Total lipid impurities	
		Residual host cell proteins		Residual solvent	
				Additional residual impurities	
QAs		Residual DNA			Appearance
					Viscosity

[a]Abbreviations: AS, active substance; CQA, critical quality attribute; DP, drug product; dsRNA, double-stranded RNA; LNP, lipid nanoparticle; poly(A), polyadenylation; pCQA, potential critical quality attribute; QA, quality attribute.
[b]RNA yield and RNA recovery can be identified as key performance indicators and not CQAs, but they remain central in the process control strategy and are also indicators of process consistency and hazardous deviations from normal operating conditions.

More specifically, activation of the innate immune system is a central factor in assessing RNA-based biologics' safety and efficacy. Inflammatory reactions are indeed related to most frequent and rare adverse events [21,32]. Although immunogenicity can be considered *a priori* as an advantageous feature for product efficacy, providing adjuvant-like properties, it could simultaneously reduce RNA translation as a result of the activation of stress genes and a cellular trade-off between innate immune and translation machinery mechanisms [33]. Although minimizing inflammation seems to emerge as the best approach for prophylactic vaccines and a requirement in gene therapies, this stimulation remains promising in cancer treatment because type I interferon activation is correlated with favorable disease outcomes [34]. More indirectly, inflammation can affect potency by limiting the dose regime, as occurred in the second phase of CureVac's COVID-19 vaccine trials [16].

Regarding the drug product, *in vivo* administration of naked RNA and empty LNP triggers immune stimulation, confirming the immunogenic nature of both structures [35]. Particular emphasis should be placed on polyethylene glycol (PEG) lipids suspected to be related to the observed anaphylactic reactions [36,37]. On the one hand, potential lipid–RNA reactions and hybridizations also require careful consideration because they have been observed during the mRNA-1713 manufacturing process and could lead to inactivation of the active substance potency and enhanced degradation [20,34]. On the other hand, numerous RNA-related impurities are also potentially immunogenic (Table 1), and longer double-stranded RNA (dsRNA) species represent one of the major risks [38]. These heterogeneous byproducts are potent pathogen-associated molecular patterns and can completely deplete RNA translation. Structural elements, such as 5′-cap and polyadenylation [poly(A)] tail, and their integrity play another key role in product reactogenicity and translational efficiency [39]. While the criticality of RNA primary sequence is obvious, the importance of the RNA secondary and tertiary structure is still being assessed [40]. The higher-order structures of BNT162b2 active substance have been evaluated during

characterization studies and are expected to influence the RNA thermostability and half-life [23,41]. Ultimately, LNP structural characteristics, such as the lipid content, size distribution, and surface charge, are of critical importance in determining immunogenicity, biodistribution, cellular uptake, endosomal escape, and circulation time [42].

Although risks are now thoroughly identified, there are still major knowledge gaps in our understanding of product structure, inflammatory pathways, and their links with clinical performance. First, as described in Box 1, a diversity of LNP structural and morphological features, potentially affecting product activity, have been observed, but very few of them have been tested or screened during development. Once better understood, the consistency of the LNP morphology could also be checked during initial process development, scale-up, or technology transfer. Second, elucidating the activation of certain inflammatory pathways, including on- and off-target effects, would be of great help to optimize safety and efficacy profiles [43]. For instance, clinical studies of RNA vaccines inform us that a Th1-type bias response and specific cytokine signatures could be predictors of a potent antibody response [44,45]. This will also help us to define appropriate endpoints and design relevant models and assays for activity testing, which remain fundamental in current process development and quality control strategy [28]. Finally, in line with the scope expansion of the RNA technology, identifying optimal product characteristics for a given route of administration, therapeutic field, or targeted organ will be crucial. While the optimal immunogenic profile and LNP morphology are both likely to be highly variable, targets for other CQAs could also vary [46]. Among others, while neutral particle surface is desired in current RNA vaccine, surface charge appears to be a crucial parameter in organ targeting and in determining lymph node- and mucus-penetrating ability [47–49]. In addition, the LNP molecular composition and size both play an evident role in product biodistribution and activity [50–52]. Besides, the desired immunogenicity of the active substance can also vary [22,53]. This is especially important in the case of repeated RNA administration or high-dose regimens, such as in chronic administration or protein replacement therapy [54,55]. Taken together, addressing these issues could reshape future product and process development.

Knowledge assessment of product–process interactions

The next step in the QbD approach is to assess the relationships between identified CQAs and CPPs within all critical unit operations. The large-scale **Current Good Manufacturing Practice (cGMP)** manufacturing processes and corresponding CPPs are mapped in Figure 2, while relevant methodology and details behind individual CQA–CPP relationships are displayed in Tables S4–S11 in the supplemental information online. Despite the scarcity of large-scale and RNA-specific data, numerous in-process risks can still be identified due to the repurposing of multiple unit operations and our increased mechanistic understanding of RNA and LNPs as biophysical systems [56–58]. Although in-process stability is not yet precisely characterized, there are well-defined degradation pathways described in the literature for nucleic acid- and lipid-based delivery systems. RNA molecules, and especially longer ones such as self-amplifying RNA (saRNA), are shear sensitive and prone to hydrolysis [59,60]. On the nanoparticle side, LNP degradation is limited not just to LNP-related impurities but also to particle aggregation, fusion, RNA leakage, or other structural modifications such as lipid phase transitions [13,14].

First, numerous manufacturing options appear in upstream processing, which can significantly affect the quality of the active substance. The most striking one is the RNA capping strategy: although Pfizer-BioNTech has opted for a cotranscriptional capping using the recently developed CleanCap system, Moderna has adopted an enzymatic capping approach. This last option requires extensive intermediate purification steps, impacting overall recovery and RNA integrity, but yields almost 100% capped RNA, even for hard-to-cap structures [62]. Second, *in vitro*

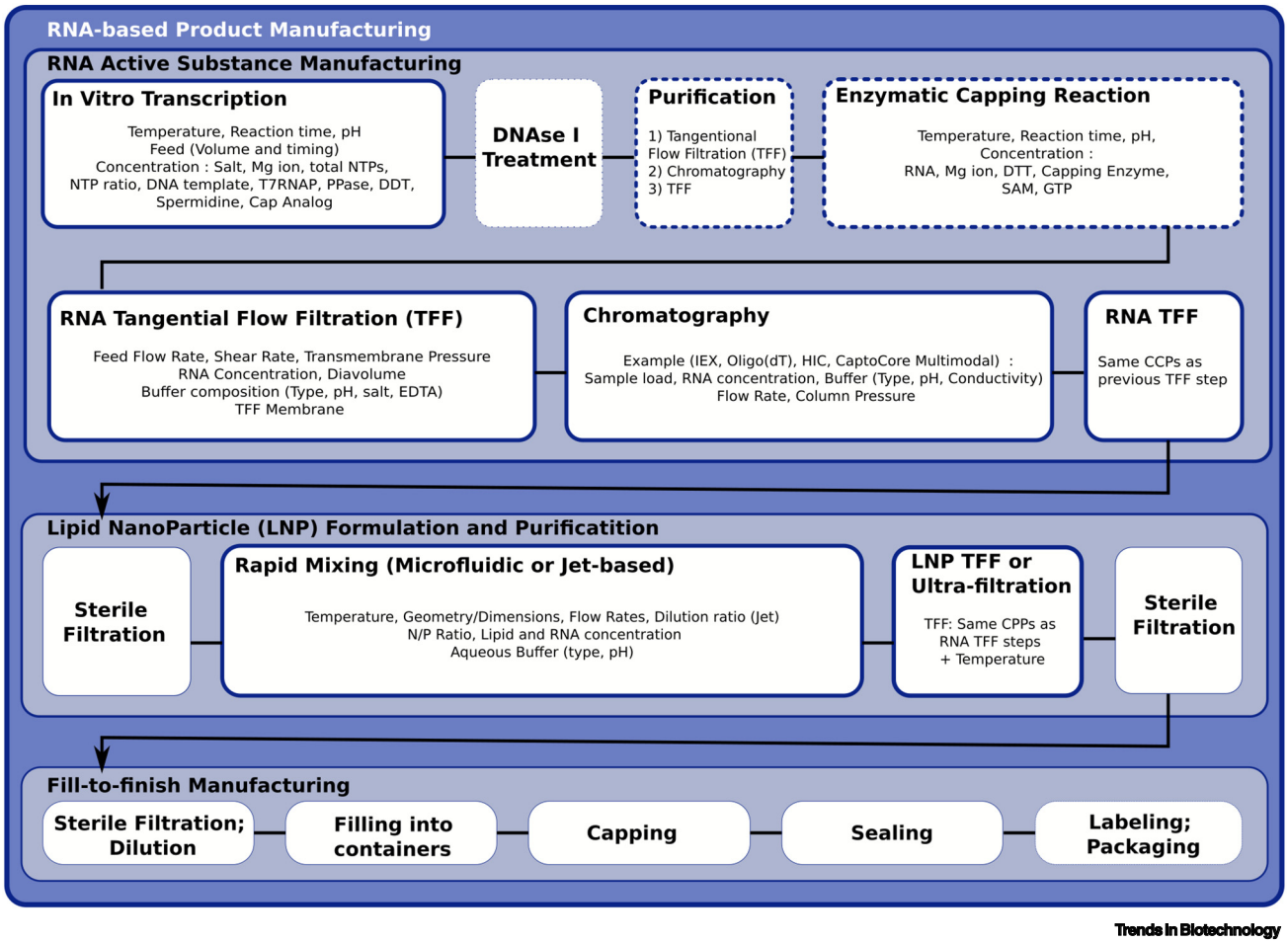

Figure 2. Current RNA manufacturing production processes and associated critical parameters. RNA vaccine manufacturing can be divided into three main phases: (i) production of the RNA active substance (or naked RNA), (ii) formulation of the RNA-lipid nanoparticle (LNP) intermediate, and (iii) drug product manufacturing (also known as 'fill-to-finish'). The latter is out of the scope of the present review, as it has less scope for innovation and often takes place at a different manufacturing site. The active substance manufacturing starts with the synthesis of single-stranded RNA from a DNA template in a processe called *in vitro* transcription (IVT). In current protocols, the RNA polyadenylation tail is plasmid encoded, while RNA 5′ capping can be performed either co- or post-transcriptionally. An optional DNase I treatment can then be performed on the 5′-capped RNA to digest the DNA template [61]. The next task is to purify the IVT process- and product-related impurities. Current setups are composed of a combination of a tangential flow filtration (TFF), followed by a chromatographic step and a second TFF. Following this, the production enters its second phase: the RNA formulation. This stage is currently based on the mixing of a liquid stream containing the four lipids in ethanol [or three, as polyethylene glycol (PEG)-lipid can be subsequently added] with another stream containing RNA in an aqueous low-pH buffer. Two options are the most appropriate for Current Good Manufacturing Practice manufacturing: microfluidics and jet mixing. Finally, LNPs are concentrated and purified using a final TFF.

transcription (IVT) can be performed in either batch or fed-batch mode. While nucleotide feeding increases the amount of RNA produced by a DNA template by two- to threefold, many RNA-related attributes are deteriorated by prolonged reaction time [63]. Besides these options, IVT process condition ranges are currently wide, as protocols have not been optimized for long RNA molecules with therapeutic application. Potential optimization paths focus on increasing RNA quality and, crucially, avoiding the formation of RNA-related impurities. In a QbD approach, Moderna, for instance, has developed an optimized protocol limiting the prevalence of fully dsRNA by focusing on nucleotide ratio [33]. Temperature and magnesium ion concentration also play a pivotal role in controlling T7RNAP activity and preventing dsRNA formation [64,65]. More disruptive approaches, such as the synthesis of tethered T7RNAP-DNA complexes,

could further decrease this risk by redefining the potential IVT **design space** and notably increase the salt concentration [66]. Recent innovations in the field also recommend performing IVT and enzymatic capping reactions at higher temperatures either to prevent dsRNA formation or to decrease capping enzyme concentration [64,67]. The adoption of these approaches is likely to require thorough studies on the temperature-dependent behavior of underlying chemical and physical processes.

Regarding downstream processing, the main risks can be identified from existing protein and other nucleic acid purification experiments, as the same phenomenon of gel formation and membrane fouling has been described for RNA tangential flow filtration (TFF) [68]. The choice of membrane and optimal purification buffer is specific to RNA molecules and needs to prevent phosphodiester ion formation, RNA precipitation, and denaturation [69]. Although high RNA recovery is currently reported, this step is likely to be risker when longer RNA, such as saRNA, needs to be purified or if TFF is performed continuously. Following TFF, no single chromatographic method has currently emerged, as they all display advantages and drawbacks, and the efficient HPLC methods used previously in RNA vaccine development are poorly scalable [70]. The exact chromatographic setups used at commercial scale are not publicly disclosed, but potential methods are reverse phase, hydroxyapatite, oligo-dT, ion exchange, hydrophobic interaction, multimodal, or cellulose-based chromatography [71,72]. Of particular interest in a multiproduct manufacturing platform is CaptoCore multimodal chromatography, a scalable, efficient method for purifying both saRNA and mRNA species with a small footprint [73]. While process conditions in hydroxyapatite, reverse phase, or oligo-dT chromatography could be detrimental for RNA integrity, undesired binding is likely to occur in CaptoCore chromatography [74,75].

During RNA formulation, the complex interplay between manufacturing and nanoparticle structure goes beyond the choice of the encapsulation technique. In both jet-based and microfluidics-based techniques, the flow rates, lipid, and nucleic acid concentration strongly affect LNP size distribution and encapsulation efficiency. A diversity of morphological features can also be generated by only varying the process parameters [12]. For example, nucleic acid concentration influences the transition from a multilamellar to an electron-dense morphology [76], while pH and temperature are key factors in the formation of hexagonal or lamellar structures [13,14]. Crucially, recent structural studies on core-dense LNPs indicate that encapsulated RNA is still in an aqueous environment and thus prone to hydrolysis [77]. Therefore, the operating conditions that dictate LNP morphology and aqueous buffer composition may be critical for both RNA and LNP stability. Investigative studies on the stability of formulated RNA would be required to adopt a system approach of LNP-formulated RNA. Recent research further confirms the complex nature of this system, establishing a link between LNP chemistry, formulation, and RNA-based modification strategy [78]. Ultimately, despite accumulating knowledge and data, a complete framework is still far from complete, given the complexity and diversity of LNP-RNA systems, the numerous unknowns surrounding formation mechanisms, and our lack of specific and easy-to-use characterization tools. The interactions between formulation composition and process parameters further complexify this knowledge assessment [79]. Adopting generic LNP systems would thus be helpful with a view to better characterize the LNP formation process and transfer knowledge among RNA products against different disease targets. Finally, LNP purification should not be overlooked, as this step can significantly impact particle physical attributes and product thermostability [80,81].

In summary, despite the high number of identified and possible CQAs, this mapping of CQA–CPP interactions can provide guidance on the prioritization of future experiments. First, RNA hydrolysis and dsRNA formation should be placed at the center of a refinement of the IVT design space,

further decreasing downstream burden and intensiveness. In subsequent purification steps, optimization should decrease the process time, shear stress, and thus RNA exposure to denaturing conditions. Finally, the development of relevant biological assays and models remains central in formulation process development. While ensuring an appropriate level of homogeneity, RNA encapsulation, and stability, the ability to tune and control LNP morphology, size distribution, surface charge, and immunogenic properties within a preliminary design space would be fundamental in a multiproduct platform development. In an iterative manner, this capability will help elucidate the relationships between these attributes and clinical performance, enabling a more rational design of RNA-LNP products.

Quality by Digital Design for future RNA manufacturing

Once CQAs and CPPs are identified, efforts should be made to establish mathematical relationships between them to support design space identification, process optimization, and in-process control (Figure 1). Reliable and accurate measurements are needed, however, to build, feed, and validate these models. Additionally, new analytical methods can extend our knowledge toward new attributes and mechanisms, which are precious for further development, update of CQAs and CPPs, and root cause analysis [82]. While at commercial scale current specification measurements are mostly based on offline measurements, analytical capabilities could be rapidly enhanced by the introduction of advanced tools (Box 2). Existing alternatives can be found in **process analytical technology (PAT)** systems [83]. By enabling real-time or timely quality assurance and release testing, they open new avenues for streamlining production process and automation [84]. PAT also provides tools and reliable data to develop advanced modeling techniques, notably through the identification and characterization of underlying kinetic parameters [85]. Eventually, the combination of knowledge and enhanced analytical capabilities can reshape the **process design** and **control strategy** by enabling the use of model-based process design and predictive control.

First, the relative simplicity of RNA upstream processing offers an outstanding advantage for ambitious model-based process design and control. Successful modeling of the effective RNA yield has been achieved by integrating multiple differential kinetic equations describing mechanistically RNA synthesis, degradation, and precipitation [100]. In a filed patent, Moderna subordinates IVT feeding strategies to a model predicting RNA yield, poly(A) tail, and capping level in real time [63]. The expansion of these models to RNA impurities, and particularly dsRNA, would further refine process design and control strategy. Our increased mechanistic understanding of the different dsRNA formation mechanisms is particularly promising [65,66,101]. The use of **mechanistic modeling** can be advantageous in an initial data-scarce environment to direct experimental effort as well as ease technology transfer or adapt the process to product innovations [102].

Second, RNA downstream production processes can greatly benefit from the recent advances in protein and cell purification modeling tools. The flow dynamics and membrane behaviors are extensively characterized in TFF systems through notably mass transport and balance equations [103,104]. Multiple chromatographic modeling options can also be rapidly explored. For example, studies on CaptoCore ligands and shells detail their binding, adsorption, and diffusion properties and enable the development of accurate predictive models characterizing the capture of proteins as a function of their size [66,92,105].

Regarding formulation, various studies establish a clear correlation between mixing and nanoparticle diameter and size distribution [106]. Current mixing processes are thoroughly characterized, going from simple mathematical estimations of bulk flow turbulence to high-resolution **computational fluid dynamic** simulations [107,108]. Compared with the nanoprecipitation process occurring in

Box 2. Enhanced analytical capabilities for RNA-based product

Although UV/visible (UV-Vis) spectroscopy is currently applied offline for measuring RNA concentration, inline UV spectrometers are readily available and could be implemented for process design and product release in IVT and all purification steps [86]. Automated high-throughput circular dichroism (CD) is an additional promising tool to rapidly monitor the RNA primary, secondary, and tertiary structures [87]. This device has already proved its accuracy and versatility in protein folding analysis [88] and is able to monitor RNA secondary structure following its encapsulation [89]. A rapid path toward PAT is to equip IVT and downstream processing steps with autosamplers in order to integrate liquid chromatography and multiple detectors, such as UV-Vis or CD spectrometry, into the process path. RNA integrity and lipid and nucleic acid impurities could thereby be routinely quantified. A similar system has been developed successfully by Moderna to characterize IVT and monitor mRNA, nucleoside triphosphates (NTPs), cap analog, plasmid DNA, and enzyme concentration [63]. Ultimately, RNA integrity and lipid and nucleic acid impurities could, in theory, be quantified using such devices.

Relatively simple real-time particle size analyzers, such as those based on inline or online dynamic light scattering (DLS) methods, are implementable for both the LNP formation and purification steps [90]. A combination of multiangle light scattering, UV and refractive index detectors, coupled with size exclusion chromatography, is able to measure size-dependent RNA content in less than 1 h [91]. Alternatively, an asymmetric flow field-flow fraction method has recently been adapted to LNP-RNA inherent instability and is another robust and versatile approach for deeper LNP physical characterization [92,93]. More detailed information on LNP structure can be provided by other techniques (Table I). Raman spectroscopy, for instance, is able to monitor LNP structure in real time and can provide insight on lipid content and state as well as nucleic acid distribution [94]. Using small-angle X-ray scattering (SAXS), the temporal evolution of some LNP morphological features can also be followed [95]. More established methods are cryo-electron microscopy (CryoEM), small-angle neutron scattering (SANS), and nuclear magnetic resonance (NMR), all providing deeper structural insights [11,77,96].

In addition, chemical biology is a buoyant field and recently allowed the development of new screening tools for IVT. Indeed, two distinct biosensors provide analytical solutions for high-resolution testing of capping efficiency and T7RNAP activity at laboratory scale [86,97]. Fluorescence-based assays have also been developed for measuring intracellular and extracellular degradation of formulated RNA, opening avenues for alternative stability assays [98].

Table I. Analytical methods and process analytical technology systems for main RNA-based product attributes and process parameters[a,b]

Quality attribute/process parameters	Current offline analytical methods	Potential process analytical technology alternatives	Suggested acceptance criteria
RNA yield, RNA recovery, RNA content	UV spectroscopy, fluorescence-based assay, anion exchange chromatography	Inline or online UV, automated (high-throughput) CD	>1.5 g/l, 95–70%
RNA sequence identity	RT-PCR, Sanger sequencing, NGS	Automated LC-MS/MS	N/A
RNA structure integrity	CD spectroscopy, SEC-MALS, DSC	Automated high-throughput CD spectroscopy	N/A
5′ capping efficiency	Analytical LC-UV/MS, LC-MS, nuclease digestion followed by MS/MS, ribozyme assay with CE	Fluorescence-based molecular sensor	>50–85%
Poly(A) tail length and distribution	RNA electrophoresis, analytical LC-MS, LC-UV/MS, RP-HPLC, UP-HPLC		100–120 bp
Poly(A) tail level	Analytical LC-MS, LC-UV/MS, MS, ddPCR	At-line or online HPCL/UPLC with autosampling and embedded detector	>70%
RNA purity, shorter RNA	RNA electrophoresis, analytical RP-HPLC, RP-UPLC, IEX-HPLC, western blot oligonucleotide mapping		>50%
dsRNA content	Immunoblot, dot blot, ELISA, analytical LC		<1 ng/µg RNA
Residual DNA content	qPCR, fluorescence-based assays		<330 ng/mg RNA
Residual enzymes, host cell proteins	NanoOrange, Ph. Eur. 2.5.33		<300–500 ng/mg RNA
RNA encapsulation	Ribogreen assay, IEX-HPLC, RP-HPCL, CryoEM, SEC-SLS/UV	Raman, UV-Vis spectrometry, SEC-MALS-UV/RI	>80%
LNP size, polydispersity, stability	DLS, NTA, MALS, SEC-MALS, CE, FFF-MALS-UV-dRI	Inline or online DLS, online MALS, SEC-MALS-UV/RI	<100–200 nm, <0.3
LNP charge	ELS, PALS, CE	–	±20 mV
Lipid identity and content, lipid-related impurities	UPLC-CAD, HPLC-CAD, LC-MS, FFF-MALS-UV-dRI	SEC-MALS-UV/RI,	N/A
LNP morphology	CryoEM, SANS, SAXS, FFF-MALS-UV-dRI, DSC	SEC-MALS-UV, NMR, NIR, SEC-SAXS/SANS	N/A

[a]Abbreviations: CAD, charged aerosol detector; CD, circular dichroism; CE, capillary electrophoresis; ddPCR, droplet digital PCR; DLS, dynamic light scattering; dRI, differential refractive index; DSC, differential scanning calorimetry; ELS, electrophoretic light scattering; FFF, asymmetric field-flow fractionation; IEX, ion exchange; LC, liquid chromatography; LNP, lipid nanoparticle; MALS, multiangle light scattering; N/A, nonapplicable; NGS, next-generation sequencing; NTA, nanoparticle trafficking analysis; PALS, phase-analysis light scattering; Poly(A), polyadenylation; RI, refractive index; RP, reverse phase; SEC, size exclusion chromatography; SLC, static light scattering.
[b]This table was compiled based on [86,94,99]. The suggested acceptance criteria mainly reflect current quality requirements for RNA-based vaccines administered intramuscularly.

jet-based mixer, the mechanistic characterization of the LNP assembly process is less advanced in microfluidics settings [57]. Self-assembly modeling, capturing only thermodynamic aspects, is still being explored, and the higher degree of control over mixing conditions enables the development of efficient statistical models predicting LNP bulk attributes [109,110]. The ability to predict and control the LNP assembly process, including the LNP inner structuring and RNA–lipid interactions, is one of the major future modeling challenges. Even though more intense research is required, this complex process could be approached by more detailed simulations at the molecular level. Recent **molecular dynamics** studies illustrate the potential of this approach, offering new mechanistic insight into lipid clusters, membrane structuring, LNP stability, and RNA folding [111–113]. At the end, all these computational tools could be integrated to monitor, optimize, and control the kinetic and thermodynamic aspects of the LNP-RNA formulation process. A structural model relating RNA microenvironments, detailed LNP structure, and formation mechanism could be placed at the core of future design strategy.

The use of these advanced simulation techniques is also bridging the gap between product and process development [114]. Ideally, the choice of lipids and the optimal control over lipid and RNA molecular interactions should consider simultaneously the impact on RNA encapsulation, stability, and LNP structure, as well as on the expected immune stimulation, cellular uptake, or endosomal escape. Similarly, a powerful LNP degradation model, embracing thermodynamic stability, aggregation, and physical degradation, could predict both in-process and *in vivo* stability. Following the same logic, the available RNA sequence design strategy, underpinned by various data-driven, hybrid, and molecular dynamic modeling tools, could consider the sequence manufacturability in addition to RNA stability and translation [41,115–117]. The secondary structure is indeed known to impact in-process degradation and capping accessibility and could play a potential role in LNP formation [37]. These potential sequence-specific models would be more challenging to implement but could be considered as a longer-term objective in process and product continuous improvement.

Even though process models should initially be developed individually for each step, when integrated with PAT, they can also serve as a basis for coupling unit operations. CQAs or any other outputs from a unit procedure can then be used as further inputs to subsequent processing steps. Thus, a model for the entire production process can be obtained, quantitatively summarizing existing knowledge in alignment with the QbD framework. This will enable design space and normal operating range definition, as well as optimization, at the full flowsheet level. Furthermore, the present knowledge assessment showcases that analytical and modeling techniques could be rapidly deployed, adapted, or repurposed for RNA technology, making the development of such holistic digital process replica, or process digital twins, possible (Figure 3, Key figure). These could notably rely on the use of PAT measurements and initial process models to build data-driven or hybrid algorithms, which can rapidly predict process performance and detect anomalies [118]. In addition, the use of real-time adaptative process control will enable the digital process replica to meet Quality Target Product profiles within the normal operating region.

Toward a versatile platform technology

QbD could be a key enabler for the development of versatile and disease-agnostic production processes. In theory, adoption of the QbD framework offers the flexibility and quality assurance level to cope with the heterogeneities and manufacturing challenges of new RNA-based vaccines and therapeutics. We anticipate that QbD implementation will first allow industry to navigate confidently within the process design space. Process parameters can thereby be optimized and tune product attributes and structural or immunogenic characteristics for the chosen route of administration, disease, or organs while maintaining a high level of product quality assurance. Second, the

Key figure

Potential future RNA platform technology

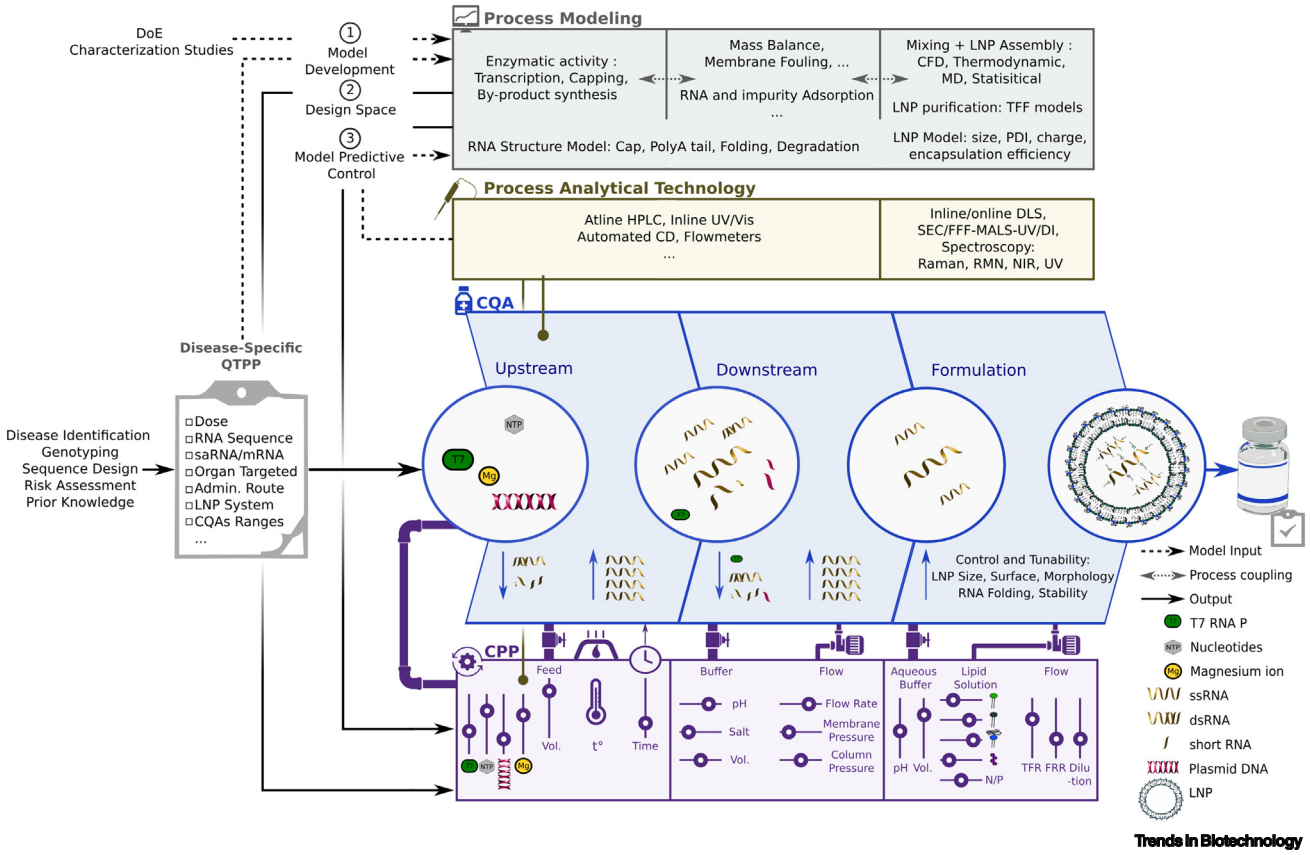

Figure 3. The proposed future disease-agnostic RNA platform relies on the use of a holistic Quality-by-Design (QbD) approach for product development, control strategy, and life-cycle management. An enhanced QbD approach will be crucial to reduce process variability and testing requirements and to enhance process capability, transferability, and understanding. Process modeling and analytical technologies could cover the entire manufacturing process and be applied for initial process design and model-based predictive control. Three major developmental paths can be distinguished. First, process modeling can help control the RNA-related impurity formation, with double-stranded RNA (dsRNA) identified as a priority, and streamline RNA purification by coupling *in vitro* transcription (IVT) and all the subsequent purification steps together. This model-based approach could also decrease the need for intensive purification methods, enable chronic administration of RNA, or fine-tune RNA-based product adjuvant-like properties. Second, lipid nanoparticle (LNP) optimization may be unlocked by increasing our fundamental understanding of LNP structure–activity relationships. Thus, new process designs that can rationally manipulate crucial LNP properties while maintaining the same level of quality assurance regarding other critical quality attributes (CQAs) would be essential to build this knowledge. Third, controlling RNA higher-order structure throughout the process and during encapsulation could play a key role in product thermal stability and future product development.

RNA length can vary greatly, depending on the protein of interest and the RNA technology used (saRNA or mRNA), affecting IVT process conditions, purification process operation, product stability, and RNA encapsulation within LNP. Additional differences include chemical modifications of nucleotides and types of lipids in the LNP system [119]. All of this could lead to a more dynamic concept of design space, with these product-specific characteristics as fundamental inputs to process design and control. Later, the RNA sequence and predicted secondary structure could also be integrated in this strategy. Third, the integration of model-based predictive control with PAT would add a final layer of control and assurance to ensure an optimal level of quality and performance, regardless of the RNA sequence. By enabling knowledge transfer and rational

product design and enhancing product knowledge, it would also promote and speed up the expansion of the RNA technology toward new therapeutic fields.

In parallel, the development of such knowledge-based approaches is likely to be a canonical requirement for the regulatory approval of a standardized, disease-agnostic manufacturing process. Within this framework, the process design, manufacturing, and control strategy could be developed and approved in a disease-agnostic manner so that validation may be restricted to limited studies and comparability checks for each new product. This platform 'pre-qualification' will greatly reduce approval timelines, thereby enabling rapid mass production and response. For instance, this could support pandemic preparedness and enable rapid sequence update to deal with the emergence of new variants. The current regulatory landscape, including regulatory submissions for COVID-19 vaccines, the guidelines for industry regarding severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) variants, and World Health Organization guidance on RNA prophylactic vaccines, holds great promise for significant knowledge transfer between products [28].

Finally, this framework, associated with a preapproved platform technology, would underpin the development of integrated and distributed manufacturing, potentially transforming current global manufacturing approaches. First, model-based process automation associated with PAT and potential real-time release testing can make technology transfer seamless and reduce facility requirements. In fact, this would be a requirement for continuous manufacturing, which would further reduce facility footprint and production costs [120,121]. Therefore, instead of a centralized manufacturing approach, numerous disease-agnostic, affordable, automated, and small RNA manufacturing platforms could be distributed where they are most needed [60]. Multiproduct facilities with shared development costs are anticipated to further enhance the economic viability of this approach [122]. Production can be rapidly repurposed to produce large quantities of needed products in emergencies, leading to a more coordinated and regionally focused response to future outbreak threats. In addition, the same platform can produce a wide range of RNA-based products for clinical trials and adapt to regional needs. Altogether, this could offer greater autonomy and better equity in vaccine and therapeutic access. The distribution of such versatile facilities would also foster innovation, facilitating future process development and clinical trial enrolment worldwide. Beyond vaccine products, this QbD framework could also support the deployment and approval of more affordable personalized medicine units.

Concluding remarks and future perspectives

The emergency development of SARS-CoV-2 vaccines has been a turning point for RNA technology, demonstrating that it is not just a promising technology but also an effective and safe one. Now, QbD principles can be placed at the center of a long-term development vision. This review outlined current knowledge on CQAs of RNA-based products, with product immunogenicity and instability being the main risks, as well as the intimate relationships between product and process. Existing assessments are sufficient to ensure high safety and efficacy profiles in COVID-19 RNA vaccines. The next task is to make these attributes more controllable or tunable and to assess their criticality and potential target as a function of the type of disease, organ targeted, or route of administration. To do so, the characterization of product and process interactions is central. The main relationships are highlighted herein, but further research will be required to confirm them. The present CQA–CPP mapping provides an initial roadmap for future experiments and **Design-of-Experiment (DoE)** implementation, with IVT characterization and innovation set as priorities.

No vaccines have yet been developed under a full and integrated QbD framework. However, the transferability of knowledge between products and the relative simplicity of the production process,

Outstanding questions

The recent growth of the RNA industry will enhance future research capacity and innovation, with new laboratories developing their own vaccines and filing patent applications. In the midterm to the long run, will this lead to harmonization or diversification of RNA-based products and manufacturing processes?

How will industrial collaborations, licensing partnerships, and the patent landscape evolve after the COVID-19 pandemic?

What will be the future regulatory framework for the RNA platform technology? Will it leave sufficient room for the 'pre-qualification' of a versatile platform technology? How easily would data and knowledge be shared between these future products to support validation?

Future innovations are likely to modify the current technological landscape. Will saRNA-based products demonstrate their potential in future clinical trials? What will be the requirements for switching from batch to continuous processing? Will PAT and digital tools be developed rapidly enough to enable industry transformation?

combined with our increased analytical and computational capabilities, make the implementation of such an approach an ambitious but realistic objective. This would be a key milestone toward the development of deployable, automatable, cost-effective, and transferable platform production processes. The application of this new QbD framework will also enhance product development by creating fundamental knowledge and expanding the potential therapeutic scope of RNA-based products. This path will certainly require a shift in research and asset allocations. To develop a robust and versatile platform, future studies should rapidly focus on a multiproduct approach to expand knowledge and design space characterization by integrating, for instance, RNA molecules of different size, structure, or chemical composition. Going to the extreme at the early stage of platform design will indicate the magnitude of future manufacturing and modeling challenges to better integrate product heterogeneities. In the same perspective, analytical methods should rapidly be developed and validated under a similar analytical QbD framework to support extended and seamless data transfer among diverse products and should later be integrated within a preapproved manufacturing platform [123] .

Finally, the future industrial cooperation and regulatory landscape is likely to determine the fate of the highlighted manufacturing platform (see Outstanding questions). The requirements and the scope of platform 'pre-approval' will also largely depend on these unknowns, and it will certainly be crucial to rapidly establish standardized guidelines and approaches. To conclude, the application of QbD principles to RNA manufacturing delivers a hopeful message for the development of a disruptive production platform technology, which requires rapid and coordinated efforts to become a concrete reality.

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Declaration of interests

The authors have no interests to declare.

Supplemental information

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Resources

[i]www.ema.europa.eu/en/medicines/human/EPAR/comirnaty

[ii]www.ema.europa.eu/en/medicines/human/EPAR/Spikevax

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