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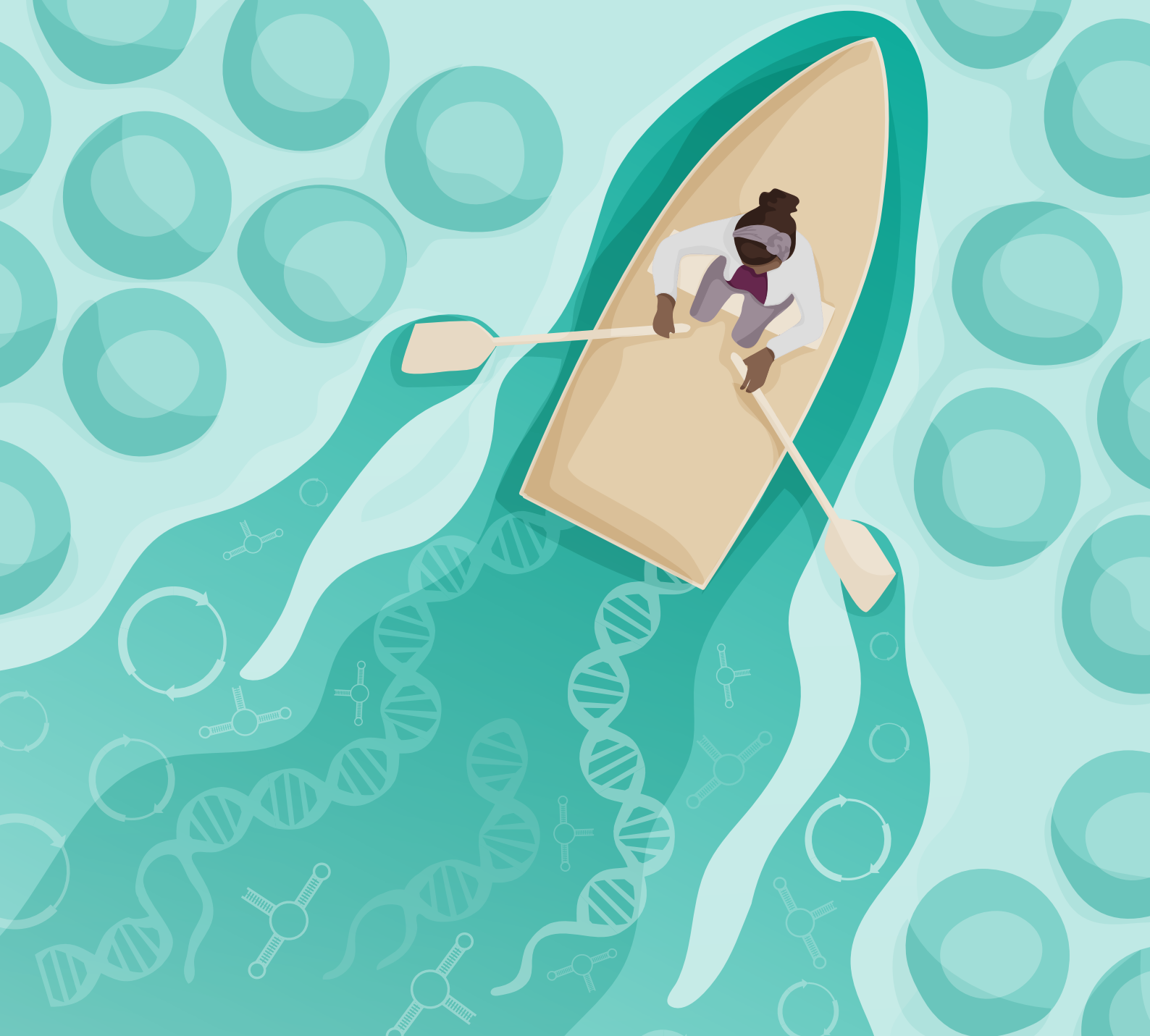
NUCLEIC ACID INSIGHTS

SPOTLIGHT
Upstream processing and analytics



NUCLEIC ACID INSIGHTS

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UPSTREAM PROCESSING AND ANALYTICS

SPOTLIGHT

REVIEW

Integrated continuous cGMP platform for end-to-end mRNA manufacturing

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mRNA has evolved from a niche research technology into a major therapeutic platform, with its impact clearly demonstrated during the COVID-19 pandemic. While mRNA vaccines achieved unprecedented development timelines and strong clinical outcomes, limitations in production capacity, supply chains, and batch scalability constrained global access as witnessed during the pandemic. As applications expand to personalized cancer vaccines, protein replacement therapies, and advanced immunomodulators, the need for agile and reliable manufacturing platforms is pressing. To address these limitations, a team of experts from academia and industry supported by regulatory specialists developed a fully integrated, end-to-end continuous platform for cGMP-compliant mRNA manufacturing, encompassing both a micro-factory (~25–50 mg/h) and a pilot plant (>1 g/h). The platform integrates mechanistic, data-driven, and hybrid models to optimize *in vitro* transcription, precipitation-based purification, tangential flow filtration, lipid nanoparticle formulation, and continuous lyophilization. Real-time monitoring of critical process parameters and quality attributes is incorporated throughout, enabling predictive process control and enhanced product consistency. By coupling modular, scalable operations with digital twin frameworks, the platform reduces reagent consumption, accelerates development timelines, and strengthens supply-chain resilience. Ultimately, this proof-of-concept work demonstrates that continuous, end-to-end mRNA manufacturing is feasible and can deliver high-quality therapeutics at flexible scales, providing a generalizable framework to expand global access to mRNA-based medicines and support rapid responses to emerging health challenges.

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mRNA has emerged as a powerful therapeutic modality in modern medicine. Its potential became particularly evident during the COVID-19 pandemic, when mRNA vaccines enabled the rapid development of effective disease protection [1,2]. Whereas traditional vaccine development typically requires 6 months to several

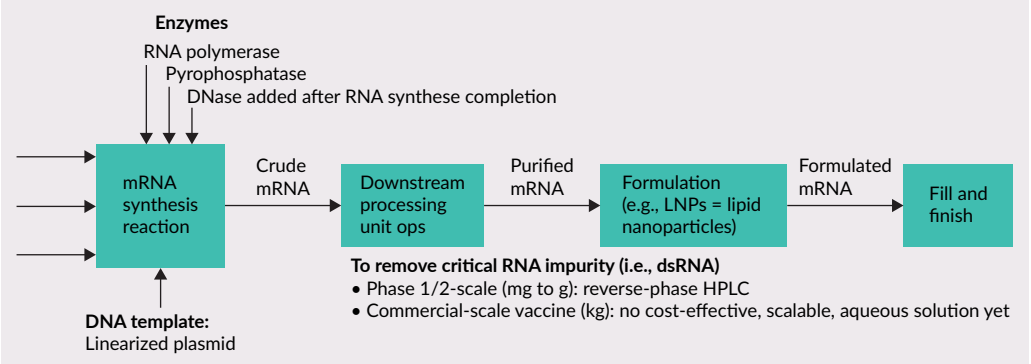
years, mRNA-based platforms can generate clinical-grade material within weeks [3]. Although scale-up and regulatory approval may extend the overall timeline. Beyond infectious diseases, the modular design of mRNA supports a broad and expanding range of applications, including rapid responses to emerging pathogens, personalized cancer vaccines, protein replacement therapies, and gene-editing strategies. These advances position mRNA as a promising platform for future pharmaceutical therapies [4,5].

The rapid growth of mRNA therapeutics in recent years has revealed critical limitations in existing manufacturing approaches [3,6]. As mRNA technologies expand beyond vaccines into a broad range of therapeutic applications, the need for production platforms that are flexible, reliable, and scalable has become increasingly urgent. Despite this growing demand, mRNA manufacturing remains largely dependent on centralized, batch-based processes [5]. Each batch is handled independently, constraining throughput and process flexibility while causing variability in product quality [5]. These limitations result in inefficient use of expensive raw materials, increased operational costs, and the formation of production bottlenecks. All these challenges become particularly acute during periods of high demand.

The mRNA manufacturing begins with an *in vitro* transcription (IVT) reaction, in which a linearized DNA template, typically derived from plasmids, is transcribed by RNA polymerases such as T7 to assemble nucleotide triphosphates (NTPs) into the target RNA sequence (Figure 1). During or following transcription, the mRNA is capped either co-transcriptionally, using cap analogs, or post-transcriptionally through enzymatic methods to ensure transcript stability and efficient translation. This step is inherently expensive: both NTPs and cap analogs require complex, multistep chemical synthesis involving challenging phosphorylation reactions and must be supplied in molar excess to achieve high capping efficiency and RNA quality. In addition to its cost, the IVT reaction generates a complex mixture containing residual nucleotides, enzymes, DNA templates, and byproducts such as double-stranded RNA. These impurities must be removed during downstream purification (Figure 1), adding further complexity and cost to the process. Consequently, the IVT step represents one of the most resource-intensive stages of mRNA manufacturing and a major driver of overall production expense. Downstream processing typically involves multiple unit operations, including filtration and chromatography steps such as size-exclusion, affinity, ion-exchange, and

FIGURE 1

High-level mRNA synthesis, separation, and formulation process.



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ion-pair reverse-phase chromatography. While significant advances have been made, a fully standardized and broadly scalable purification strategy has yet to emerge, leading to substantial variability in process design across manufacturers and products. Following purification, the mRNA is encapsulated into lipid nanoparticles (LNPs), which both protect the RNA from enzymatic degradation and enable efficient cellular delivery. The final stages of manufacturing involve fill-and-finish operations to ensure long-term stability for an acceptable shelf life of the formulated product **(Figure 1)**.

Beyond process-intrinsic challenges, mRNA manufacturing is further constrained by external factors, including limited availability of GMP-grade reagents, reliance on patent-protected technologies, and slowly evolving regulatory frameworks that slow the technology adoption and complicate global deployment. Moreover, most current mRNA formulations require ultra-cold storage (−20 to −80 °C), imposing significant logistical and infrastructure burdens, particularly in resource-limited settings. The COVID-19 pandemic starkly exposed the consequences of these limitations. As global demand for mRNA vaccines surged, the production capacity was pushed to its limits, the batch-based operations proved inflexible, and the fragile supply chains hindered timely and equitable access to life-saving therapies. These experiences made clear that existing manufacturing paradigms are not only costly and resource-intensive but also insufficient to meet the scale, speed, and adaptability required for global healthcare. All these challenges underscore the need for a paradigm shift in mRNA production. The development of flexible, scalable, and cost-efficient manufacturing platforms is essential to reduce reagent consumption and operational costs, improve reproducibility, strengthen supply-chain resilience, and enable equitable access to mRNA therapeutics worldwide. Addressing these limitations is critical to fully realizing the promise of mRNA technology and ensuring its rapid and effective deployment in response to both emerging health threats and long-term medical needs. In general, incremental improvements to individual unit operations needed for transcription, purification, formulation, and fill-finish will not be sufficient to overcome the structural limitations of today's batch-based workflows.

The modular and comparatively simple architecture of mRNA production makes it particularly well suited for continuous processing, enabling the adaptation of proven strategies from small-molecule and conventional biologics manufacturing while supporting faster, more reliable, and economically sustainable production. Despite these advantages, continuous processing places stringent demands on the precise control of key process parameters, making advanced strategies for real-time monitoring and regulation essential. Building on lessons learned from continuous manufacturing in other sectors, this work outlines a roadmap for translating these principles to mRNA production. Central elements include: (i) tight integration of upstream mRNA synthesis with downstream isolation and purification, (ii) implementation of real-time monitoring of critical process parameters and quality attributes, and (iii) application of quality-by-design principles to ensure robust and reproducible performance. These advanced pharmaceutical manufacturing efforts are further supported by the development of mechanistic, data-driven, and hybrid models to enable deep process understanding, capture workflow dynamics, and ultimately support model-driven process control [7].

This contribution describes an ongoing initiative that established a fully integrated, continuous platform for cGMP-compliant mRNA manufacturing in collaboration with Recipharm, encompassing both a micro-factory (25–50 mg/h) and a pilot

plant (>1 g/h) [8]. The project combined academic research, industrial expertise, and regulatory guidance to create a generalizable framework that is robust, scalable, and cost-efficient. Key achievements include the construction of an operational cGMP facility and the development of a regulatory submission framework to support future commercial translation (Figure 2).

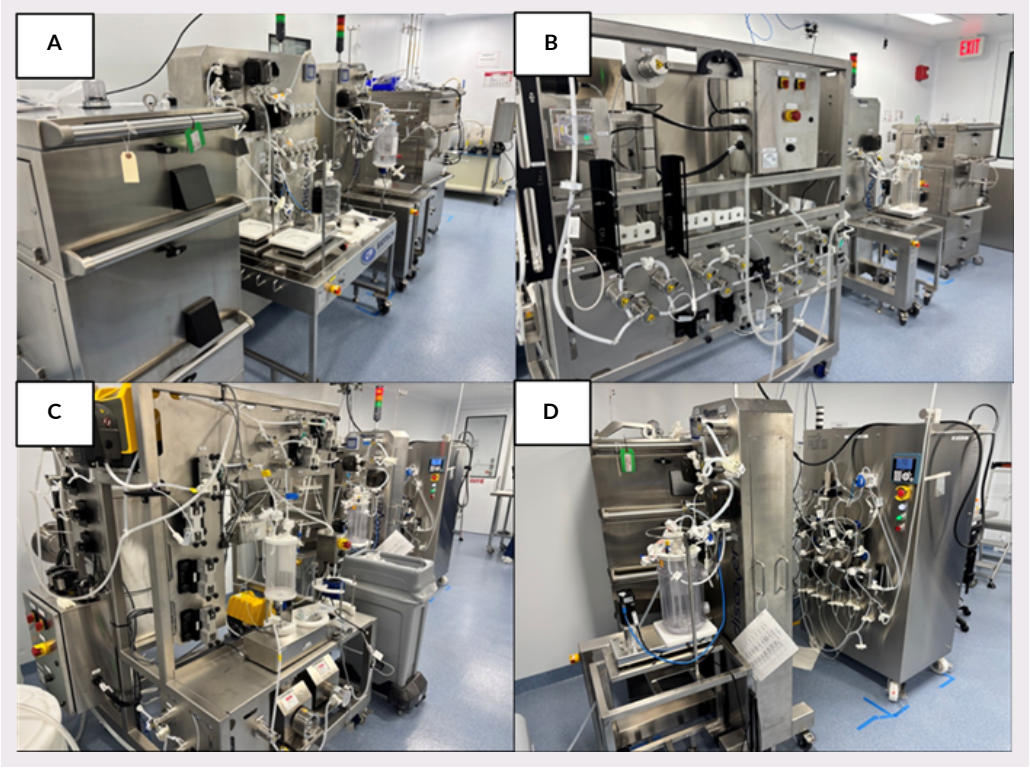
The initiative addresses critical gaps in current mRNA manufacturing, redefining production workflows, accelerating development timelines, strengthening supply chain resilience, and expanding global access to mRNA therapeutics. The following sections will detail the mRNA manufacturing processes from IVT synthesis to fill-and-finish leading to the cGMP facility (Figure 2), while highlighting innovations across the entire production chain. Integrated process monitoring and control strategies have been incorporated to enhance efficiency, ensure product quality, and enable scalable, end-to-end continuous mRNA manufacturing. Herein, the reported process sections reflect individually optimized unit operations developed stepwise towards the implementation of a continuous manufacturing platform.

mRNA SYNTHESIS REACTION

IVT offers a flexible, cell-free method for generating mRNA, allowing rapid

FIGURE 2

cGMP-compliant mRNA manufacturing facility from Recipharm.



(A) IVTR and PIVT skids, shown installed with the associated PAT Box skids post-commissioning, used for real-time monitoring and control of transcription reactions. **(B)** Chromatography skid, employed for purification of mRNA intermediates. **(C)** Drug substance TFF skid, used for tangential flow filtration and buffer exchange of the purified mRNA. **(D)** LNP formulation skid, for encapsulation of mRNA into lipid nanoparticles suitable for drug substance production. DSTFF: drug substance TFF; IVTR: *in vitro* transcription reactor; LNP: lipid nanoparticle; PIVT: post-IVT; PAT: process analytical technology.

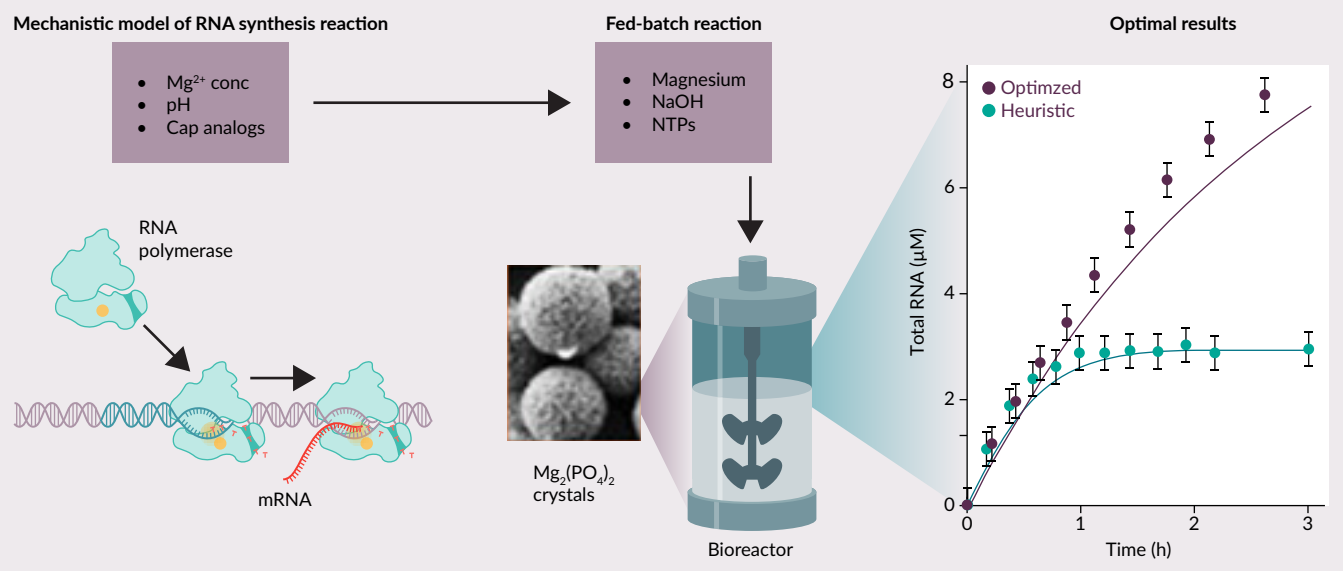
production of many different sequences directly from a DNA template. Because the reaction occurs outside of living cells, scaling the process is straightforward. Researchers can adjust reagent concentrations, reaction duration, or reactor format without needing to manage complex cellular growth requirements. This modularity makes it easy to switch between mRNA products simply by providing a new DNA template, supporting diverse applications such as vaccines, gene-editing tools, and protein-replacement therapies. The IVT reaction follows the classical steps of initiation, elongation, and termination. During initiation, RNA polymerase recognizes and binds the promoter region of the linearized DNA. The process advances along the DNA template, incorporating NTPs to synthesize a complementary RNA strand, and ultimately releases a single-stranded mRNA upon reaching the termination sequence. Magnesium ions are essential cofactors throughout this process. However, the inorganic pyrophosphate generated during nucleotide addition can bind to magnesium and DNA to form insoluble complexes that sequester the template and impede polymerase activity. To prevent these undesired complexes, pyrophosphatase is routinely added to degrade inorganic pyrophosphate, maintaining magnesium availability and supporting continuous RNA synthesis.

To better understand these molecular events, mechanistic models were constructed to describe both the initiation and elongation phases of IVT [9,10]. These models help rationalize experimental observations that were previously difficult to interpret. For example, reduced RNA yields can result from crystallization of magnesium–pyrophosphate–DNA complexes, which limits template availability for transcription and ultimately decreases RNA production [11,12]. The models also aid explaining the formation of double-stranded RNA impurities under certain conditions and provide a quantitative framework for optimizing reaction variables.

In addition, the mechanistic models have clarified key factors that influence IVT efficiency, particularly in relation

FIGURE 3

Schematic illustration of model-based optimization of fed-batch *in vitro* transcription.



Using a mathematical model of enzyme kinetics and solution thermodynamics for the IVT reaction, optimized fed-batch strategies are developed to reduce the resource costs of mRNA synthesis while maintaining critical quality attributes for *in vivo* efficacy. Adapted from [13].

to capping. Because co-transcriptional capping competes directly with the natural NTPs for incorporation at the 5′ end, high cap analog concentrations are traditionally required to ensure that the cap is incorporated preferentially over a standard nucleotide. This competition makes capping one of the most reagent-intensive, and costly, steps in conventional batch IVT. Model-guided process design has shown that fed-batch operation can address this challenge **(Figure 3)** [13]. By gradually feeding NTPs into the reaction, the relative concentration of cap analog remains higher at the moment of initiation, improving its likelihood of incorporation without requiring excessive amounts. This strategy preserves RNA yield and cap quality while substantially lowering cap analog consumption, making fed-batch a more economical and scalable alternative to traditional batch processes. Building on the success of fed-batch operation, further efforts are currently directed toward the development and implementation of fully continuous IVT processes. These same mechanistic insights have advanced the development of continuous IVT processes, where maintaining productivity and reducing off-specification species are major priorities. The models help explain improvements observed with pyrophosphatase addition and increasingly serve as the foundation for digital-twin systems designed to predict and optimize upstream IVT behavior in real time.

DOWNSTREAM

After the IVT, the mRNA undergoes a series of downstream purification steps to achieve clinical-grade purity by combining typically chromatographic methods with membrane-based filtrations [14]. Chromatography, including size-exclusion, anion exchange, and affinity capture, remains today the most commercially established strategy. In particular, affinity capture using oligo(dT) resins is highly effective, selectively binding the poly(A) tail of mRNA to remove most of the impurities. However, this traditional approach is limited by high resin cost, low binding capacity, mass transfer limitation and high sensitivity to buffer conditions [15,16]. Tangential flow filtration (TFF) complements chromatography by enabling both solution concentration and buffer exchange [17]. The TFF performance depends on various factors such as transmembrane pressure, membrane molecular weight cutoff, and fouling [17]. In addition, these parameters are driven by mRNA adsorption to the membrane, representing a significant limitation to achieve extended operations or even continuous processing. To address these shortcomings, single-pass TFF has emerged as an attractive alternative, eliminating recirculation and enabling steady-state operation, which reduces process times and can help minimize product degradation [18,19]. However, it requires larger membrane areas and careful control of concentration polarization to maintain optimal performance. In addition, mRNA requires pretreatment to ensure efficient binding to chromatography resins and maintain adequate solubility. The pretreatment typically entails a combination of elevated temperature treatment followed by buffer exchange to prevent blockages in the chromatographic systems and filtration units [20,21]. Yet, repeated buffer exchanges and exposure to elevated temperatures can compromise mRNA stability and make continuous purification processes difficult to implement.

To address these shortcomings, precipitation-based purification has emerged as a practical alternative, offering a route to overcome many of the constraints of chromatographic and membrane-based methods, particularly for continuous operation [22,23]. To date, precipitation has been widely demonstrated for mRNA isolation

and purification at the laboratory scale, but its potential for large-scale applications remains largely unexplored. Recent studies have shown that combining polyethylene glycol (PEG) with sodium chloride (NaCl) can selectively precipitate mRNA directly from crude IVT mixtures, efficiently removing enzymes, unincorporated nucleotides, template DNA, and dsRNA (double-stranded RNA) contaminants [24]. Systematic optimization of precipitation conditions, including PEG concentration and the type and concentration of salts, has enabled recovery yields of 80–93% and purities of approximately 80–83%, while preserving mRNA integrity and biological activity in cell transfection assays [24]. Building on prior work reporting precipitation of conventional biotherapeutics such as monoclonal antibodies [25,26], mRNA precipitation was adapted and implemented in a fully continuous purification process in this project. The workflow begins with optimized mRNA precipitation using PEG 6000 and NaCl in a tubular reactor equipped with static mixers with a 20 min residence time. The precipitated mRNA then passes through two sequential continuous TFF steps for washing and buffer exchange, before being redissolved in a formulation-ready buffer at the desired concentration for direct lipid nanoparticle encapsulation. At the benchtop scale (~0.006–0.15 g/h), the system delivers mRNA with over 95% purity, 92% overall yield, and 97% full-length sequences, while keeping dsRNA below 0.01%. When scaled to industrial levels, the platform can produce ~1.5–2.4 g/h, maintaining the laboratory proven high-quality standards while outperforming conventional chromatography methods (Table 1) [8].

Additionally, the process is monitored in real time, with turbidity and UV absorbance sensors to ensure efficient mRNA precipitation and to adjust the final mRNA and buffer concentration, respectively, to aid optimal LNP formulation thereafter. Compared with conventional chromatography and TFF strategies, this mRNA capturing by precipitation provides higher yields and purities, while offering improved process robustness and potential for integration into current RNA workflows (Figure 4). The purified mRNA can be seamlessly integrated from the IVT reaction and directly formulated into LNPs without additional conditioning (Table 2). Thus, the precipitation-based platform represents a scalable and flexible alternative to chromatography, suitable for incorporation into end-to-end continuous mRNA manufacturing (Figure 4).

TABLE 1

Comparison of chromatography- and precipitation-based mRNA purification, and suggested quality attributes and process parameters for mRNA-based products [24].

Quality attribute/ Process parameter	Suggested acceptance criteria	Chromatography	Precipitation
Recovery yield	97–70%	n.d.	92%
Purity	–	93%	95%
Fragment purity	90%	93%	97%
Residual enzymes	<5.0 µg/mL	1.96 ug/mL†	b.d.l.
dsRNA content	<1%	<1% *	0.016%

*Reported by the industrial partner and determined using dot blot analysis. †Values were measured using the NanoOrange assay. ‡Values were obtained by qPCR. b.d.l.: below detection limit; n.d.: not determined.

Further efforts beyond developing a fully continuous precipitation–TFF platform were directed toward accelerating the process development and ensuring scalability for such a system. Despite being advantageous compared to chromatography as detailed above, the precipitation development still remains relatively time-consuming, material-intensive, and costly. This leaves room for optimization, important particularly given the high value of mRNA. To this end, a predictive thermodynamic model was established to estimate mRNA solubility as a function of sequence under varying conditions, including temperature, precipitating agents, and concentration [27]. In parallel, a high-throughput image analysis platform was developed to systematically characterize precipitate morphology, including particle size, shape, and volume. Filterability of precipitates is highly dependent on salt composition, as different salts and ionic strengths modulate nucleation and particle growth pathways, leading to distinct particulate morphologies that directly influence filtration performance. Leveraging this relationship, the platform guided the rational selection of salts and additives to improve both precipitation efficiency and downstream filtration steps. This integrated approach enabled rapid screening of up to 96 conditions in seconds without performing actual filtration experiments, reducing material consumption by 95 % and supporting a robust, continuous mRNA purification process [28].

FORMULATION: LNPs FORMULATION

After purification, mRNA is encapsulated into LNPs, generating the final drug product while ensuring transcript stability and efficient cellular uptake. LNPs are typically 50–150 nm vesicles composed of an ionizable lipid that complexes with mRNA and promotes endosomal escape, helper lipids such as phospholipids and cholesterol that provide structural stability, and PEG-lipids that reduce aggregation [29,30]. The formation of LNPs occurs by rapidly mixing the aqueous mRNA solution with an ethanolic lipid mixture, a polarity shift that drives self-assembly into nanoparticles [31]. To achieve consistent particle size and uniformity, microfluidic devices such as T-junctions or impinging-jet mixers are employed, providing the rapid and controlled mixing required for reproducible LNP production [31]. Critical process parameters, including the flow rate ratio between the aqueous and lipid phases, the total flow rate, lipid composition, and buffer conditions, are carefully optimized to ensure reproducible nanoparticle formation [32]. This is essential since the quality

▶ TABLE 2

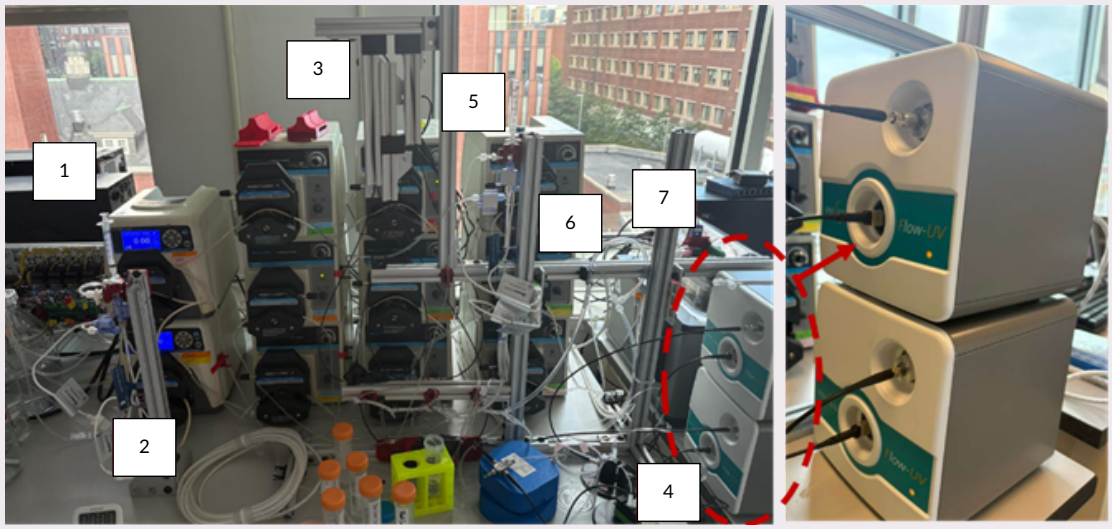
The lyophilization stage is not included in this table, as experimental scale-up data are not yet available.

Operation	*In vitro* reaction		Continuous precipitation		LNP formulation	
Scale	Laboratory	Industrial	Laboratory	Industrial	Laboratory	Industrial
Flow (mL/min)	0.1–0.5	5	0.5–2	5	20–50	200–500
Concentration mRNA (g/L)	1–5	5–8	1–3.5	5–8	100–50 µg/L	100–50 µg/L
Cross sectional area – tubular reactor (mm^2)	–	–	3.20	10	–	–
Filter membrane (cm^2)	–	–	13	~170–350	–	–

Scale up and integration scenario from laboratory to full industrial scale for *in vitro* reaction, continuous precipitation, and LNP formulation.
LNP: lipid nanoparticle.

FIGURE 4

Modular continuous precipitation–TFF platform for mRNA purification.



1. In house process control
2. Tubular reactor for precipitation
3. Precipitation PAT–OD sensor
4. Redissolution PAT–UV sensor
5. Hollow fiber module for microfiltration
6. Water bath for redissolution
7. Temperature control

The fully automated setup enables continuous operation with integrated PAT for real-time monitoring and control of critical process parameters and product quality attributes. PAT: process analytical technology; TFF: tangential flow filtration.

of the LNP will play a major role for the therapeutic potency of the mRNA drug [31]. Key parameters include 1) particle size for proper biodistribution, 2) mRNA encapsulation efficiency to reduce transcript loss, and 3) a stable composition for long-term storage. Studies have shown that suboptimal mixing conditions generate larger, polydisperse particles with reduced encapsulation [33,34]. In contrast operating within the optimal regime yields ~100 nm LNPs with nearly complete mRNA loading (encapsulation efficiency >80%) and low polydispersity (PDI < 0.3) and across the explored operating conditions, demonstrating the importance of a particle engineering approach for the control over the formulation conditions [35]. Following the mixing, the organic solvent, typically ethanol is removed, and the LNP dispersion is exchanged into the formulation buffer, a step commonly achieved through TFF [36].

Generally, the assembly of LNPs is a highly dynamic process, driven by rapid nucleation and growth of lipid structures as the aqueous and lipid phases mix. Capturing the mechanistic details of this self-assembly is challenging, given its multiscale nature, with molecular interactions occurring on the millisecond timescale to form nanoscale particles. LNP self-assembly starts with the formation of small lipid–mRNA clusters, which expands as additional molecules join. During this process, particles may combine to form larger structures or fragment into smaller ones, dynamically determining the final size and distribution of the nanoparticles. To better understand and predict the impact of process parameters on LNP formation, a population balance model (PBM) has been developed, linking the dynamics of nucleation, growth, aggregation, and breakage to the resulting particle size distribution. Therefore, insights from the PBM were leveraged to develop strategies for controlling LNP manufacturing, allowing precise modulation of particle size, encapsulation efficiency, and stability [37]. Such strategies include multi parameter optimization of lipid composition, comprising ionizable lipids, phospholipids, cholesterol, and PEGylated lipids, controlled mixing techniques, and environmental parameters like pH and ionic strength, as

well as post-assembly purification methods. Together, these approaches provide a rational framework for reproducible and scalable production of LNPs with tailored physicochemical and therapeutic properties.

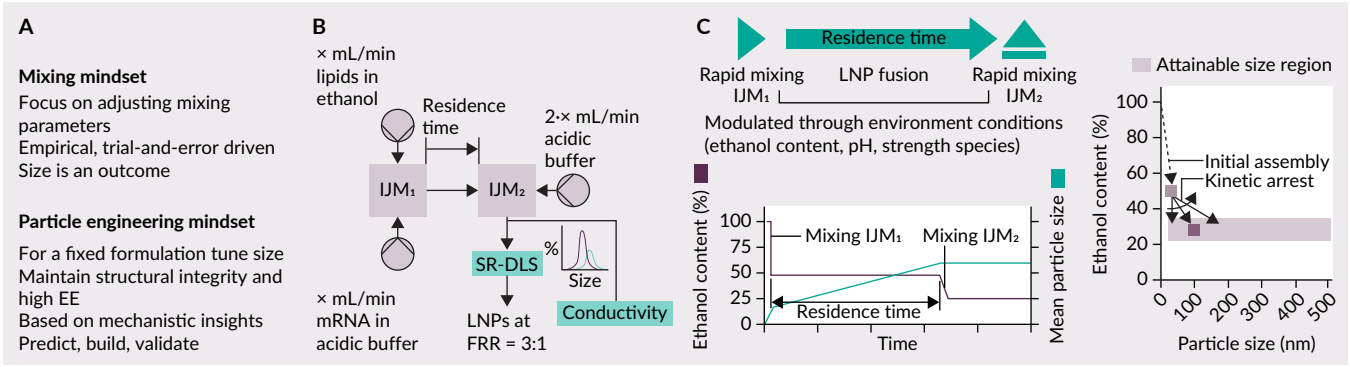
To date, developing LNP manufacturing processes for drug delivery is resource- and time-intensive because the development relies on steady-state sampling and offline particle characterization. To overcome these limitations, an automated platform has been developed in the project that integrates an impinging jet mixer for nanoparticle synthesis with spatially resolved dynamic light scattering for real-time particle monitoring (Figure 5) [38]. Synchronized control software, a structured database, and a graphical user interface enable seamless operation and data management, while Bayesian optimization and automated Design of Experiments accelerate process exploration. The proof-of-concept platform using model LNP formulations demonstrated the feasibility to rapidly identify critical process parameters, enhanced process efficiency, reproducibility, and scalability, while minimizing material consumption and supporting robust drug delivery process development [38].

CONTINUOUS LYOPHILIZATION

After encapsulation, the product can be subjected to lyophilization to improve long-term stability and shelf-life. Lyophilization, or freeze-drying, is a preservation process in which a solution is first frozen and then dried by removing ice through sublimation under reduced pressure. The ability to stabilize biologics that are notoriously unstable in solutions makes lyophilization particularly relevant for emerging therapeutics. For instance, maintaining integrity during storage and transport is a major challenge for mRNA vaccines. Both the mRNA and lipid components in LNPs are prone to degradation in the typical aqueous formulations. Consequently, ultra-cold storage condition (−20 to −80 °C) are required, contributing to the limited shelf-life and complex supply-chain requirements of the mRNA vaccines. In contrast, lyophilized mRNA–LNP formulations have been shown to remain stable for extended periods at refrigerated conditions (2–8 °C), and in some cases even at room temperature, substantially alleviating storage, transport, and handling requirements [39]. In practice, lyophilization is typically performed using batch-operated processes,

FIGURE 5

Particle size–control strategy.



(A) Shifting from a traditional mixing-focused approach to a particle-engineering perspective aimed at precise size regulation. **(B)** Experimental setup used to implement the size-control method; and **(C)** mechanistic principle: symmetric mixing (FRR = 1:1) leverages rapid lipid fusion and solution sensitivity, followed by kinetic arrest at a defined residence time to lower ethanol content and lock nanoparticles at the target size. Adapted from [35].

where vials are frozen and subsequently dried under tightly controlled conditions. Cryoprotectants or lyoprotectants are often included to preserve the structural integrity of LNPs during freezing and drying. Despite these precautions, today's common batch operated lyophilization processes remain highly sensitive to operational parameters and can exhibit significant batch-to-batch variabilities [40,41]. Critical factors such as shelf temperature and chamber pressure govern the sublimation rate, while vials positioned at the periphery of the lyophilizer chamber are prone to non-uniform heat transfer [42]. This 'edge-vial effect' can lead to fluctuating drying rates, variations in residual moisture, and differences in cake morphology, potentially compromising product consistency and quality [42]. This issue becomes even more critical in industrial settings, where larger batches of vials (often in the tens to hundreds of thousands per lyophilization run) must be processed compared to laboratory settings, leading to product quality concerns, which restricts the supply chain.

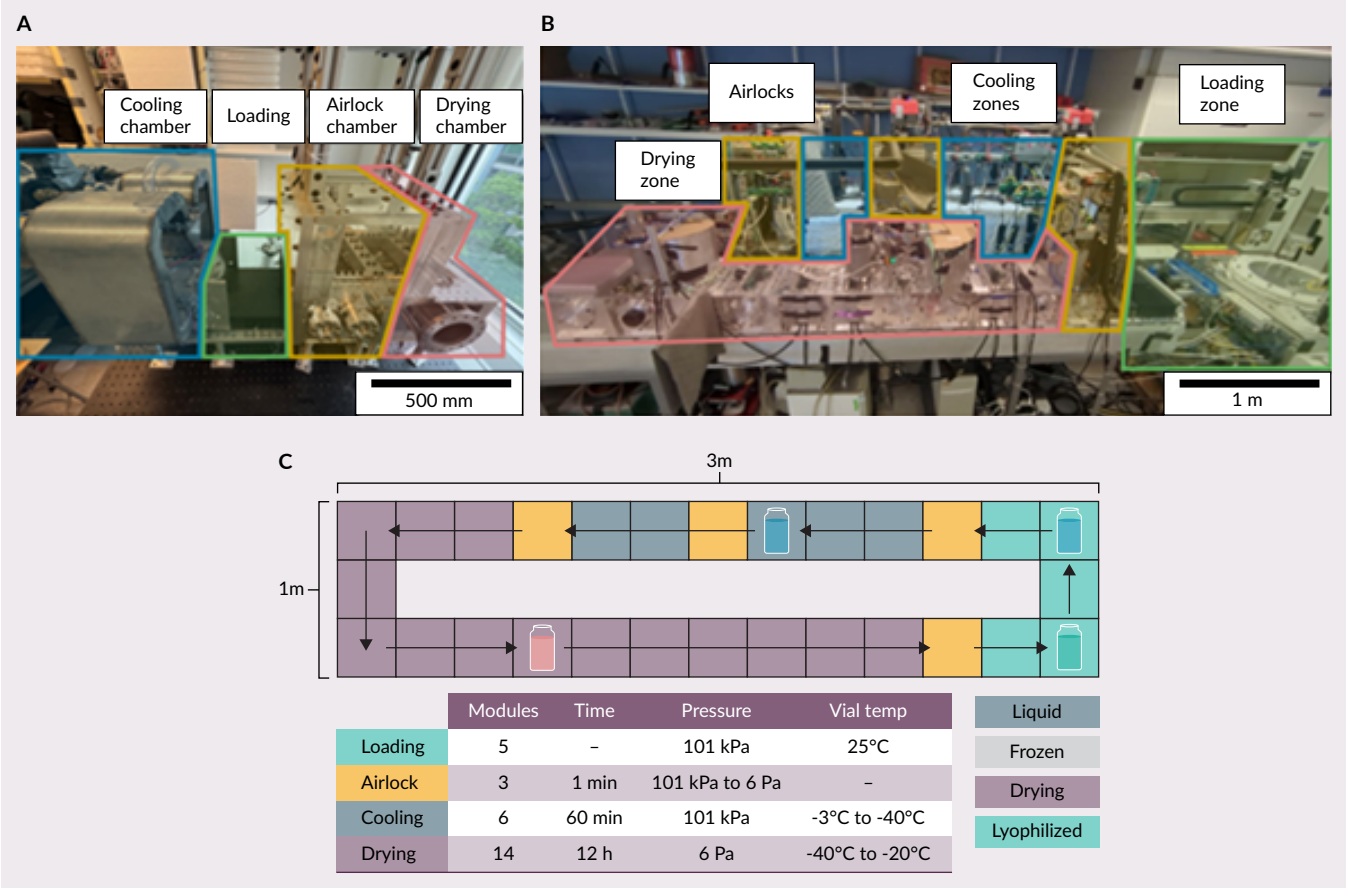
To overcome these limitations of traditional batch lyophilization, a new modular continuous platform was developed in house (Figures 6A and 6B) [43]. The system separates freezing, sublimation, and drying into specially and timely distinct stages (Figure 6), allowing vials to move continuously through each step under tightly controlled process conditions, which improves uniformity and consistency [43,44]. Briefly, an in-line weighing module provides real-time monitoring of residual moisture with a precision of up to 5 mg, thus, about 3% in a typical 150 mg dose, enabling tighter control of product dryness without interrupting the process cycle [44,45]. A rapid nucleation step by thermal-shock induces ice formation at the vial walls rather than at the free surface of the liquid, potentially accelerating sublimation and enhancing drying efficiency. Moreover, the platform is compatible with current pharmaceutical manufacturing workflows and scalable, from laboratory (e.g., lab and pilot lyophilizers with shelf areas of ~0.1–5 m^2 and batch capacities of up to ~100–150 kg) to full production size systems (industrial lyophilizers with shelf areas of ~10 m^2–40 m^2 and batch capacities of hundreds of kilograms) [43]. In addition, a comparison of lyophilization timescales for a 3 mL 10 wt% mannitol solution further highlights the efficiency of the continuous system, which reduces total process time to ~16 h compared with ~20.5 h at laboratory batch scale and ~35 h at pilot/industrial batch scale. The successful proof-of-concept using a prototype built at MIT demonstrates improved throughput, operational flexibility, and product quality compared with conventional batch freeze-drying systems (Figure 6). This comparison of the continuous with the standard batch lyophilizer highlights the potential for a next-generation lyophilization platform for biologics. Overall, the platform could be integrated into the advanced mRNA manufacturing workflows detailed in the sections above, potentially enabling scalability from laboratory experiments to full-scale end-to-end production settings by continuous operation (Figure 1) [43].

DIGITAL TWINS

The complexity of continuous biomanufacturing, particularly for novel therapeutics such as mRNA vaccines, demands robust strategies to ensure consistent product quality and efficient processes. While continuous operations offer significant advantages over traditional batch processes, they also introduce new challenges, including the need for real-time monitoring, currently an underexplored area [46–48]. The latter is essential to ensure precise control of critical process parameters (e.g. particle size, concentration monitoring, etc.). Digital twins, virtual representations of physical systems

FIGURE 6

Laboratory-scale system used to validate hardware components.



(B) Overview of the continuous lyophilization system with key process zones highlighted. **(C)** Schematic view of the continuous lyophilization system with typical operating conditions. Adapted from [43].

built on mechanistic and data-driven models, offer a flexible and powerful framework for predicting process behavior, guiding decision-making, and optimizing performance. By integrating experimental, historical, or real-time data, often provided by PAT tools, they enable early-stage process optimization, predictive control, and the ensuring of critical quality attributes, while supporting the transition from lab-scale development to industrial production. Integrating digital twins within QbD frameworks further enhances flexibility, efficiency, and reliability in the manufacturing of mRNA therapeutics. Despite their potential, the adoption of digital twins in biopharmaceutical manufacturing, in general, remains limited [46]. To date, only few studies have explored end-to-end implementations for biotherapeutic processes [23,46,49–51], leaving a significant gap that represents both a challenge and an opportunity for innovation in data-driven, robust, and scalable production (advanced pharmaceutical manufacturing) [7]. Within the scope of this project, a platform for continuous, end-to-end cGMP mRNA manufacturing was established with the digital twins for precipitation currently under development to complete the workflow (Figure 7). The platform integrates multiple mechanistic models into a modular software architecture that supports plug-and-play functionality and includes a graphical user interface for designing, running, and visualizing the complete mRNA

production pipeline without the need for programming [14]. The framework currently integrates all major unit operations, IVT, TFF, continuous chromatography, LNP formulation, and lyophilization, enabling flexible end-to-end simulation and optimization of the manufacturing process [14]. To further enhance process understanding, Morris-based global sensitivity analysis was applied to each unit operation, identifying critical input factors and guiding both process optimization and model validation. Because the study is a proof-of-concept, the digital twins are utilized for simulations. These analyses provide predictive identification of key optimization variables and operating regions to guide future experimental implementation. By combining these capabilities, the platform accelerates process development, reinforces QbD principles, and lays the groundwork for future closed-loop control and operator training. Overall, this work demonstrates how digital twins can be utilized for continuous mRNA manufacturing to support fully integrated, data-driven production with improved scalability, reliability, and responsiveness to global healthcare demands.

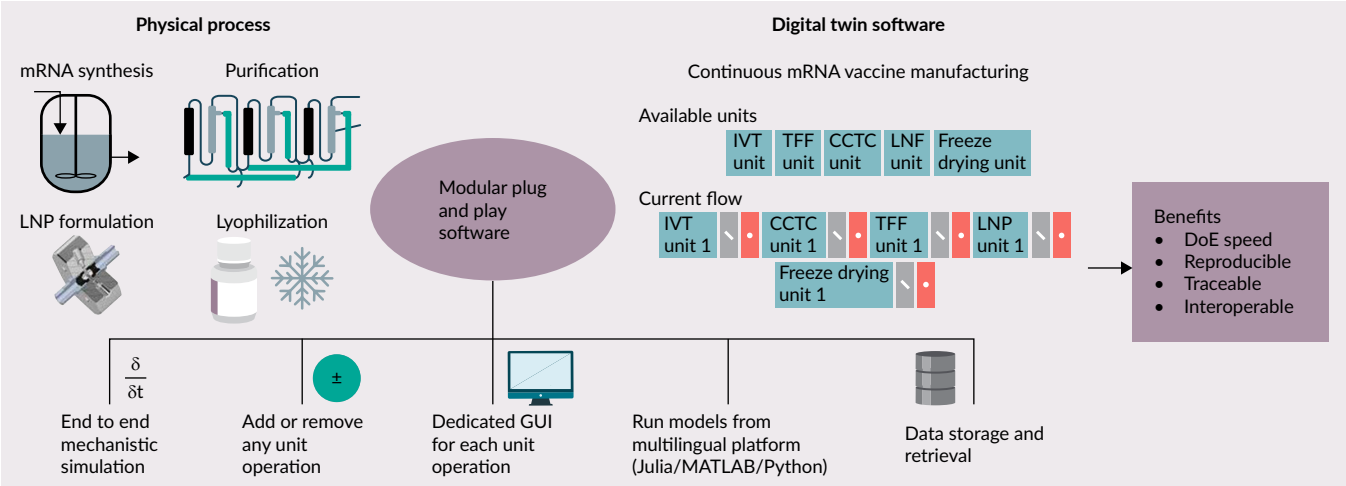
TRANSLATIONAL INSIGHTS

The mRNA field is rapidly emerging as a transformative modality in therapeutics, offering unprecedented potential for vaccines and next generation of medicines. However, realizing the full promise of mRNA manufacturing requires overcoming significant technical and economic barriers across the entire production workflow, including the IVT reaction. While fed-batch operation improves reagent use, strategies such as enzyme or template immobilization and new purification approaches remain underexplored and require systematic evaluation for continuous, high-productivity manufacturing. While demonstrated using representative constructs, future work will evaluate performance across a wider range of transcript sizes to further demonstrate platform versatility for diverse vaccine and therapeutic applications.

Supported by the needed mechanistic models, such processes can be guided by a predictive framework that explains how reaction conditions, catalyst concentrations, and process dynamics influence byproduct generation. Yet, the practical deployment of model-guided continuous

FIGURE 7

Digital twin framework for continuous mRNA vaccine manufacturing.



Adapted from [14].

mRNA manufacturing remains a challenge due to the limited availability of suitable PATs for real-time monitoring of, e.g., the IVT reaction. This need highlights future research opportunities aimed at enabling advanced pharmaceutical manufacturing for mRNA. Integrating the PAT recorded data into models within digital twin frameworks will minimize waste, enhance enzyme and template utilization, and increase overall process efficiency, supporting the broader therapeutic deployment and equitable access.

To make continuous mRNA manufacturing robust, commercially viable, and globally accessible strengthening the supply networks is essential. To date large-scale mRNA commercialization remains vulnerable to fragile supply chains for the specialized raw materials, exposing production to volatility, delays, and disruptions. Addressing these constraints requires flexible manufacturing, with modular and decentralized production enabling localized, resilient supply and improved equitable access. At the same time, sustainability pressures, including energy-intensive cold-chain logistics and extensive reliance on single-use consumables, increase both environmental impact and cost.

Alongside technological and infrastructural developments, regulatory frameworks for continuous and advanced mRNA manufacturing also need to evolve further. Entities such as the FDA and ICH (International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use) need to continue to establish guidance for industry to define expectations for implementation, validation, and lifecycle management of such continuous production systems. While these efforts are intended to support innovation and strengthen supply reliability, the transition from traditional batch manufacturing to continuous or distributed models requires not only extensive validation, compliance with cGMP standards, and coordination across international regulatory systems but also a change in mindsets within the industry as witnessed for small pharmaceutical molecules. As a result, commercial deployment of continuous and advanced mRNA manufacturing technologies is expected to proceed progressively.

CONCLUSIONS

This project represents a paradigm shift in mRNA therapeutics, bridging cutting-edge research with practical, industry-ready applications. Conventional batch manufacturing approaches often impose limitations on speed, scalability, and flexibility, creating bottlenecks that slow the translation from laboratory discoveries to patient-ready therapeutics. Recognizing these challenges, a team of experts from academia and industry supported by regulatory specialists have advanced the development of a fully integrated, continuous, cGMP-compliant mRNA manufacturing platform. This platform is designed to bring together upstream IVT synthesis, downstream purification, automated LNP formulation, and continuous lyophilization (fill and finish) into a seamless, automated workflow, creating a unified process that addresses the inherent inefficiencies of traditional batch production of mRNA-based therapeutics.

The advances reported in this project on each individual step in the end-to-end manufacturing value chain of mRNA therapeutics create a platform that simultaneously enhances throughput, reduces operational costs, and minimizes environmental impact through lower reagent consumption and energy use. Beyond technical efficiency, the framework is designed to be flexible and scalable, providing a generalizable strategy that can accelerate development timelines, expand global accessibility, and support the broader adoption of mRNA therapeutics across diverse medical applications, from vaccines to gene therapies and personalized medicine.

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NUCLEIC ACID INSIGHTS

INTERVIEW

SPOTLIGHT ON • Upstream processing and analytics

Addressing sustainability and scalability bottlenecks in oligonucleotide manufacturing

Ewan Moody

INTERVIEW

"There is some really exciting work coming out in the enzymatic field, but for the time being, it still seems that chemistry has the upper hand."

Jokūbas Leikauskas, Editor, *Nucleic Acid Insights*, speaks with **Ewan Moody**, Postdoctoral Research Associate, The University of Manchester, about the key sustainability and scalability bottlenecks in oligonucleotide manufacturing, focusing on solvent use, purification waste, and emerging chemical and enzymatic strategies for greener large-scale production.

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Q What are you currently working on?

EM After finishing my PhD in the Lovelock Research Group, I continued working there on a short postdoc, working on a directed evolution

"Typically, about 50% of waste material is produced during oligonucleotide purification, which is an obvious area for improvement."

campaign, engineering DNA polymerases for activity towards phosphorothioate nucleotide triphosphates. I then later joined Patrick Cai's group, where I now work on creating synthetic plant genomes – in essence, I have gone from working with very small DNA to very large DNA!

Q From your perspective, what currently limits scalability in oligonucleotide manufacturing?

EM I feel like the answer to this has not changed much over the last few years. There is an excellent 2020 review that covers the sustainability challenges and opportunities, which I think sums up the issues in this field very well [1].

Obviously, the biggest problem is the very high reliance on acetonitrile. The number often quoted is 1,000 kg of solvent per kg of product, which has a huge impact on process mass intensity and sustainability. Furthermore, there are certainly downstream purification and separation issues at the end of the phosphoramidite process. Typically, about 50% of waste material is produced during oligonucleotide purification, which is an obvious area for improvement.

Q How is the nucleic acid therapeutics field addressing scale-up difficulties, high solvent consumption, and increasing environmental impact concerns?

EM Following on from the previous question, much of the work focuses on acetonitrile and solvent use. There was a great paper by the Livingston group published in December 2025 [2] about liquid-phase oligonucleotide synthesis using membrane filtration to 'fish' the desired products out of the solution entirely in the liquid phase, which is an exciting piece of work, especially if it can be demonstrated on a larger scale.

Linked to that, there has been recent work on acetonitrile recycling, either by completely removing waste material from it for re-use in synthesis steps or, if it is still slightly contaminated with water, using it for wash steps [3].

A large amount of waste is produced just in producing the modified monomers for oligonucleotide synthesis. Looking at making monomer synthesis 'greener', it has been interesting to see the work from Phil Baran's group, where phosphorous (V) monomers are derived from limonene, demonstrating that renewable feedstocks can be incorporated into oligonucleotide synthesis [4].

Regarding enzymatic approaches for oligonucleotide manufacturing, which is obviously positioned as a greener alternative to traditional chemical synthesis, some of the most interesting work recently has been in the enzymatic synthesis of the nucleotide monomers. Recent research from the Lovelock and Turner groups at the University of Manchester demonstrates engineered enzymes that enable access to molecules previously only accessible by chemistry, which represents a great step forward in terms of realising a wholly enzymatic oligonucleotide synthesis platform [5,6].

Q Enzymatic synthesis is often positioned as a greener manufacturing alternative – where do you think the environmental benefits are most compelling, and where does the reality still lag behind the promise?

EM Again, the most obvious benefit is removing the reliance on acetonitrile. However, there is still so much waste produced from synthesising the monomers that can be addressed. If, for example, you look at TdT-based systems, producing a 3'-NH^2 blocked monomer can require a multi-step chemical synthesis to prepare that single monomer. It takes an awful lot of chemistry to make something that is then described as 'green.' Reiterating the earlier point, making these monomers in a less wasteful way is really the crux of the problem.

Using large-scale unmodified DNA synthesis as an example, it's clear that enzymatic approaches must be shown to genuinely compete with established chemical processes before stakeholders will consider investing the capital required to replace phosphoramidite chemistry. Despite generating a powerful TdT variant through many rounds of evolution, Codexis and Molecular Assemblies' enzymatic DNA synthesis platform has not managed to challenge existing chemical approaches. Maybe in the current climate it is not viable to fully embrace enzymatic synthesis, but as the field improves, I hope it will become more attractive.

More of a commercially successful example of an enzymatic platform is the nucleotide-tethered TdT approach developed by Ansa Biotechnologies, which emerged from Jay Keasling's group. They are not really focused on oligonucleotides but on much larger DNA synthesis. They are currently promising 50 kb constructs with no reliance on sequence structure, which is something that current chemical synthesis simply cannot do. In essence, there is some really exciting work coming out in the enzymatic field, but for the time being, it still seems that chemistry has the upper hand.

Q In your experience, what are the most challenging aspects of evolving polymerases specifically for non-natural or modified nucleotides used in therapeutics?

EM The hardest thing is finding the initial starting activity. There is a lot of literature about removing the steric gate, which helps with 2'-blocked monomers. It is relatively straightforward to get started with activity that you can then evolve. It is much more difficult with more non-natural substrates.

I have had experience with phosphorothioates, where you can get incredible polymerase activity towards one diastereoisomer and absolutely zero towards the other. It is very difficult to find a starting point for an evolution campaign.

Even once you find that, another major challenge is setting up a robust assay. Codexis have said that in their experience, the best option is to set up a fluorescence assay because it offers the highest throughput and reliability. But that is not always easy, especially when working with poorly accepted substrates. It can be very difficult to get something to stand out above the background. Ultra-high throughput approaches such as microfluidics are incredibly powerful but are often challenging to establish; evolution campaigns will likely speed up as these alternatives to traditional evolution techniques become more widespread.

From a practical standpoint, the cost of modified monomers is also prohibitive for many laboratories. Even tiny amounts are extremely expensive. To test at scale, you need gram

quantities, which are simply out of reach for many groups. That certainly limits the number of people working in the field.

Q Looking ahead, which tools or process innovations do you think will be most important for enabling reliable, large-scale oligonucleotide manufacturing over the next 5–10 years?

EM Some of the most interesting developments are actually around increasing the efficacy of the medications themselves, so that you do not need as much oligonucleotide in the first place. There is a lot of work on encapsulation and conjugation. Attaching an oligonucleotide to a targeting molecule that directs it to a specific tissue is a powerful approach for treating specific tissues. The N-acetylgalactosamine (GalNAc) conjugates targeting the liver are well established, but targeting other tissues remains challenging. As that technology improves, we may be able to work with much smaller doses, reducing the pressure on establishing large-scale manufacturing platforms.

Similarly, the pharmacokinetics effects of stereochemistry in phosphorothioate oligonucleotides remain poorly understood. It is difficult to determine which molecules are efficacious or even active in a mixture of hundreds of thousands of diastereomers. As synthesis methods and analytical techniques improve, the overall efficacy of these medications should improve, which is exciting.

Acetonitrile recycling will also become more feasible at a very large scale. It is not currently worth doing for the primers most of us order, but for a multi-ton scale synthesis of oligonucleotides, it will likely become economically attractive.

Furthermore, improvements in purification are also important. The process still relies heavily on traditional reverse-phase high-performance liquid chromatography (HPLC). Developing alternative purification strategies would be beneficial. There has been work on performing synthesis in acetone, and other non-HPLC purification approaches are emerging.

Finally, the continued development of enzymatic methods and further directed evolution of polymerases will be important. Even in the last few weeks, there has been a paper from the Chaput Lab on engineering a DNA polymerase to accept RNA-like monomers, showing that there is still plenty of research being done in the optimisation of enzymes for oligonucleotide synthesis [7].

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BIOGRAPHY

Ewan Moody earned his Master's degree in chemistry from the University of Sheffield in 2019 before completing his PhD 'a versatile biocatalytic platform for therapeutic oligonucleotide synthesis' with Sarah Lovelock at the University of Manchester, where he developed a novel one-pot process for the enzymatic synthesis of modified oligonucleotides (DOI: 10.1126/science.add5892; DOI: 10.1126/science.adl4015). He is currently a PDRA in Patrick Cai's group at the University of Manchester working on the design and construction of synthetic plant genomes.

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UPSTREAM PROCESSING AND ANALYTICS

SPOTLIGHT

Sustainable and scalable oligonucleotide manufacturing: addressing operational and CMC challenges

Fred Briones

INTERVIEW

"The hardest things to control at scale are sequence-related impurities and batch-to-batch consistency."

Jokūbas Leikauskas, Editor, *Nucleic Acid Insights*, speaks to **Fred Briones**, Senior Director, CMC, City Therapeutics, about the challenges and opportunities of scaling oligonucleotide manufacturing to commercial volumes, with particular emphasis on sustainability, process innovation, and regulatory alignment in RNA interference (RNAi) therapeutics.

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Q What are you currently working on?

FB I am currently a Senior Director of CMC at City Therapeutics, where I oversee both the oligonucleotide drug substance and product manufacturing. City Therapeutics focuses on combining novel, potent RNAi trigger designs

with advanced delivery capabilities. In essence, we aim to develop the next generation of RNAi therapeutics.

There is still significant innovation in the RNAi space that we believe we can leverage, and my role is to discover novel manufacturing technologies. I spend a lot of time bridging discovery, process development, and regulatory expectations, especially as we consider scaling up these new technologies.

Q What are the main technical and operational hurdles when scaling oligonucleotide manufacturing to commercial volumes?

FB When we reach commercial scale, it comes down to batch-to-batch consistency, as you work to maintain yields and control impurities across multiple runs. It is crucial to avoid making purification and analytics a bottleneck for production. Concurrently, you are continuously improving your process, and as you do, you must keep regulators informed.

When introducing new technologies, it is critical to engage regulators early to ensure they are comfortable with the change. The siRNA field is now well established, and regulatory agencies are accustomed to conventional manufacturing methods. However, in order to scale up with a different technology, you need to be prepared to answer all their questions.

Q Solid-phase oligonucleotide synthesis is notorious for solvent and reagent intensity. How can the manufacturing of these advanced therapeutics be made more sustainable?

FB Solid-phase oligonucleotide synthesis, based on phosphoramidite chemistry, is a mature, well-established platform that has been in use for decades. I think the biggest sustainability win would not be in drastically changing the core phosphoramidite chemistry, but rather in improving operational efficiency. That includes reducing unnecessary solvent use where possible and implementing robust solvent recovery and recycling for unavoidable uses. For example, at the peak of the COVID-19 pandemic, there was a global shortage of acetonitrile as many laboratories suddenly began producing oligonucleotides to try to help address the pandemic. However, because these solvents are highly specialized and expensive, this became a significant issue. If a company implements recycling, it can address these issues. At a commercial scale, efficient solvent recovery can significantly reduce footprint and costs, but it requires strict controls to avoid introducing variability or impurity risks.

Many companies mention solvent recycling in talks and at conferences, but it is often a major challenge for CDMOs, both operationally and economically. Additionally, many clients remain skeptical about using recycled solvents. However, if the right controls and advanced analytical methods are in place to determine that you are not introducing new impurities into these solvents, I think this is a good way to make manufacturing more sustainable.

Beyond recycled solvents, many are addressing these challenges by exploring greener, more sustainable technologies and more efficient manufacturing processes. This includes

"Traditional solid-phase oligonucleotide synthesis will always be limited in terms of scale."

approaches in the synthesis area, such as enzymatic technologies, and in the purification area, where people are moving toward continuous rather than batch purification.

Q Enzymatic synthesis is frequently cited as a more sustainable and scalable alternative for oligonucleotide manufacturing. What are the key barriers that are preventing it from being more widely adopted?

FB I believe enzymatic oligonucleotide synthesis is more sustainable and potentially scalable, but it is not yet widely adopted because it is not yet mature. I once worked at a company focused on gene-editing technology for almost 4 years, and at the time, we were developing very long oligonucleotides. The biggest barriers were control, compatibility, and manufacturing readiness – this approach requires different equipment and controls.

This manufacturing process involves introducing enzymes, and achieving precise single-base control over very long sequences remains difficult. You need engineered enzymes and similar solutions to address those issues. Additionally, enzymatic transformations are limited by the modifications they can incorporate. For example, during my early days at Moderna, I remember working on new chemical modifications, and the polymerase simply would not incorporate them. Furthermore, the GMP precedents for enzymatic synthesis remain far behind phosphoramidite chemistry, which is already well established.

Therefore, I think enzymatic synthesis is promising, but it is not yet a drop-in replacement. That said, I think the field is moving in that direction, possibly sooner rather than later. For example, Alnylam Pharmaceuticals, a leading RNAi company, recently announced a new enzymatic synthesis platform, siRELIS, that is expected to substantially increase manufacturing capacity and reduce production costs [1].

What was particularly encouraging was that this platform was well-received by the US FDA and included in its Emerging Technology Program, which provides fast-tracked global regulatory engagement. That is an important step forward for adoption, because many groups are hesitant to be the first to test the waters. Seeing companies like Alnylam move in this direction is highly encouraging for others to follow suit.

Q When you think about different synthesis modalities, how do you decide which is best suited for a given oligonucleotide or therapeutic application?

FB Enzymatic synthesis and other new technologies can genuinely increase capacity and lower costs, and early engagement with the FDA helps de-risk them. This is especially true in the siRNA field, where we are seeing more and more

programs targeting large indications, which may ultimately require metric tons of siRNA to be manufactured. Traditional solid-phase oligonucleotide synthesis will always be limited in terms of scale. For many companies, it is important to explore this as early as possible and to determine how you will solve the problem if you suddenly need to produce 100 kg of material. That may be a 'good' problem to have, but it still requires planning. Large pharmaceutical companies can sometimes afford to take on more platform risk and longer timelines. For smaller biotechnology companies, speed, predictability, and very low regulatory risk are usually critical.

In practice, we tend to see a staged rollout. For a first program, companies may choose a more conventional approach simply to get things moving. Afterwards, as I mentioned earlier, they engage with regulators early and discuss new technologies. Ultimately, it comes down to company priorities. If they have the flexibility, they are more willing to take risks and adopt innovative technologies. If not, they are more likely to be risk-averse, given the need to deliver quickly.

Q From a CMC and quality perspective, what are the most difficult attributes to control as oligonucleotides move to larger scales?

FB The hardest things to control at scale are sequence-related impurities and batch-to-batch consistency. As you scale up, truncations and closely related impurities become harder to separate and fully characterize. This problem becomes exponentially larger when you are dealing with very long oligonucleotides – for example, in gene editing, oligonucleotides are often longer than 100-mers. On top of that, chemical modifications can introduce additional impurities.

Ultimately, what matters is not just overall purity but a stable, well-understood impurity profile. More recently, regulators have also become increasingly focused on diastereomeric profiling. This is something we are seeing more and more, as many oligonucleotide therapeutics contain phosphorothioate modifications that introduce chiral centers. You are controlling not only the impurity level but also the stereochemical distribution. That becomes very complicated from an analytical standpoint – if you have 26 diastereomers, that is a large number of variables to manage.

Batch-to-batch consistency then becomes critical, particularly when you introduce changes to raw materials or processes. That is where comparability and control strategies become extremely important. At scale, CMC is less about making material once and more about making it the same way every time. Again, this requires further development of advanced analytical methods, particularly for oligonucleotides with chiral centers and complex diastereomeric profiles.

Q Looking ahead, what tools or process innovations do you think will have the biggest impact on enabling reliable, large-scale production of oligonucleotides over the next 5–10 years?

FB I think it will be a combination of improved purification, analytics, and profile-based control strategies. Alternative synthesis platforms, such as

ligation or enzymatic synthesis, will slot in where they are strongest. Additionally, the biggest impact will come from making purification faster and higher yielding, and from making high-resolution analytics more routine. There is also a need to better align control strategies with regulators' increasing focus on diastereomeric composition. For shorter oligonucleotides, including siRNAs and antisense oligonucleotides, we can make a meaningful impact.

For longer oligonucleotides, such as CRISPR guide RNAs, synthesis still requires significant improvement. We know that coupling efficiency starts to drop once you get beyond around 60-mers, and impurities grow exponentially as you approach 100-mers. There is room to improve upstream processes, and this is where ligation strategies can play a role: using shorter oligonucleotides to ligate into a single guide RNA.

Furthermore, it does not stop at synthesis. We still need robust purification and high-resolution analytics, which are particularly challenging for long oligonucleotides due to their complex secondary structures. You may need to denature them, but then you are dealing with base-labile RNA modifications, which adds further complexity. This also complicates analytical characterization, especially when you are trying to determine impurities and maintain a consistent impurity profile from batch to batch. That part of the field is still in its infancy, but that is what makes it exciting. For chemists, these are exactly the kinds of problems we want to solve. As more CRISPR-based technologies move into the clinic and toward commercialization, these processes should be addressed as early as possible to ensure batch-to-batch consistency.

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BIOGRAPHY

Fred Briones is an experienced leader in nucleic acid therapeutics with over a decade of experience spanning research, process development, and global manufacturing. Trained as a synthetic organic chemist, he earned his doctorate degree from Emory University, Atlanta, USA, and completed postdoctoral research at Texas A&M University, and AstraZeneca R&D in Waltham, Massachusetts. He has contributed to the advancement of innovative genetic medicines at Moderna, Alnylam Pharmaceuticals, and Tessera Therapeutics across multiple therapeutic platforms. He currently serves as Senior Director of CMC at City Therapeutics, a Cambridge, Massachusetts-based biotechnology company engineering the future of RNAi-based medicine and advancing the next generation of RNAi therapeutics. In this role, he leads strategic and technical efforts to translate cutting-edge RNAi science into robust development and manufacturing programs.

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NUCLEIC ACID INSIGHTS

mRNA
DOWNSTREAM PROCESSING
AND PURIFICATION

COMMENTARY

Scaling up RNA manufacturing through adaptable downstream processing

Manoj Pohare, Bojan Kopilovic, and Zoltán Kis

Despite rapid advances in RNA-based vaccines and therapeutics, downstream processing remains a primary constraint on scalable, cost-effective manufacturing. In this commentary article, three researchers at the University of Sheffield discuss key downstream challenges across mRNA and self-amplifying RNA (saRNA), including purification, pre- and post-lipid-nanoparticle processing, as well as analytical limitations, and highlight adaptable, low-shear, digitally enabled platforms as critical enablers of robust RNA manufacturing at scale.

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We are an ambitious, dynamic RNA manufacturing group of young, highly motivated researchers working under the supervision of Professor Zoltán Kis at the University of Sheffield, UK. Our work focuses on digitalized RNA platform processes that enable rapid, scalable, and cost-effective production of high-quality RNA vaccines and therapeutics. By integrating cutting-edge experimental and computational approaches, we aim to transform RNA manufacturing. Our research is driving the development of an advanced manufacturing platform, which we call RNAbox™, capable of delivering RNA-based medicines at scale, quickly, affordably, and without compromising quality.

ADDRESSING DOWNSTREAM BOTTLENECKS IN SCALABLE RNA MANUFACTURING

The scalability of RNA-based therapeutics manufacturing is strongly limited by several downstream processing bottlenecks. Firstly, the purification of long and fragile RNA molecules remains inefficient, as existing chromatography and filtration technologies offer limited capacity and selectivity for separating RNA from closely related product impurities, both in terms of chemistry and size, such as double-stranded RNA (dsRNA), truncated (abortive) transcripts, residual DNA, and enzymes, without inducing degradation, especially at large scale. Secondly, the purification of

BIOINSIGHTS
TECH SHOWCASE

STREAMLINING QUALITY ASSESSMENT ACROSS IVT mRNA PRODUCTION

Addressing key analytical challenges in IVT mRNA production

A guide to the BioPhase 8800 system for quality assessment during IVT mRNA production by Tingting Li
Manager, Cell and Gene Therapy Applications, SCIEX

Our new TECH SHOWCASE provides concise, interactive overviews of emerging technologies shaping cell and gene therapy. Combining a short video with an at-a-glance summary, we highlight new platforms, tools, and capabilities from industry innovators, giving you a quick way to understand how the technology works and where it may add value.

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RNA-containing lipid nanoparticles (LNP) represents a major and often unacknowledged bottleneck, as current approaches rely predominantly on tangential flow filtration, which is effective for buffer exchange and removal of residual solvent and excess lipids, but has limited selectivity for removing free RNA and empty LNPs, while preserving particle integrity at scale. Finally, these technical challenges are compounded by poor process robustness and limited real-time monitoring and control, resulting in batch-to-batch variability and, consequently, additional costs at scale, in addition to the already high cost of raw materials.

Overcoming these challenges requires the development of high-capacity, RNA- and RNA-LNP-selective purification methods, the implementation of continuous and intensified downstream processes, and more efficient use of materials through recycling of unused IVT components, a process that can be further enhanced by immobilizing either RNA polymerase or DNA template or both. This strategy reduces RNA manufacturing costs by allowing the reuse of expensive reagents, such as proprietary capping reagents, RNA polymerase, and DNA templates. In addition to immobilization, improving RNA polymerase performance to minimize the formation of process-related impurities, such as dsRNA, will further reduce the burden on downstream purification methods. Lastly, the integration of digital tools, mechanistic modelling, and real-time analytics is crucial to enable robust, scalable, and cost-effective RNA manufacturing.

DOWNSTREAM PURIFICATION DIFFERENCES BETWEEN mRNA & saRNA AT SCALE

Downstream purification of mRNA and saRNA generally involves similar unit operations, such as enzymatic digestion, filtration, chromatography, and ultrafiltration; however, the physical properties of the RNA fundamentally shape how those steps must be designed and operated. mRNA molecules are relatively short (typically 1–6 kb), mechanically more tolerant, stable, and sufficiently distinct from many process-related impurities, allowing purification to emphasize efficient separation using robust capture methods such as oligo-dT affinity and conventional ion-exchange chromatography. In contrast, saRNA molecules are much larger (~9–12+ kb), highly shear-sensitive, and structurally complex, with impurities such as dsRNA that closely relate to saRNA in size, charge, and hydrodynamic behavior. As a result, saRNA purification places greater emphasis on preserving molecular integrity while discriminating among very similar species, forcing operation at lower flow rates and pressures, and often requires alternative capture strategies and additional orthogonal polishing steps, especially for dsRNA removal.

These differences become most consequential at scale, where physical stress, mass-transfer limitations, and cumulative yield losses are amplified. Capture chromatography and filtration are particularly limiting for saRNA, as low flux and fouling constrain throughput and extend cycle times, requiring larger equipment sizes and higher costs. Small variations in feed quality or operating conditions that are easily tolerated in mRNA processes can shift impurity co-elution with saRNA, reducing robustness and reproducibility. Consequently, while mRNA downstream processing scales primarily with cost considerations, saRNA downstream processing is constrained by throughput, yield, and process control, making scale-up substantially more challenging despite superficially similar process flows.

Overall, saRNA synthesis and downstream processing typically result in lower final yields than mRNA; however, the 10–100-fold lower dose requirements of saRNA can offset these productivity losses.

DOWNSTREAM RNA PROCESSING STRATEGIES BEFORE VERSUS AFTER LNP ENCAPSULATION

The downstream processing strategy for RNA before LNP formulation is fundamentally focused on the selective purification of RNA molecules from a complex *in vitro* transcription matrix. At this stage, the primary objectives are the efficient removal of closely related product-derived impurities while preserving RNA integrity and biological activity. In addition, the intrinsic sensitivity of RNA to shear, interfaces, and chemical stress represents a significant contributor to product loss.

In contrast, post-encapsulation downstream processing shifts from molecule-centric to a particle-centric strategy. Once encapsulated, the RNA is partially protected, and downstream objectives focus on maintaining LNP size distribution and encapsulation efficiency, while removing excess lipids, residual solvents, unencapsulated RNA, and improperly formed particles. Current approaches are dominated by tangential flow filtration and related membrane operations, which are effective for buffer exchange and solvent removal but offer limited selectivity for resolving free RNA and empty LNPs. As a result, post-encapsulation downstream processing is often constrained by trade-offs between purification efficiency, product integrity, and scalability, highlighting the need for more selective, robust, and intensified approaches tailored specifically to nanoparticle-based RNA therapeutics.

ADAPTING PURIFICATION PLATFORMS FOR DIVERSE RNA MODALITIES

Current purification platforms can be made sufficiently adaptable to handle different RNA lengths and structures by shifting from length-dependent, single-mode workflows toward modular, low-stress, and multi-modal separation strategies that are tunable rather than fixed.

At the unit-operation level, adaptability begins with shear- and pressure-tolerant designs, including low-shear pumps, wider flow paths that support laminar flow, and chromatography media with large, convective pores (such as monoliths or membranes) that reduce mass-transfer limitations for long RNAs while remaining suitable for shorter mRNA; however, a key trade-off of wider convective pores is reduced binding capacity per unit area compared to smaller pore paths. At short residence times, the apparent on-rate for poly(A)–oligo-dT binding may become limited by the need for the longer RNAs to undergo rotational and conformational diffusion so that the relatively small poly(A) segment is correctly oriented to engage surface oligo-dT ligands and form stabilizing hydrogen bonds, even when bulk mass transfer in convective pores is no longer limiting. Supplementing oligo-dT–only capture with either ion-exchange or mixed-mode capture enables a single platform to accommodate RNAs with different lengths, secondary structures, and poly(A) characteristics. These modes could be tuned by adjusting buffer composition, salt gradients, and residence time, rather than by redesigning each construct. Additional adaptability is achieved through orthogonal impurity control, in which combinations of charge-based, hydrophobic, and structure-selective separations enable effective removal of dsRNA, truncated species, and aggregates across a wide RNA size range without overstressing any single step. In parallel, flexible filtration strategies such as adjustable MWCO membranes and low-flux operation support efficient processing of large saRNA while remaining effective for smaller mRNA.

At scale, adaptability depends on platform robustness and control, not just chemistry. Real-time monitoring (e.g., UV, conductivity, and emerging RNA-specific analytics such as pH-based quantification),

shorter hold times, and standardized, single-use hardware enable rapid adjustment of operating windows as RNA length changes without re-engineering the process. Together, these improvements shift purification platforms from being optimised for a single RNA format to being tunable frameworks capable of supporting mRNA, saRNA, and future RNA modalities with minimal redevelopment. One such example is the RNAbox™ platform that we are developing in our group, which will be an automated, continuous, flexible, multiproduct, integrated system that combines enzymatic RNA synthesis, RNA purification, RNA encapsulation into LNPs, and LNP purification into a single platform for end-to-end manufacturing of mRNA vaccines and RNA-based therapeutics.

ADDRESSING ANALYTICAL CHALLENGES IN CONFIRMING RNA ENCAPSULATION & STABILITY

The key analytical challenges in confirming RNA encapsulation efficiency (EE%) and stability during downstream processing stem from the complexity of RNA–LNP formulations and the commonly used methods. Accurate determination of EE% requires techniques that can distinguish RNA protected within nanoparticles from unencapsulated RNA without perturbing the particle structure. This becomes an even more critical challenge in continuous manufacturing, where in-line monitoring may be desirable. Fluorescence-based intercalating dyes are the most widely used tools. The RiboGreen dye is highly sensitive to RNA but is membrane-impermeable; therefore, it detects only free RNA and cannot directly quantify encapsulated RNA without an additional LNP-disrupting step, which may introduce variability and compromise reproducibility. However, due to the assay's susceptibility to matrix effects, dilution conditions, and sample handling, RiboGreen EE% can be difficult to reproduce across labs and methods unless conditions (buffers, temperature, timing, LNP concentration) are tightly controlled and thoroughly qualified. SYTO9, in contrast, is a membrane-permeable dye and can penetrate intact LNPs to directly measure encapsulated RNA. However, it has lower specificity, can bind non-RNA components, and typically requires flow cytometry for detection. Both dyes, therefore, involve trade-offs among sensitivity, selectivity, and sample deviations, and careful validation is necessary to obtain reliable EE% data. Furthermore, SYTO9 enables the quantification of loading efficiency.

In terms of payload stability, RNA–LNP formulations are susceptible to membrane fouling during tangential flow filtration when process parameters are not adequately optimized. Exposure to interfacial and shear stresses can further induce nanoparticle aggregation, cargo leakage, or RNA degradation. Consequently, assessing RNA integrity post-encapsulation is critical to ensure drug product stability, introducing additional analytical challenges.

The exposed challenges complicate real-time monitoring and can lead to over- or underestimation of EE% if not accounted for. Addressing these bottlenecks requires orthogonal analytical approaches, such as combining fluorescence assays with chromatography or light scattering, as well as process optimizations, including low-shear purification, optimized buffers, and controlled flow conditions. The integration of real-time process analytics, including in-line soft sensors, can further enable dynamic monitoring of encapsulation and particle stability, thereby improving robustness and reproducibility in downstream RNA–LNP processes.

EXPLORING EMERGING RNA PURIFICATION TECHNOLOGIES

Several emerging purification technologies have the potential to be transformative for RNA downstream processing over the next

5–10 years, especially as the field moves toward longer, more complex RNAs like saRNA and other modalities.

Convective-flow innovations

Next-generation monoliths with tailored pore sizes and chemistries could combine high binding capacity with ultra-low shear, enabling efficient separation of very large RNAs without degradation. Functionalization for selective dsRNA or truncated species removal could further improve yields.

Membrane-based innovations

High-capacity, single-use membrane adsorbers or mixed-mode membrane platforms could allow scalable, rapid purification with reduced footprint and flexible tuning for different RNA lengths and secondary structures.

Affinity-based innovations

Engineered ligands that specifically bind RNA structures, dsRNA, polyA tail, caps, or sequence motifs, either individually or simultaneously (multivalent capture) could enable highly selective capture of diverse RNA constructs, minimizing the need for multiple orthogonal chromatography steps.

Liquid-liquid phase separation-based innovations

Tunable precipitation, aqueous two-phase systems, or stimuli-responsive polymers could enable scalable, low-shear separation of RNA from impurities while being broadly applicable to RNAs of different sizes.

Automated, real-time monitoring and closed-loop control

In-line analytics such as UV absorbance, light scattering, RNA-specific sensors, including pH-based soft sensors, or even nanopore-based detection could allow real-time adjustment of purification parameters, increasing adaptability across RNA types and reducing batch-to-batch variability. In parallel, RNA encapsulation efficiency could be monitored using soft sensors (e.g., IR-based) during both manufacturing and downstream purification, supporting more robust and responsive process control. Integrated chromatography-based analytical platforms can further accelerate RNA therapeutics development by enabling real-time workflow monitoring during process development.

Continuous and flow-through purification

Continuous chromatography and integrated filtration systems could minimize hold times and shear exposure, improving throughput and process robustness, particularly for fragile saRNA. Moreover, recent advances in LNP purification using monolith columns, which are uniquely suited to low-shear separations, indicate that this technology could be extended to continuous LNP purification.

Microfluidic or nanofluidic separation platforms

While still early-stage, these could provide precise, low-shear control over RNA purification, potentially allowing high-resolution separation of closely related species like full-length vs truncated saRNA. These concepts are reflected in emerging microfluidic platforms.

In combination, these technologies point toward modular, low-shear, and highly selective platforms capable of processing a wide range of RNA modalities efficiently at scale, reducing losses, and accelerating time-to-clinic for next-generation therapeutics.

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NUCLEIC ACID INSIGHTS

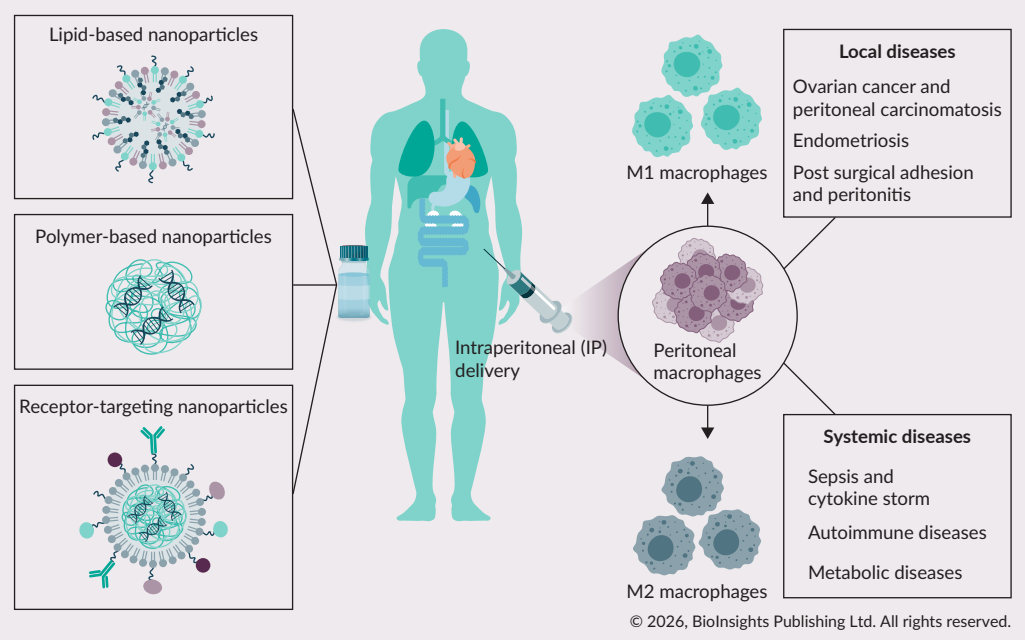
REVIEW

TOPIC FOCUS • Oligonucleotides

RNA interference therapies targeting peritoneal macrophages for local and systemic diseases

Kavita Iyer, Dhaval Oza, Ivan Zlatev, and Mansoor M Amiji

The peritoneal cavity serves as a unique immunological niche where peritoneal macrophages (PMs) play central roles in local and systemic immunity. Their abundance, accessibility, and phenotypic plasticity make them attractive targets for RNA interference (RNAi) based therapies. By silencing disease-driving gene expression via RNAi effectors, PMs can be directed towards pro- or anti-inflammatory phenotypes. While direct intraperitoneal (IP) delivery of short-interfering RNA (siRNA) offers access to PMs, variable distribution and rapid clearance reduce efficiency and underscore the need for optimized delivery strategies. Nanoparticle carriers are employed to improve the distribution and delivery efficiency of RNAi effectors and are capable of enhancing uptake into PMs. Ligand-targeting through macrophage-specific receptors further promotes specificity and cellular internalization. Collectively, these strategies utilizing RNAi technology to target large peritoneal macrophages (LPMs) represent promising approaches for the treatment of local diseases such as peritoneal cancer, endometriosis, peritonitis, and systemic diseases such as sepsis, autoimmunity, and atherosclerosis. Key challenges in therapeutic development include inefficient endosomal escape, excessive off-target uptake, and unwanted immune activation. Addressing these challenges is essential for advancing macrophage-targeted therapies toward successful clinical translation.



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THE PERITONEAL MACROPHAGE AS A THERAPEUTIC HUB

The peritoneal cavity

The peritoneum is a large serous membrane consisting of parietal peritoneum lining the abdominal wall and visceral peritoneum covering the intra-abdominal organs, playing a vital role in maintaining intra-abdominal homeostasis [1]. The peritoneal cavity is a specialized serous space between the parietal and visceral layers that functions as a dynamic immune niche, housing diverse resident and recruited immune cell populations [1]. This comprises of innate immune cells such as macrophages, natural killer cells, monocytes, and dendritic cells, alongside adaptive immune cells such as B cells and T cells, and collectively they serve as a critical site for immune surveillance, first-line defense in infections, and as a regulator of inflammation [2]. Selective modulation of immune response can be utilized as a strategy to mitigate peritoneal fibrosis, infection, and tumor metastasis.

Types of PMs

Peritoneal macrophages (PMs) are important players in regulating the immunological response and maintaining dynamic equilibrium. Macrophage differentiation within the peritoneal cavity occurs in three broad stages, which include monocyte recruitment and early differentiation, maturation, and functional specialization with retained plasticity [3,4]. Circulating monocytes enter the peritoneum under homeostatic or inflammatory cues and differentiate into immature macrophages, progressively acquiring tissue-specific transcriptional and activation markers necessary for peritoneal macrophage identity [3]. Post-maturation, peritoneal macrophages segregate into two primary subtypes: small peritoneal macrophages (SPMs) and large peritoneal macrophages (LPMs) [4]. SPMs are predominantly

short-lived, monocyte-derived, and immunologically active with enhanced MHC-II antigen presentation and cytokine production. LPMs are long-lived, embryonically derived, evolutionarily conserved macrophages representing 70–90% of the total population within the peritoneum and play an important role in rapid first-line response and efferocytosis [5]. SPMs differ from LPMs primarily in ontogeny, surface phenotype, lifespan, transcriptional programming, and functional bias toward antigen presentation and inflammatory responsiveness rather than homeostatic maintenance. LPMs demonstrate a highly selective expression of the transcription factor GATA6, which is a lineage stability factor, and the deletion of GATA6 leads to loss of characteristic phenotype and dramatic reduction of LPM population [6].

Previous studies have shown that LPMs can respond to inflammatory stimuli through the Macrophage Disappearance Reactions (MDRs), where they migrate into the lymph nodes within the omental fat [5]. Under inflammatory conditions such as MDR, the depleted LPM populations are replaced by monocyte-derived SPMs, which can adopt LPM-like phenotypes, underscoring the significant plasticity of these populations [7]. Additionally, multiple studies have confirmed that their migratory properties allow LPMs to infiltrate and respond to sterile injury or acetaminophen-induced liver injury in intra-peritoneal organs such as the liver [8,9]. Surprisingly, these LPMs have also demonstrated the ability to migrate into the lungs upon chlodronate-mediated depletion of alveolar macrophages through a systemic route [10]. Owing to their large abundance within the peritoneum, their ability to polarize readily, and their unique migratory properties, LPMs are an attractive target cell type to treat local as well as systemic diseases.

Macrophages demonstrate remarkable plasticity, allowing them to undergo polarization by responding to the microenvironment. At a resting state, macrophages exist in the Mθ state to maintain tissue homeostasis, but exposure to foreign pathogens and inflammatory stimuli such as lipopolysaccharide (LPS) or thioglycolate (TG) triggers classical (M1) activation, inducing a pro-inflammatory cytokine cascade, enhanced nitric oxide production, and increased phagocytic activity for microbial clearance [11]. However, prolonged production of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) or interferon gamma (IFN-γ) can lead to sustained inflammation and autoimmune diseases [12]. The alternative (M2) pathway is involved in wound healing and tissue repair with the release of anti-inflammatory cytokines such as interleukin-4 (IL-4) and transforming growth factor beta (TGF-ß) that create an immunosuppressive environment [11]. However, long term dysregulation can lead to fibrosis, and M2 macrophages can support tumor metastasis [11].

RNAi: an overview of gene expression silencing

RNA interference (RNAi) is an evolutionarily conserved mechanism of regulating gene expression. First identified in *C. elegans*, it demonstrated that endogenous short non-coding double-stranded RNAs can post-transcriptionally suppress complementary messenger RNAs [13]. In the early 2000s, the mechanism of RNAi was characterized by cleavage of 25-nucleotide dsRNA into 21/23 nucleotide fragments that bind to Argonaute-2 (Ago2) protein to form the RNA-induced Silencing Complex (RISC) [14–16]. The PIWI domain of Ago2 structurally resembles RNase H-type nucleases and provides the catalytic activity required for RISC-mediated endonucleolytic cleavage of target mRNAs when the guide RNA is Watson-Crick paired with full complementarity [16]. This landmark finding culminated in Andrew Fire and

Craig Mello receiving the 2006 Nobel Prize in Medicine for their discovery of the RNA interference (RNAi) pathway [13]. In the 27 years since the discovery of RNAi, the mechanism has been successfully applied to eight FDA-approved siRNA drugs [17]. Tremendous improvements in delivery, potency, and duration have enabled this technology to rapidly succeed in the clinic, demonstrating a favorable safety profile and exceptional efficacy.

The first technology supporting the delivery of this emerging therapeutic class to the liver is the lipid nanoparticle (LNP) platform, wherein the siRNA drug is encapsulated within a multicomponent lipid system that mediates cellular uptake (**Figure 1**). The LNPs formulated with an ionizable lipid acquire liver tropism post-injection through rapid adsorption of endogenous Apolipoprotein E (ApoE) from circulation that binds exposed lipids post PEG shedding [18]. ApoE-coated LNPs interact with low-density lipoprotein receptor (LDLR), highly expressed on the surface of hepatocytes, facilitating clathrin-coated endocytosis into early endosomes. The reduction in pH during early-to-late endosomal maturation promotes protonation of amine groups on the ionizable lipid, inducing destabilization of the endosomal membrane and subsequent release of the siRNA into the cytosol for RISC loading [18].

Ligand conjugates are the next frontier in RNA delivery, and the majority of currently approved drugs utilize a siRNA-conjugate platform comprising a trivalent N-acetylgalactosamine (GalNAc) conjugate moiety [17]. Ligand conjugation with higher valency enhances binding to the asialoglycoprotein receptor (ASGPR) highly expressed on the surface of hepatocytes within the liver [19]. This facilitates efficient siRNA delivery into the cell, and the favorable kinetics offered by the fast-recycling receptor promote continuous internalization and superior duration of action with GalNAc-siRNA conjugates [20,21].

Types of RNAi effectors: siRNA, shRNA, and miRNA

RNAi effectors are short non-coding RNAs that regulate the expression of a gene post-transcriptionally. There are three main types of RNA interference (RNAi) effectors, and each has their distinct structure, mechanism of action, and therapeutic advantage.

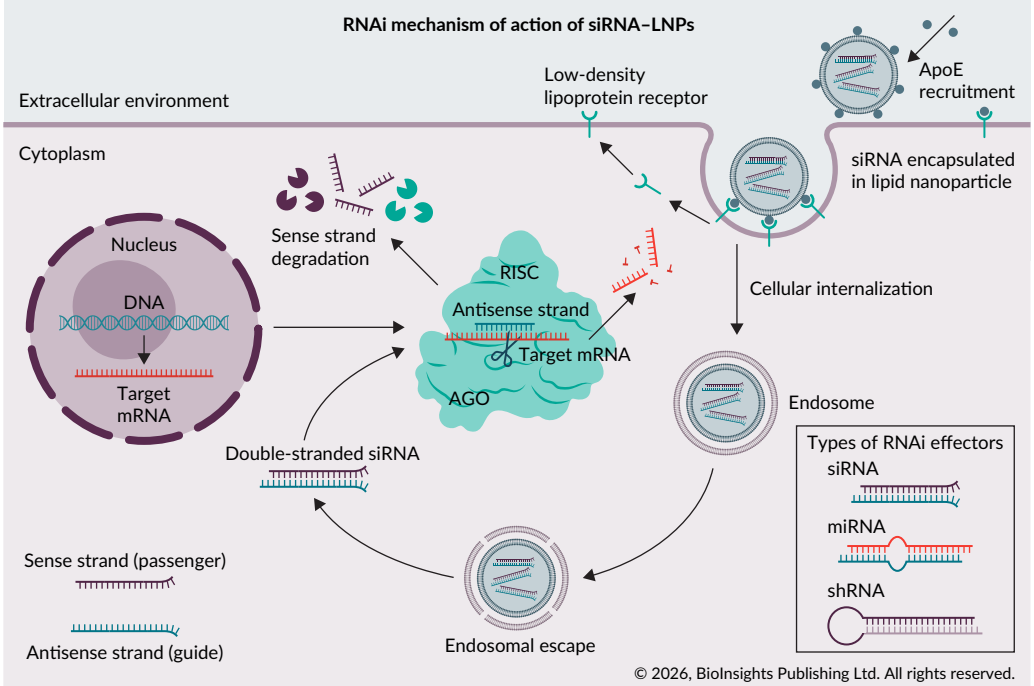
Structure, chemical design, and mechanism of action

Small interfering RNA (siRNA) is short double-stranded RNA ranging 19–25 nucleotides in length [22]. Typical siRNA designs possess full Watson-Crick complementarity to the target mRNA sequence. Canonical siRNA designs include a 19–21 base pair double-stranded RNA duplex with 2-nucleotide 3′ overhang on both ends, 5′ phosphate, and ~50% GC content to optimize RISC loading and target cleavage [23]. The antisense strand has a less stable 5′ end (A/U-rich) for preferential RISC incorporation, while the sense strand is cleaved and discarded. Synthetic siRNA drugs include sense strand (guide) and antisense strand (passenger) annealed in an equimolar ratio to form the double-stranded duplex. Chemical modifications such as 2′-O-methyl (2′-OMe), 2′-fluoro (2′-F), and phosphorothioate (PS) linkages are added to improve nuclease resistance and reduce immunogenicity without altering canonical structure [24].

Short hairpin RNA (shRNA) features a short double-stranded RNA with a specific stem-loop structure mimicking the primary transcripts (pri-miRNA) precursor. The double-stranded stem ranges 19–25 nucleotides in length [25]. Dicer, an RNase III endoribonuclease, cleaves double-stranded RNA hairpins into 21–23 nucleotide structures competent for RISC loading [26]. shRNA is processed by the Dicer resulting in siRNA-like structure and low thermodynamic stability at 5′ antisense end leads to favorable RISC loading which mediates transcriptional silencing. Alternate shRNA structures

FIGURE 1

Lipid nanoparticle-mediated RNA interference: ApoE-bound siRNA LNPs are taken up by hepatocytes via LDLR-mediated endocytosis, escape endosomes into the cytoplasm, and guide RISC to cleave complementary target mRNA, silencing gene expression.



that possess an 18–19 base pair stem with a smaller loop between 3–5 nucleotides can bypass the Dicer processing step and directly induce Ago2 slicing [27]. Clinically, shRNA strategies are pursued less often because they rely on long-term expression from DNA vectors, which complicates safety control and regulation [28]. In contrast, siRNA drugs are shortlived, nonintegrating molecules that are easier to dose, monitor, and evaluate within existing drugdevelopment frameworks.

Micro-RNA (miRNA) is a class of endogenously expressed non-coding RNA produced by the cells and functions as a tool to modulate gene expression via RNAi. miRNAs originate as pri-miRNAs with imperfectly base-paired stem-loop structures, which are further processed in the nucleus by Drosha/DGCR8 enzyme into ~60–70 nucleotide pre-miRNAs retaining the stem-loop structure [29]. Subsequently, pre-miRNAs are exported to the cytoplasm, where Dicer cleaves the substrate to form 18–25 nucleotide miRNA duplex. The guide strand loads into RISC, imperfectly base-pairing target mRNA, where a seed match of 2–8 nucleotides with the target mRNA leads to translational repression, mRNA destabilization, deadenylation, or cleavage [22]. Anti-microRNAs (anti-miR) or antagomirs are another class of synthetic molecules designed to be complementary to endogenous miRNA to inhibit their function, which is advantageous in diseases where dysregulated miRNA expression promotes pathogenesis [30,31].

Advantages of RNAi

Main advantages of RNAi effectors include high specificity to the target RNA with full-complementarity with siRNA/shRNA and partial complementarity with miRNA, thereby resulting in reduced unwanted off-target effects compared to other

modalities such as small molecules [32]. Another advantage is the transient nature of RNAi effect, and the ability to reverse the course of therapy if necessary [33]. Additionally, RNAi is inherently safer than genome editing technologies since RNAi effectors cannot integrate into the genome, thereby eliminating the risk of permanent genetic changes. Lastly, RNAi offers an avenue to target 'undruggable' proteins that lack drug-binding pockets, targeting upstream of the protein synthesis pathway and effectively inhibiting the translation of aberrant mRNA [34]. Amongst the RNAi effectors, siRNA is preferred over shRNA and miRNA because it enables precise, transient, and dose-controlled gene silencing, making it better suited for therapeutic and translational applications in a wide array of diseases.

Rationale and scope

PMs polarize toward M1 or M2 states with distinct cytokine profiles, reflecting their high plasticity [35]. This plasticity allows reversible repolarization via RNAi-mediated gene silencing, delivered directly or through nanoparticles, to modulate pathogenic gene expression. Local modulation of pro- or anti-inflammatory markers can shift macrophage phenotypes, while systemic effects alter cytokine and chemokine networks controlling broader immune responses. Transient repolarization thus offers a versatile strategy to treat both cavity-confined and disseminated diseases.

DELIVERY STRATEGIES: TARGETING & REPOLARIZING PERITONEAL MACROPHAGES

Direct IP delivery

Targeted delivery remains a major challenge for RNAi therapies and significant efforts are underway to deliver to non-hepatocyte cell populations including immune cells. Chemical modifications to the sugar and phosphate backbone of oligonucleotides have enabled prolonged activity via parenteral routes (IV, SC) [17]. Non-parenteral routes (oral, intranasal, and topical) require additional refinement since the large, highly charged, nuclease-sensitive molecules are poorly absorbed, rapidly degraded, and show limited uptake into target tissues and cells [36]. Optimized cell-targeting strategies are therefore critical to enhance bioavailability, tissue specificity, and improved PK/PD properties. Intraperitoneal (IP) administration provides a passive delivery route to the peritoneal cavity, where it is particularly effective for accessing tissue-resident LPMs. IP administration enhances accumulation on visceral surfaces, providing direct access to organs such as the pancreas and ovaries, and localized delivery minimizes off-target exposure.

Limitations of IP delivery

Key challenges for IP administration of unconjugated or 'naked' siRNA include variable distribution within the peritoneal fluid and rapid clearance by the reticuloendothelial system (RES) [37]. The peritoneal cavity contains a diverse array of cells, including mesothelial cells, fibroblasts, adipocytes, endothelial cells, and immune cells, making targeted, cell-specific uptake challenging [38]. Additionally, it has been observed that negatively charged low molecular weight cargo (<20 kDa) delivered to the peritoneal cavity is absorbed into lymphatic circulation and entrapped in the lymph nodes, resulting in loss of activity in the target tissues and cell types [37]. Active nanoparticle targeting addresses the limitations of passive delivery by guiding therapeutics to specific cell populations, improving uptake, tissue specificity, and pharmacological efficacy.

Nanoparticle-mediated delivery systems

Lipid and polymeric nanoparticle-mediated delivery systems represent an optimal

strategy for delivering RNA interference (RNAi) payload (Figure 2). They offer protection from nuclease-degradation, enhancing their stability and half-life *in vivo* and facilitate efficient cellular internalization through endocytic pathways overcoming the cellular membrane barrier to negatively charged RNA molecules. Inclusion of ionizable lipids in the formulation promotes endosomal escape in the acidic intracellular endosomal compartments [39]. Nanoparticles can be surface-modified to enhance tissue specificity and reduce off-target uptake, while maintaining low immunogenicity and favorable pharmacokinetics. Together, these properties make nanoparticles a versatile platform for rapid clinical translation of RNAi therapeutics.

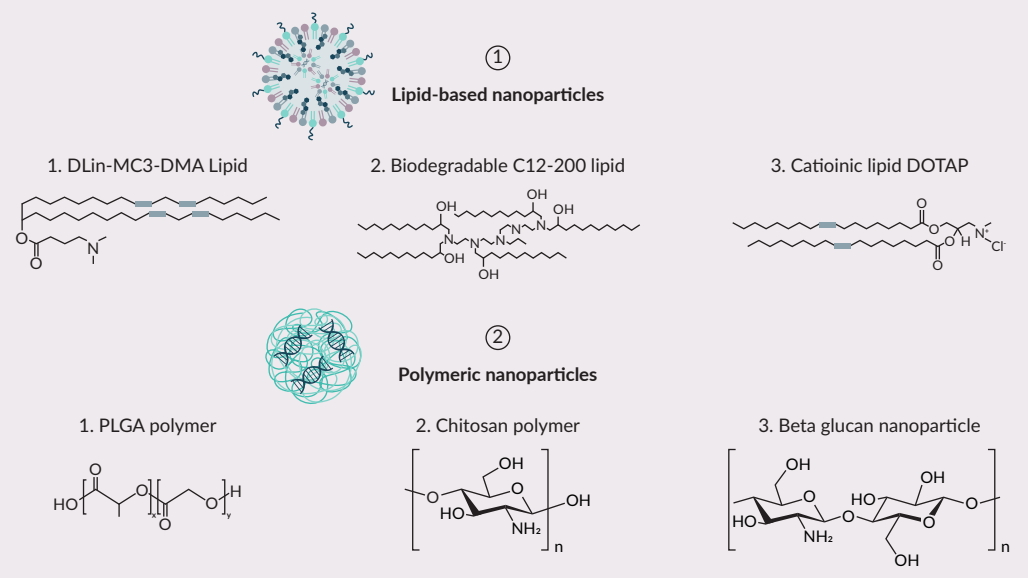
LNPs

Lipid-based nanoparticles (LNPs) are the most advanced class of nanoparticle systems used for the delivery of siRNA, mRNA, and other CRISPR-Cas9. LNPs have been extensively evaluated and validated in the clinic. The first FDA-approved siRNA therapy utilized LNP to deliver the therapeutic payload to the hepatocytes [18,34,39]. The FDA-approved RNAi drug Patisiran includes a novel biodegradable ionizable lipid Dlin-MC3-DMA (MC3) capable of enhancing delivery to the liver hepatocytes via ApoE-mediated LDLR uptake, and promotes endosomal release of siRNA into the cytosol [39]. Quantitative imaging studies have shown that only 1-2% internalized siRNA delivered by MC3-LNPs escapes the endosomal compartment; however, the small cytosolic fraction is sufficient for gene knockdown due to efficient RISC loading [40]. The successful translation of Onpattro with the LNP delivery platform in hATTR amyloidosis patients validated the delivery approach and confirmed the favorable safety, immunogenicity, and PK/PD profile and derisked the platform for future use.

The clinical validation of the LNP platform was a key enabling step for mRNA vaccine delivery during the COVID-19 pandemic. This provided a path forward for other types of nucleic acid cargo delivered with ionizable lipids and allowed Moderna and Pfizer/BioNTech to adapt the delivery

FIGURE 2

Select examples of nanoparticle formulations: lipid-based and polymeric nanoparticle delivery systems utilizing ionizable/cationic lipids or biocompatible polymers.



strategy rapidly for encapsulating the mRNA encoding spike protein mRNA of the SARS-Cov-2 virus. The determination of the ideal lipid composition, manufacturing ability, and the path to regulatory acceptance further enabled successful administration across all age populations.

Onpattro® LNP formulation: clinically validated RNAi delivery platform

MC3 LNPs have been used with various siRNA targets to treat peritoneal diseases, primarily in rodent models, with translation to higher species still unproven. However, emerging data suggest that the clinically approved Onpattro formulation could potentially be applied in this context. Weissman, *et al*. have previously shown that MC3 LNPs encapsulating mRNA cargo can be delivered intraperitoneally and possess similar half-life as the SC route with strong protein production levels inferior only to the IV route of administration [41]. However, dose-response studies revealed saturation of the uptake and translation capacity within the cells in the peritoneal cavity and higher expression in the liver that is characteristic of the systemic spread of MC3 nanoparticles.

A recent study utilized siRNA targeting fibrinogen α chain (siFga) encapsulated within MC3 LNP in a thioglycolate-induced peritonitis mouse model [42]. During peritonitis, inflammation induces a rapid influx of fibrinogen, which binds to the receptors on macrophages and impairs the migration to the sites of inflammation. The siFga-LNP successfully lowered the fibrinogen upon injection, restoring macrophage migration in mice.

Another study utilized lipid nanoparticles to deliver siRNA targeting a phagocytic receptor MerTK(siMerTK) to reverse the immunosuppressive (M2) phenotype in tumor-associated macrophages, limiting the efferocytosis (clearance of apoptotic cells) in the tumor microenvironment (TME) [43]. This construct was administered intraperitoneally in a colorectal cancer (CRC) peritoneal metastasis mouse model and displayed improved outcomes such as suppressed tumor growth, improved survival and enhanced antimetastatic efficacy when combined with immune checkpoint blockade (ICB) in both liver and peritoneal metastasis the tumor model. However, to further develop MC3 based LNPs, comparative assessment of MC3 vs alternative ionizable and permanently cationic lipids for peritoneal delivery are necessary to address ApoE mediated off-target uptake into liver, spleen and other tissues.

Alternative biodegradable ionizable lipids

Ionizable biodegradable lipidoids such as 306Oi10, 200Oi10, or 514O6,10 intraperitoneal nucleic acid delivery, potentially limiting ApoE association and thereby reducing hepatocyte uptake. Recently, ionizable lipid 200Oi10 or C12-200 was utilized for intraperitoneal delivery of siRNA targeting High Mobility Group Box 1 (siHMGB1) gene, showing primary uptake in large peritoneal macrophages following IP delivery [9]. The induction of acetaminophen-induced liver injury resulted in the migration of these repolarized macrophages into the injured liver and reduction in inflammation markers with a well-tolerated safety profile. The migratory properties of LPMs have been previously explored for dysregulated immune response in chronic inflammation associated with thermally induced sterile injury in a mouse model [44].

LPMs polarized to wound-healing M2 phenotype under the influence of interleukin-4 anti-inflammatory cytokine, demonstrating further that LPMs possess unique tropism and plasticity for treating a variety of diseases. Another study utilized biodegradable ionizable lipids L101 to encapsulate siRNA against Interferon regulatory factor 5 (siIrf5) to attenuate liver injury in a hepatitis mouse model [45]. Cellular uptake with L101 was mediated by an ApoE-independent mechanism. Future

studies evaluating the translation of this in higher species and confirmation of safety of the LNP and cargo at reasonable doses will determine feasibility of utilizing migratory macrophages.

Macrophage-mediated horizontal gene transfer

Macrophage-mediated horizontal gene transfer has emerged as a novel strategy for delivering nucleic acid cargo to distal organs. In one approach, LNPs formulated with the lipidoid 306Oi10 and the cationic helper lipid DOTAP were used to deliver mRNA intraperitoneally [46]. Peritoneal macrophages exhibited the highest mRNA uptake among cavity-resident cells and subsequently packaged the translated protein and/or mRNA into extracellular vesicles generated through endosomal multivesicular body pathways, enabling secondary transfer of genetic material to pancreatic β cells. The horizontal transfer of cargo from macrophage to pancreatic cells was observed with reporter and fluorescent protein both *in vitro* and *in vivo*; however, the transfer of disease-relevant mRNA and protein remains to be seen [46].

A similar horizontal transfer approach has been previously observed with siRNA lipoplex complexes, where transfer of cargo containing siRNA against polo-like kinase (siPLK1) was observed from macrophages to cancer cells via the Rab27a recycling pathway, resulting in tumor growth inhibition and increased cancer cell apoptosis in a murine breast cancer mouse model [47]. These studies collectively show that intraperitoneal administration of LNP complexed with nucleic acid cargo such as siRNA or mRNA can be used to treat diseases via the horizontal transfer of the cargo while minimizing exposure and localization within the liver or other immune cell types.

Overall, novel biodegradable lipids offer a promising path to deliver to peritoneal macrophages; however, developing biodegradable, non–ApoE-binding lipids is challenging because it requires balancing endosomal escape, stability, and pharmacokinetics in the peritoneal cavity while minimizing hepatocyte uptake and off-target effects.

Cationic lipids

Permanently cationic lipid nanoparticles can facilitate intracellular delivery by promoting strong nucleic acid complexation and enhanced uptake into target cells. Following internalization, these lipids support endosomal release of RNA cargo, enabling efficient cytoplasmic delivery. A study utilized DOTAP-containing permanently cationic liposomes to target peritoneal macrophages for treating the inflammasome aberration in ulcerative colitis [48]. DOTAP was combined with cholesterol, resulting in a self-assembled nanoparticle delivery system that was further combined with siRNA against NOD-like receptor family, pyrin domain 3 (siNLRP3). This targeting strategy resulted in polarization of macrophages to M2 state resulting in inhibition of inflammasome activation and ameliorated tissue injury.

LNPs containing cationic lipid DOPE complexed with PEG2000 demonstrated significant siRNA-mediated knockdown in both murine and human macrophages, as well as dendritic cells [49]. Another study utilized cationic lipid YSK05-based liposomes to deliver siRNA against cluster of differentiation 45 (siCD45) to peritoneal macrophages and showed high uptake and silencing efficacy with the intraperitoneal delivery route [50].

Challenges with cationic lipids

Despite their advantages in facilitating cell entry and cytosolic delivery, permanently cationic lipids pose several challenges, such as high toxicity, non-specific interactions with serum proteins, and rapid clearance, which limit safety and tolerability *in vivo* [51]. Due to these limitations,

cationic lipids remain experimental methods of delivering oligonucleotides.

Polymeric nanoparticles

Biodegradable polymers offer a range of delivery options for encapsulating/complexing nucleic acid cargo for macrophage-targeting strategies. Polymers such as PLGA (poly-(lactic-co-glycolic), and chitosan are employed for siRNA delivery as they minimize toxicity, immune activation, and reduce the systemic side effects of the drug delivery systems. In addition, the low immunogenicity risk is favorable for repeat dosing with RNAi-based transient approaches.

In a recent study, authors employed chitosan-coated nanoparticles (CNPs) to deliver nucleic acid cargo to promote pro-inflammatory cytokine production and macrophage repolarization [52]. Chitosan nanoparticles are cationic polysaccharide polymers with favorable biocompatibility and cytotoxicity profiles [53]. CNPs were combined with unmethylated cytosine-phosphate-guanine (CpG) oligodeoxynucleotide (ODN) to create a delivery system capable of binding toll-like receptor 9 (TLR9) on macrophages. These CpG-ODNs are typically utilized as an adjuvant to promote Th1 pro-inflammatory response and enhance the delivery of various types of cargo to isolated mouse primary peritoneal macrophages.

Another strategy combined poly(D, L-lactic-co-glycolic acid) (PLGA) nanoparticles with galactose-chitosan coating to enable TNF targeting siRNA (siTNF) delivery to macrophages [54]. The galactose sugars interact with the ASGPR receptor, promoting uptake, and the chitosan polymers improved the encapsulation efficiency of nanoparticles. Another class of polymeric particles comprising beta-glucans has been explored to deliver siTNF to macrophages upon intraperitoneal administration [55]. The beta-glucan nanoparticles were previously utilized for oral siRNA delivery, in addition to the intraperitoneal delivery route, displaying efficient gene silencing [56]. However, beta-glucans are immunostimulatory by design and result in a strong immune response in addition to aggregation challenges, limiting their use in real-world applications.

Limitations of polymeric nanoparticles

Polymeric nanoparticles offer promising drug delivery properties but remain largely experimental. Their limitations include poor pharmacokinetics, rapid clearance, and short half-life, which in turn unmasks the cargo prematurely, making it metabolically labile and reducing overall therapeutic efficacy of the drug [57]. Additionally, polymeric nanoparticles lack the effective endosomal release mechanisms, resulting in entrapment of the drug in the lysosomal compartment and subsequent degradation or exocytosis [58]. In the absence of a targeting moiety, the polymeric nanoparticles are taken up by multiple cell types due to non-specific uptake, further reducing the productive activity of the drug. Combining biodegradable carriers with active targeting can enhance productive uptake while minimizing immune activation.

Non-specific and receptor-mediated targeting

Non-specific targeting of nanoparticles leverages passive cellular uptake mechanisms, which are dependent on the size, shape, and surface charge, and rely on the phagocytic activity of macrophages [59]. Larger, positively charged, or opsonized nanoparticles are readily engulfed by macrophages, but this approach lacks selectivity, specifically in the context of different sub-populations of macrophages and other immune cells that reside within the peritoneum. To overcome this limitation, emerging strategies in nanoparticle delivery focus on incorporating targeting ligands on the NPs that can minimize unintended off-target effects. Active targeting strategies employ surface ligands on

nanoparticles that bind and activate macrophage-specific receptors, promoting specific and efficient uptake. Recent strategies focus on different types of ligands such as sugars, peptides, nanocomposite materials, and antibody fragments that can interact with specific receptors.

Glycan modified nanoparticles

Naturally occurring glycans combined with nanoparticle delivery systems can interact with specific lectin-type cell-surface receptors and aid in cellular internalization to macrophages. In addition, the unique glycosylation patterns on macrophages due to varied polarization states can improve selectivity and uptake of glycan-nanoparticles. One such example utilizes hyaluronic acid (HA) polysaccharide as the targeting ligand, coupled with a cationic polymer, polyethyleneimine (PEI), which promotes self-assembly of the conjugate with negatively charged nucleic acid cargo [60,61]. This nano-delivery construct specifically binds to the Cluster of Differentiation 44 (CD44) cell surface receptor overexpressed in macrophages. The HA-PEI nanoparticle system was utilized to deliver a microRNA-125 b (miR-125b) responsible for macrophage activation via intraperitoneal injection, resulting in the repolarization of tumor-associated macrophages (TAMs) to an antitumor M1 phenotype [61].

Mannose monosaccharide has been heavily investigated as a targeting ligand since it binds to the macrophage mannose receptor CD206, also highly expressed in TAMs [62,63]. The earliest evidence of mannosylated nanoparticle delivery combined mannose sugar with curdlan or beta-(1,3)-glucan nanoparticle to administer siRNA targeting TNF (siTNF), demonstrating CD206-mediated uptake [62]. Mannose-conjugated nanoparticles encapsulating different types of siRNA cargo are a promising delivery strategy to modulate the polarization of pro-tumoral macrophages to an anti-tumoral state (Table 1) [63–67].

Ligand-functionalized nanoparticles

Ligand-modified nanoparticles exploit the surface receptors for intraperitoneal delivery to resident peritoneal macrophages, enhancing uptake via receptor-mediated endocytosis over passive diffusion (Figure 3). TAMs express high levels of scavenger receptors (SR-A). Ligands such as M2pep, a homing peptide specific to M2 macrophages,

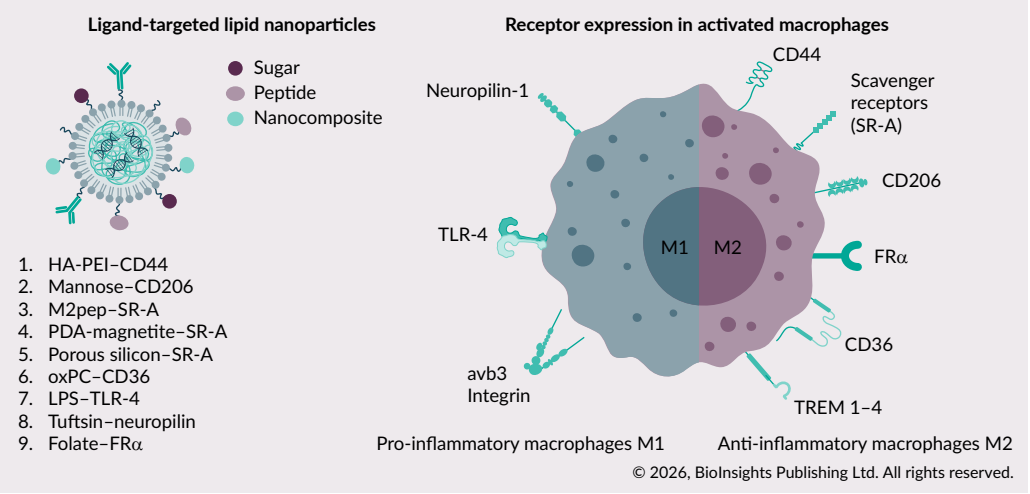
TABLE 1

Delivery strategies to utilize mannose-conjugated nanoparticles.

Ligand conjugate	Delivery and siRNA target	Receptor-mediated uptake mechanism	Repolarization strategy	Reference
Chemically modified mannose (CMM4)	CMM4-branched linker directly conjugated to siHPRT1/siB2M	CD206 (mannose receptor) endocytosis with high-affinity binding and selective uptake in $CD206^{+}$ macrophages	Strong ligand-valency dependent uptake, gene silencing, and protein downregulation in M1/M2 macrophages and DCs	[65]
Mannose-modified trimethyl chitosan cysteine (MTC)	MTC conjugated tripolyphosphate (TPP) nanoparticles with siTNF	Caveolae-mediated endocytosis and macropinocytosis	Multifunctional MTC NPs promote siRNA-mediated gene silencing in macrophages via oral delivery	[66]
Mannose-functionalized polyplex	Diblock copolymer with DMAEMA and BMA with mannosylated-PEG encapsulating siIκBα	Mannosylated polyplex uptake and binding is CD206 dependent with higher uptake in M2-polarized human and mouse macrophages	Therapeutic siRNA induced macrophage repolarization from an M2 towards an M1 phenotype	[67]

FIGURE 3

Peritoneal macrophage targeting strategies.



(Left) Ligands for receptor mediated nanoparticle delivery systems and (right) receptors expressed in pro- versus anti-inflammatory macrophage state. © 2026, BioInsights Publishing Ltd. All rights reserved.

have been combined with gold nanoparticles for delivery to pro-tumoral M2 macrophages [68,69]. Nanocomposite materials such as porous silicon nanoparticles have also been explored in combination with peptide and fusogenic lipids to target scavenger receptors SR-A [70]. Another strategy utilized magnetic nanoparticles to repolarize TAMs using polydopamine-coupled magnetic nanoparticles (PDA-MNPs) with siRNA against protein kinase R-like ER kinase (siPERK) [71].

Scavenger receptor CD36 is responsible for high lipid uptake and metabolism in TAMs, and the combination of oxidized phosphatidylcholines (oxPCs) with liposomes has been investigated as a ligand for the CD36 receptor [72]. A different strategy for repolarizing TAMs relies on toll-like receptor 4 (TLR-4) agonists such as LPS containing liposomal nanoparticles to increase the phagocytic properties and boost immunostimulatory effects [73].

Neuropilin-1 receptor (NRP1) is abundantly expressed on macrophages, with vascular endothelial growth factor (VEGF) as its main ligand. However, the short tetrapeptides such as Tuftsin can bind to specific motifs on this receptor and have been utilized to deliver IL-10 plasmid DNA into macrophages, facilitating their repolarization toward the anti-inflammatory M2 phenotype [74]. Integrin receptors, particularly αvβ3 and β1 subtypes highly expressed on macrophages, can be exploited for targeted siRNA delivery via RGD peptide or antibody functionalization of nanoparticles [75].

Folate conjugation to nanoparticles targets folate receptor (FRα), overexpressed on activated macrophages and cancer cells, enabling receptor-mediated endocytosis for enhanced siRNA delivery and gene silencing. This strategy has been employed in folate-targeted dendrimer PAMAM to repolarize macrophages in an arthritis mouse model; however, combined therapy with RNAi effectors in nanoparticles remains to be explored [76].

Efforts are underway to identify new receptors that can be exploited for delivery to macrophages, and one example, triggering receptor expressed on myeloid cells 2 (TREM2), has been investigated for its role in mixed phenotype macrophage repolarization [77]. However, its role in peritoneal macrophage delivery and combinatorial targeted ligand strategies needs to be further explored.

Although significant progress has been made in developing macrophage-specific ligands (e.g., CD36, folate-FRβ), *in vivo* validation in the peritoneal cavity remains limited, with outstanding questions in ligand optimization, biodistribution, and translation to human inflammatory diseases. Importantly, the therapeutic success of ligand-targeting strategies depends not only on delivery efficiency but also on the underlying disease biology, highlighting that advances in targeted delivery and disease-specific therapeutic design must be pursued in tandem to effectively address complex local and systemic disorders.

THERAPEUTIC APPLICATIONS: FROM PRECLINICAL SUCCESS TO CLINICAL PROMISE

Targeting local peritoneal diseases

LPMs are often referred to as the immune cell guardians of the peritoneal cavity [78]. While their prominent role in policing the abdominal cavity is essential for maintaining homeostasis and clearing cellular debris and pathogens, their pathophysiological roles, particularly in certain diseases occurring within the peritoneal cavity, have been known to promote diseases manifesting in the abdomen. In addition to homeostatic maintenance, key indications influenced by LPMs include ovarian cancer, endometriosis, and surgical adhesions **(Figure 4)**.

Ovarian cancer and peritoneal carcinomatosis

Targeting LPMs is a promising strategy for treating ovarian cancer and peritoneal carcinomatosis because these cells promote tumor growth, metastasis, and immune evasion. Therapeutic approaches range from depleting these pro-oncogenic macrophages in the tumor microenvironment, repolarizing them to a pro-inflammatory, anti-tumor state, or blocking their recruitment and tumor-promoting functions [79,80].

The role of LPMs in cancer

LPMs are a major cell population in the peritoneal cavity that originate primarily from embryonic precursors and maintain their population through self-renewal [5]. In the context of ovarian cancer, these macrophages tend to adopt an anti-inflammatory, pro-tumor phenotype and contribute to disease progression by promoting metastasis, where they help cancer cells form spheroids, adhere to tissues, and colonize the omentum, the fat-rich tissue in the abdomen [81]. LPMs suppress immunity and create an immunosuppressive environment that inhibits the activity and proliferation of anti-tumor T cells [82–84]. They facilitate the survival and growth through secretion of growth factors (like VEGF and IGF1) and use specific metabolic pathways (such as high oxidative phosphorylation and autophagy) that support tumor cell survival and accumulation of malignant ascites (fluid buildup) [82–84].

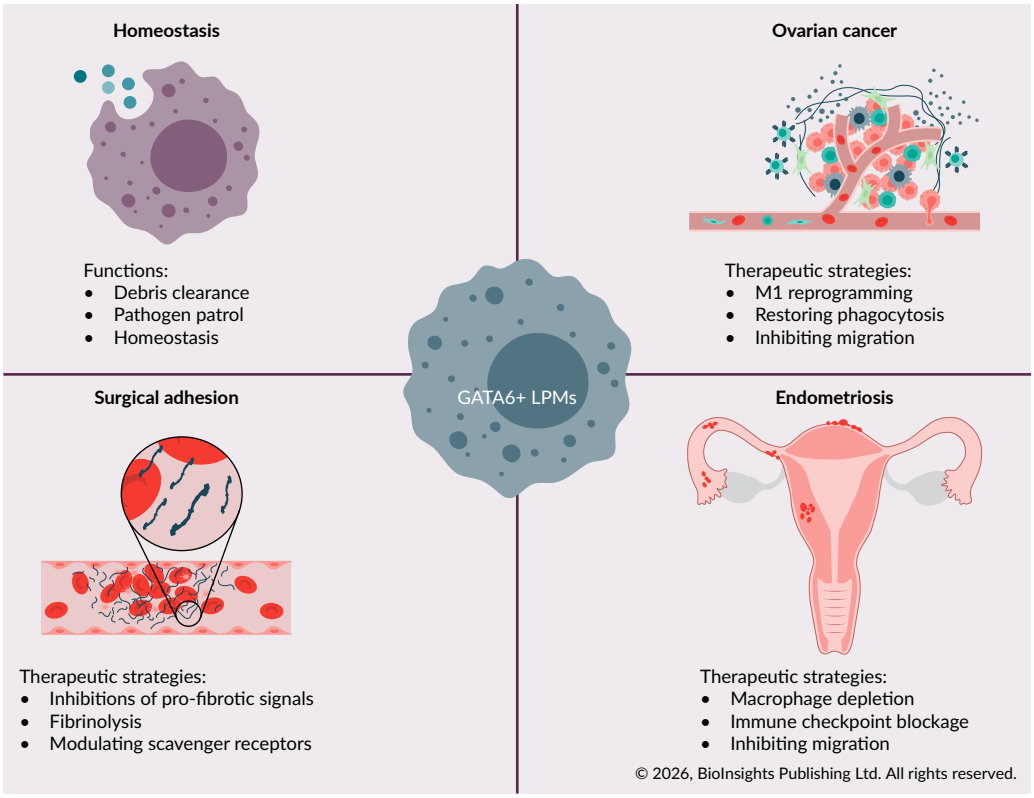
RNA therapeutics to target LPMs in ovarian cancer

LPMs occupy a privileged immunological niche and are uniquely positioned to influence both local and systemic inflammatory responses. Advances in RNA interference (RNAi) delivery, particularly through LNPs, polymeric carriers, and ligand-guided targeting, have enabled efficient, selective silencing of key genes within the macrophage pathways [85]. By modulating PMs toward anti-inflammatory, anti-fibrotic, or anti-tumor phenotypes, RNAi-based strategies show substantial therapeutic promise across a broad spectrum of diseases [9,10,61,85]. Current research is exploring several approaches to manipulate these macrophages for therapeutic benefit [79].

A notable example is the use of hyaluronic-acid–based nanoparticles delivering miRNA mimics (e.g., let-7b), which successfully reprogram tumor-associated macrophages toward a pro-inflammatory phenotype and enhance chemotherapy

FIGURE 4

Peritoneal Indications Influenced by LPMs: physiological and pathophysiological roles of LPMs within the peritoneal cavity and therapeutic strategies to target them.



responsiveness in ovarian cancer models [86]. Importantly, intraperitoneal delivery achieves high macrophage exposure while minimizing systemic toxicity. Beyond miRNA approaches, siRNA targeting transcriptional regulators and survival pathways specific to LPM identity (e.g., GATA6-regulated programs, autophagy mediators, oxidative phosphorylation genes) may present as a rational strategy to selectively disable pro-tumor macrophage functions without complete immune depletion [82–84,87].

Clinical translation

While these strategies show strong potential in preclinical studies, most macrophage-targeted therapies are still in early-phase clinical trials and are not yet part of routine clinical management. A deeper understanding of macrophage diversity in human patients is needed to develop more specific and effective treatments for ovarian cancer and peritoneal carcinomatosis [81].

Endometriosis

Endometriosis is increasingly recognized as an inflammatory disease of the peritoneal cavity, where resident LPMs are one of the key drivers of disease progression [88]. Resident LPMs, which normally clear refluxed endometrial debris, become functionally impaired and instead promote lesion persistence, angiogenesis, neurogenesis, and chronic pain [88]. In a healthy peritoneal cavity, resident LPMs primarily serve a protective function, including clearing apoptotic cells and endometrial debris, often called the ‘policemen of the abdomen’ [89]. In endometriosis, this normal function is compromised, and macrophages exhibit dysregulated behavior

that drives disease progression by reduced phagocytosis, secretion of pro-angiogenic factors such as vascular endothelial growth factor (VEGF), and promotion of fibrosis and lesion expansion through an M2-polarized phenotype [8,88–90].

RNAi-mediated targeting can offer several disease-modifying entry points based on existing disease pathophysiology and the role of LPMs. Although current therapeutic strategies for endometriosis largely rely on small-molecule or antibody-based approaches, RNA-based interventions offer a compelling complementary paradigm. Strategies like suppressing pro-angiogenic signaling, like VEGF-driven programs, or repolarization of macrophages away from M2-like, more to a pro-inflammatory M1-macrophage using miRNA or siRNA mediated delivery could be especially well suited for this indication [91–93].

Emerging neuroimmune mechanisms, such as nociceptor-to-macrophage communication via CGRP/RAMP1 signaling driven macrophage recruitment and activation highlight a neuroimmune axis with strong translational potential. CGRP/RAMP1 signaling drives both endometriosisassociated pain and lesion progression in mice; because this axis is conserved in humans and directly links sensory neuron activity to immunemediated pathology, it represents a highly promising translational target – for example, via RNAi or receptor blockade – to selectively disrupt pathogenic neuroimmune crosstalk without broadly immunosuppressing patients with endometriosis [94].

Key translational advantage

RNA therapies allow local, non-hormonal modulation of disease-driving macrophage programs, addressing a major unmet need in endometriosis management.

Post-surgical adhesion and peritonitis

Targeting LPMs is a promising therapeutic strategy for preventing and treating post-surgical adhesions (PAs) and peritonitis, as these cells play a pivotal regulatory role in both normal wound healing and fibrotic scar formation [94,95]. The goal would be to modulate, rather than fully deplete, LPM activity to encourage normal tissue restoration over adhesion development.

Role of LPMs

LPMs are the primary immune cells in the peritoneal cavity under healthy conditions, acting as the 'first responders' to injury or infection. LPMs offer protective functions during minor injury. LPMs rapidly aggregate at the site, forming a temporary cellular barrier that physically seals the damage and prevents inflammation from spreading, like platelet function [96]. They also clear pathogens and cellular debris through phagocytosis.

Adhesion promotion in severe injury/infection

During severe or persistent injury (e.g., from major surgery or peritonitis), LPM aggregates can become 'super-aggregates' [97,98]. If the immune response is not effectively resolved, these structures can serve as a scaffold for fibrin deposition and collagen production by myofibroblasts, eventually leading to permanent, pathological adhesions [97]. LPMs play a modulatory role and secrete key mediators like transforming growth factor-β1 (TGF-β1) and vascular endothelial growth factor-α (VEGF-α), which influence whether the repair results in complete restoration or fibrotic scarring [99].

RNAi-based therapeutic strategies for surgical adhesions

Therapeutic approaches aim to manage the delicate balance of inflammation and fibrinolysis, often by augmenting the beneficial functions of LPMs or inhibiting their pro-fibrotic pathways [97]. Prevention of surgical adhesions might be among the most compelling therapeutic applications

of RNAi. Foundational study has revealed that GATA6$^+$ LPMs exhibit platelet-like behavior, rapidly aiding formation of fibrin clots and cellular aggregates. However, when chronically activated, this same mechanism can drive fibrin clot formation, fibroblast recruitment, and pathological clot formation. A couple of attractive targets amenable to RNAi can be explored to be silenced, offering a unique, highly precise approach to tilt the balance to fibrinolysis from fibrosis.

Some of these targets include Plasminogen activator inhibitor-1 (PAI-1), which is a powerful regulator of fibrin-formation; silencing PAI-1 could essentially trigger fibrinolysis and help dissolve clots [100]. Another target, Urokinase plasminogen activator (uPA), is similar to PAI-1, where silencing u-PA could essentially aid fibrinolysis [100]. Notably, uPA is a well-established downstream target of the master transcription factor GATA6, which is highly specific to LPMs [3]. Given GATA6's central role in regulating fibrin-forming pathways, selectively silencing this axis could promote fibrinolysis and reduce postoperative adhesion and fibrosis.

Key translational advantage

Selective LPM-delivery of RNAi-offering a short-term silencing of these targets would perfectly align with peri-operative use-cases, where transiently silencing PAI-1 or u-PA could nudge the balance in a desirable manner.

A gateway for treating systemic diseases

Recent evidence has pointed out the unique migratory role of LPMs outside the peritoneal cavity. Additionally, along with migration, it has been seen that these LPMs can also be therapeutically harnessed to mitigate and treat inflammatory indications [9,10,61]. The combination of LPMs' migratory capacity and emerging nanoparticle-based siRNA delivery raises an intriguing question: can these endogenous cells be leveraged as a 'Trojan horse' for targeted therapeutic delivery? Below are some possible therapeutic avenues that can be explored by utilizing this unique ability of LPMs **(Figure 5)**.

Sepsis and cytokine storm

Peritoneal macrophages, both LPMs and SPMs, have been studied as a therapeutic target for sepsis and cytokine storm through strategies that involve modulating their function and using their components. These approaches include repolarizing macrophages from a pro-inflammatory to an anti-inflammatory state using nanoparticles, or using components derived from macrophages, such as extracellular vesicles (EVs), to deliver anti-inflammatory cargo [101,102].

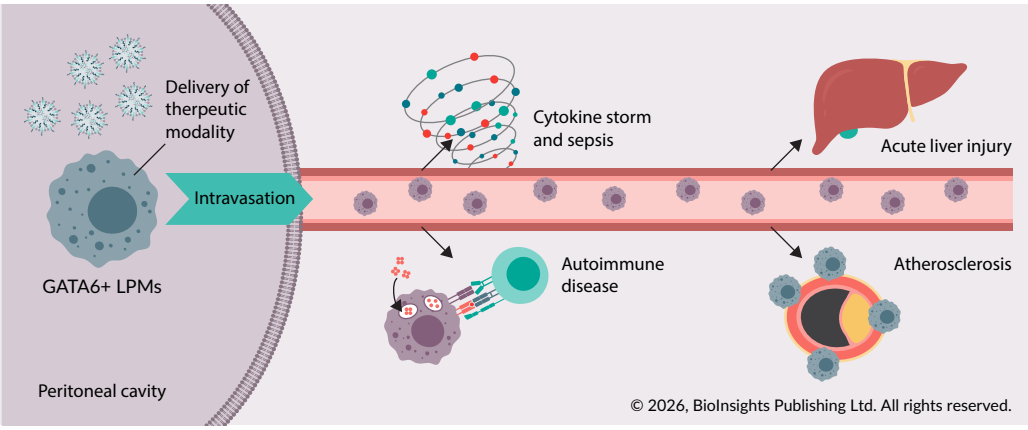
One compelling study provides proof-of-concept that peritoneal macrophages contribute to the propagation of sepsis, and that blocking their migration via administration of the fatty acid-binding protein Fh15 can mitigate disease severity [103]. Additionally, although in a different inflammatory context, silencing the DAMP high mobility group box-1 (HMGB1) using an LNP-encapsulated siRNA produced robust protection against overwhelming inflammation and fulminant hepatic failure in a mouse model of acetaminophen-induced liver injury [9]. In this study, it was compelling to see that the modulation of macrophage polarization mitigated downstream inflammation post liver injury.

Autoimmune diseases

Macrophages have a dual role in autoimmune diseases, acting in both the induction and the suppression of immune responses. Their high degree of plasticity allows them to change their function based on microenvironmental cues, making them a promising target for therapeutic modulation [104]. However, considering the dual nature of macrophages in autoimmune diseases,

FIGURE 5

Extra-peritoneal indications influenced by LPMs.



LPMs migrate beyond the peritoneal cavity and can contribute to systemic inflammatory, autoimmune, and metabolic diseases. Selective LPM-targeting with a therapeutic modality and their migratory behavior make them promising potential 'Trojan horse' carriers for nanoparticle oligonucleotide-based therapies in distal tissues. © 2026, BioInsights Publishing Ltd. All rights reserved.

it has been historically difficult to target them to address some of these indications.

While current therapeutic strategies rely on polarizing these macrophages to an M2 (anti-inflammatory) phenotype outside the body and then reintroducing them into the patient to suppress chronic inflammation, recent work involving LPM migration has great potent-ial to change the paradigm of treating autoimmune diseases, considering that these cells could themselves be used as 'Trojan horses' and reprogrammed and migrate from the peritoneum to systemic sites of inflammation [10,105].

RNAi-based approaches enable context-dependent suppression of inflammatory pathways (TNF-α, IL-6, NLRP3) without permanently ablating macrophage populations [106]. Combining our understanding of these disease-specific changes along with novel therapeutic approaches by utilizing LPMs as trojan horses of endogenously delivered oligonucleotide therapies helps in designing targeted immunotherapies. While the 'Trojan horse' macrophage strategy – using macrophages to deliver therapeutics to target tissues – shows strong preclinical promise, it remains clinically limited because controlling macrophage migration, homing, and therapeutic dosing in patients is challenging; addressing these factors will be critical for safe and effective clinical translation.

Metabolic diseases

Perhaps the most exciting application of LPM migration would be to see whether these fascinating cells could impact chronic-inflammatory and metabolically manifested diseases like atherosclerotic cardiovascular disease (ASCVD). Lipid metabolism in 'plaque-associated macrophages' has been a key pillar of research to derive novel therapies for ASCVD [107]. Despite advances in lipid lowering therapies, there is still a huge unmet need to reduce the overall risk of cardiovascular outcomes suggesting exploring different therapeutic paradigms besides just lipid lowering [108]. Considering the rapid migrating property of LPMs, several different mechanisms can be tapped into to target LPMs to mitigate ASCVD. In this context, the capacity of LPMs to traffic from the peritoneal compartment into the systemic and vascular compartments raises the intriguing possibility of exploiting this pathway to deliver RNAi-mediated immunomodulation directly into atherosclerotic plaques.

CONCLUSION

In conclusion, this review aims to summarize the advancements in RNAi delivery to peritoneal macrophages and the therapeutic avenues that can benefit from these strategies. While preclinical studies support the therapeutic promise of macrophage-targeted approaches, their limited clinical advancement reflects an incomplete understanding of human macrophage heterogeneity, which remains a key barrier to designing specific, effective, and translatable treatments. The key challenges in delivery remain endosomal escape where destabilization of endosome with certain carriers can add toxicity risks and limit therapeutic application window. Some delivery approaches can induce innate immune activation by triggering TLR-inflammatory pathways, and mitigating risks remains a high priority in advancing delivery carriers. Minimizing non-target cell uptake and optimizing siRNA designs to limit off-target gene knockdown is critical to ensuring high specificity. Peritoneal macrophages are not a uniform cell population, and this poses additional challenges in targeting a single subset. Future directions rely on chemistry optimizations with advanced combination strategies such as dual-targeting ligands and cell-penetrating peptide conjugated to the strands, capable of driving cellular uptake and endosomal release in a single construct. Beyond RNAi, other technologies such as CRISPR-Cas9 editing, base editing, prime editing, antisense oligonucleotides, and mRNA approaches can also utilize similar delivery approaches to achieve outcomes for macrophage engineering. Combination therapies with siRNA and small molecules can provide additional avenues for synergistic effects in immunotherapy. Overall, while challenges in delivery and biological translation remain, ongoing innovation in nanomedicine and immunology is paving the way for novel treatments for a wide range of intractable local and systemic diseases.

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AUTHORSHIP & CONFLICT OF INTEREST

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INDUSTRY INSIGHTS♦ MARCH 2026

Clinical and regulatory momentum for antisense and mRNA therapeutics

CLINICAL TRIALS AND RESEARCH ♦ REGULATORY CHANGES AND UPDATES ♦ COLLABORATIONS, PARTNERSHIPS, AND ACQUISITIONS ♦MARKET TRENDS♦ TOOLS AND TECHNOLOGIES

Jokūbas Leikauskas
Commissioning Editor, *Nucleic Acid Insights*

Across February and March 2026, activity in the nucleic acid therapeutics field highlighted continued clinical progression of antisense oligonucleotides (ASOs) alongside expanding regulatory momentum and strategic industry developments. Dyne Therapeutics initiated a Phase 3 trial of its ASO candidate DYNE-101 in myotonic dystrophy type 1, while Vico Therapeutics and Stoke Therapeutics advanced early-stage clinical studies targeting polyglutamine disorders and autosomal dominant optic atrophy, respectively. Regulatory updates included the US FDA IND clearance for Innorna's mRNA therapy in refractory gout and Fast Track designations for RNA-based therapies from Argo Biopharma and ARTHEx Biotech. Industry activity also featured Novartis' acquisition of Avidity Biosciences, a collaboration to advance personalized RNA medicines, new company formation in mRNA, and funding to support mRNA therapeutics and delivery platforms.

Dyne Therapeutics initiated a Phase 3 trial of DYNE-101 for myotonic dystrophy type 1; **Clinical Trials and Research, page 137.**
Credit: stock.adobe.com.

CLINICAL TRIALS AND RESEARCH

Dyne Therapeutics initiated a Phase 3 trial of DYNE-101 for myotonic dystrophy type 1 [1]

Dyne Therapeutics announced the initiation of the global Phase 3 HARMONIA trial evaluating z-basivarsen (DYNE-101), an ASO conjugated to a transferrin receptor 1-binding fragment, in approximately 150 patients aged ≥16 years with myotonic dystrophy type 1. The randomized, double-blind, placebo-controlled study will assess intravenous dosing at 6.8 mg/kg every eight weeks over 48 weeks. The primary endpoint is the change from baseline in the five times sit to stand (5xSTS) test at week 49, with secondary endpoints including muscle function measures and patient-reported outcomes. The trial design has been aligned with the FDA and is intended to support confirmatory evidence for potential regulatory approval.

Vico Therapeutics dosed patients in a Phase 1/2 trial evaluating VO659 across polyglutamine disorders [2]

Vico Therapeutics announced patient dosing in an expanded cohort of its ongoing Phase 1/2a trial of VO659, an ASO targeting CAG repeat expansions, in Huntington's disease, spinocerebellar ataxia type 3, and type 1. The study is evaluating a twice-yearly intrathecal dosing regimen over 12 months for safety, tolerability, pharmacokinetics, and

pharmacodynamics. Early data showed a 38% reduction in cerebrospinal fluid mutant huntingtin and a 2.5% reduction in neurofilament light chain at four months. VO659 has been reported as well-tolerated with no serious adverse events to date. The program is ongoing in Europe, with FDA clearance of an Investigational New Drug (IND) application to support planned US trial initiation.

Stoke Therapeutics dosed the first patient in the Phase 1 trial of STK-002 for autosomal dominant optic atrophy [3]

Stoke Therapeutics announced the first patient dosing in the Phase 1 OSPREY study of STK-002, an ASO designed to upregulate OPA1 protein expression in autosomal dominant optic atrophy. The open-label, dose-escalation trial is enrolling patients aged 6–55 years with confirmed OPA1 mutations to evaluate safety, tolerability, and pharmacokinetics following single ascending doses. Secondary endpoints include changes in visual function, ocular structure, and quality of life. The study is recruiting in the UK and Germany, with additional European sites planned. STK-002 aims to address haploinsufficiency by increasing wild-type OPA1 expression to potentially modify disease progression.

Harness Therapeutics nominated HRN001, a first-in-class drug candidate for Huntington's disease [4]

Harness Therapeutics announced the nomination of HRN001, an ASO designed to upregulate FAN1, as its lead candidate for Huntington's disease. The therapy leverages the company's microRNA site-blocking ASO platform to modulate expression of a DNA repair nuclease implicated in suppressing somatic CAG repeat expansion. Preclinical studies demonstrated increased FAN1 expression, reduced somatic expansion, and favorable pharmacokinetic and tolerability profiles. The company also established a clinical advisory board to support advancement toward first-in-human studies, planned for 2027. Ongoing preclinical development is expected to continue through 2026, with broader applications of the platform under exploration in additional repeat expansion disorders.

Innorna received FDA clearance of an IND for mRNA therapy IN026 in refractory gout; **Regulatory Changes and Updates, page 138.** Credit: stock.adobe.com.

REGULATORY CHANGES AND UPDATES

Innorna received FDA clearance of an IND for mRNA therapy IN026 in refractory gout [5]

Innorna announced that the FDA cleared its IND application for IN026, an mRNA-based therapy for refractory gout, enabling the initiation of a Phase 1 clinical trial. IN026 uses a lipid nanoparticle (LNP) platform to deliver mRNA encoding urate oxidase to the liver, facilitating systemic uric acid breakdown. The upcoming study will evaluate safety, tolerability, pharmacokinetics, and pharmacodynamics in patients with limited treatment options. The candidate is positioned as a potential first-in-class mRNA protein-replacement therapy designed for repeat administration and long-term disease control in chronic metabolic disorders.

Argo Biopharma received FDA Fast Track designation for siRNA therapy BW-20805 in hereditary angioedema [6]

Argo Biopharma announced that the FDA granted Fast Track designation to BW-20805, an investigational small interfering RNA (siRNA) therapy targeting prekallikrein for the treatment of hereditary angioedema. The designation is intended to support accelerated development and regulatory review.

Novartis completed the acquisition of Avidity Biosciences to expand its RNA therapeutics pipeline; **Regulatory Changes and Updates, page 139.** Credit: stock.adobe.com.

BW-20805 is currently being evaluated in a global Phase 2 open-label study in adult patients, with primary completion expected in the second half of 2026 and plans for a subsequent Phase 3 trial. Early clinical data presented from open-label studies indicated reductions in plasma prekallikrein levels and decreases in time-normalized attack rates.

ARTHEx Biotech received FDA Fast Track designation for RNA therapy ATX-01 targeting myotonic dystrophy type 1 [7]

ARTHEx Biotech announced that the FDA granted Fast Track designation to ATX-01, an investigational RNA-based therapy for myotonic dystrophy type 1. ATX-01 is designed to inhibit microRNA-23b, thereby increasing muscleblind-like protein levels and addressing RNA mis-splicing associated with disease pathology. The therapy has demonstrated increased protein expression and reduction of toxic RNA foci in preclinical models. ATX-01 is currently being evaluated in the Phase 1/2a ArthemiR study. The Fast Track designation enables enhanced regulatory interaction to support development in a condition with no approved disease-modifying treatments.

COLLABORATIONS, PARTNERSHIPS, AND ACQUISITIONS

Novartis completed the acquisition of Avidity Biosciences to expand its RNA therapeutics pipeline [8]

Novartis announced the completion of its acquisition of Avidity Biosciences, adding its antibody–oligonucleotide conjugate platform and three late-stage neuromuscular programs to its pipeline. The transaction, valued at approximately $12B in equity and $11B enterprise value, results in Avidity becoming a wholly owned subsidiary. Avidity's platform enables targeted delivery of oligonucleotide therapies to muscle tissue, supporting Novartis' RNA therapeutics strategy. The deal includes a cash payment of $72.00 per share to Avidity shareholders. The acquisition is intended to strengthen Novartis' late-stage neuroscience portfolio and expand capabilities in developing therapies for genetic neuromuscular diseases.

Therna announced collaboration with Charles River to advance personalized RNA therapeutics for ultra-rare diseases [9]

Therna Biosciences announced a collaboration with Charles River to develop single-patient RNA therapeutics for ultra-rare disorders. Initial programs include an mRNA therapy targeting a rare lung fibrosis and ASOs designed to treat Lamb-Shaffer syndrome. Therna's AI-enabled platform was used to design and validate the mRNA candidate within months, while Charles River will lead preclinical development with the aim of submitting a single-patient IND application. The collaboration focuses on accelerating individualized treatment approaches where traditional clinical trials are not feasible, integrating RNA design with preclinical capabilities to support rapid development timelines.

MARKET TRENDS

BioNTech founders announced their departure and plans to establish a new mRNA-focused company [10]

BioNTech co-founders Ugur Sahin and Özlem Türeci announced plans

to step down from their roles and establish a new biotechnology company focused on next-generation mRNA technologies. The transition is expected to occur by the end of 2026, with BioNTech initiating a search for successors. The new entity will receive certain mRNA technologies and related rights from BioNTech, which will retain a minority stake and may receive milestone and royalty payments. A binding agreement is anticipated in the first half of the year. The move comes as BioNTech advances its late-stage pipeline, including multiple Phase 3 oncology studies expected by the end of 2026.

BreezeBio raised $60M Series B to advance the mRNA-based diabetes candidate toward clinical development [11]

BreezeBio announced a $60M Series B financing to support the advancement of its lead mRNA therapeutic, BRZ-101, into IND-enabling studies for diabetes. The candidate delivers mRNA-encoded autoantigens and tolerogenic factors to antigen-presenting cells to induce antigen-specific regulatory T cells and address autoimmune drivers of disease. Proceeds will also support expansion of the company's NanoGalaxy delivery platform, comprising hydrophilic nanoparticles designed for targeted nucleic acid delivery. The funding round included new and existing investors and is intended to support manufacturing scale-up, preclinical development, and progression toward clinical evaluation, as well as additional pipeline programs in autoimmune disease and oncology.

TOOLS AND TECHNOLOGIES

UAlbany researchers developed a laser technique to assess mRNA encapsulation in LNPs [12]

Researchers at the University at Albany reported the development of a Raman spectroscopy-based method to evaluate whether messenger RNA is properly encapsulated within LNPs. The non-destructive laser technique analyzes chemical composition to assess mRNA integrity and packaging, which are critical for therapeutic stability and delivery. In a study published in *Analytical Chemistry*, the method demonstrated potential for rapid quality assessment of mRNA-based therapeutics and vaccines. The approach is intended to support improved characterization of LNP formulations, addressing key challenges related to stability, delivery efficiency, and overall product performance.

etherna platform advanced to IND-enabling studies for mRNA/LNP cancer therapy [13]

etherna announced that its intratumoral mRNA–LNP platform has advanced with the nomination of LAD116, a candidate targeting non-melanoma skin cancer, by its collaborator Almirall. The therapy is designed to induce an immune response within the tumor microenvironment. The program will progress into IND-enabling studies, with a focus on manufacturing GMP-grade material to support planned Phase 1 clinical trials. The advancement reflects the ongoing development of mRNA-based cancer immunotherapies using localized delivery approaches.

***Nucleic Acid Insights'* Commissioning Editor Jokūbas Leikauskas** holds a background in science communication and digital publishing, focusing on advancing the nucleic acid therapeutics field by commissioning and shaping high-impact, open access content for *Nucleic Acid Insights*. He leads the development of interviews, expert articles, and industry perspectives that highlight emerging advances across mRNA, DNA, oligonucleotide, and drug delivery modalities. Jokubas is driven to translate complex scientific topics into engaging, accessible content while maintaining strong connections across the nucleic acids community.

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INTERVIEW

Meet the *Nucleic Acid Insights* Editorial Board

Floris Engelhardt is Co-Founder & CEO of Kano Therapeutics, an MIT spin-out developing circular single-stranded DNA – a new class of non-viral gene templates that address payload toxicity and scalability. Trained as a DNA nanotechnologist, she has conducted research across the UK, Germany, and the US on DNA-protein interactions and 3D DNA nanostructure design. She has been recognized as a rising leader in biotech by MIT, Pillar VC, and the Boston Business Journal. Recently, she was awarded with the prestigious Termeer fellowship for 1st-time CEOs.

INTERVIEW

Floris Engelhardt, Co-Founder and CEO, Kano Therapeutics

Jokūbas Leikauskas, Editor, *Nucleic Acid Insights*, welcomes Floris Engelhardt, Co-Founder and CEO, Kano Therapeutics, as a Senior Editor of *Nucleic Acid Insights*. In this article, they explore pivotal developments that have shaped the field of DNA-based nanotechnologies over the past decade, including the manufacturing scale-up catalyzed by the COVID-19 pandemic, advances in non-viral delivery and genome engineering tools, and the growing infrastructure supporting nucleic acid-based modalities. They also discuss the scientific, regulatory, and commercialization challenges that must be addressed to advance DNA-based nanosystems, as well as the importance of clear, translational communication in shaping the future of advanced therapies.

Nucleic Acid Insights 2026; 3(2), 107–112 · DOI: 10.18609/nuc.2026.013

Q What inspired your journey into the nucleic acid therapeutics field?

FE It is the 'double power' of nucleic acids that is so captivating: they store information (like text in a book) while also having a physical structure that performs a function (like the book as an object). This combination of carrying genetic code, performing complex cellular tasks, and interacting with the environment is unique. Already during my sophomore year in college, I vividly remember discussing with friends the potential of that dual nature for bioengineering. I started by studying DNA-protein interactions and later moved into the field of DNA nanotechnology. Now, years later, I am still excited by every new functionality being uncovered. Nucleic acid therapeutics are an emerging blockbuster field in medicine, and I am lucky to be part of shaping it.

Q From your background and current role, what aspects of DNA nanotechnology and biomolecular systems excite you the most today?

FE In my current role, I am no longer working in basic research on DNA origami or DNA nanotechnology, but I remain very close to it and keep up to date. For example, I loved seeing Anna Romanov's recently published paper in Science on positioning antigens on the outer shell of a 3D DNA origami structure to interact with the immune system [1]. In that case, a three-dimensional DNA structure is used as an architectural scaffold. This is a huge opportunity to create systems that steer the immune system in the right direction. The paper shows exactly that. Precise distances between the antigens optimize and enhance the immune response to this new vaccine modality. This works because the DNA scaffold itself is not immunogenic and can be reused for different types of vaccines. Anna and her co-authors were able to test this approach to create a human B cell repertoire in an animal model, using HIV as a test system. It is very exciting to see these completely new types of use cases emerging from engineering nucleic acids.

Q In your view, what has been the most pivotal development in the DNA nanotechnology or nucleic acid engineering space as a whole in the past decade?

FE The COVID-19 pandemic was the most pivotal moment in the field of nucleic acids over the past decade. Not necessarily only because of the RNA vaccines, but specifically because of the scaling up of nucleic acid manufacturing and the process development that went into it. For example, plasmid DNA (pDNA) and mRNA production were scaled up and optimized because they were required for vaccine development. This created an infrastructure layer that moved nucleic acid-based medicines from a small niche to the forefront, enabling them to treat millions of people.

For example, Moderna raised over $1.3 billion in 2020 to scale its manufacturing, and that money has since been invested in infrastructure [2]. For us, as a startup, this is important because we can access an experienced talent pool, leverage CDMOs or

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work with partners with experience in nucleic acids in a way that simply did not exist 10 years ago.

Q What do you see as the next major scientific or technological leap that will push DNA-based nanosystems into mainstream therapeutic or industrial use?

FE We need to start seeing nucleic acid products more differentiated. If you read current market research reports for nucleic acids, they only mention pDNA, RNA, and oligonucleotides, and completely fail to reflect the product diversity. This has to change – the exploration and showcasing of novel biomaterials are key. For example, long single-stranded DNA products open new markets for high-efficiency insertion and non-integrating *in vivo* therapies, respectively. But this modality fits neither the pDNA nor the oligonucleotide bucket.

The next push on commercialization requires manufacturing scalability. We need robust processes that are cheaper and yield more material, reducing the final product cost. That will require a clear assessment of whether new modalities and therapies address unmet needs and fit specific use cases.

The DNA-based nanosystems industry has grown beyond a niche, and the focus is now on its specific strengths. Acknowledging that no single approach is universally applicable is crucial for this complex, growing market.

Moving forward, DNA-based nanosystems must evolve from simple, single-function systems to ones that leverage structural design and dynamic functionalities, mimicking the balanced, push-and-pull dynamics of biological evolution. For cell and gene therapies, that means that we grow beyond single targeting toward gene circuits and synthetic signaling systems. This means designing sensing systems that integrate environmental inputs using logic-gate designs, which will unlock the field's true potential.

Q In your view, what are the biggest obstacles the field must overcome to reach its full potential – whether scientific, regulatory, manufacturing, or delivery-related – and how might these be addressed?

FE We cannot focus solely on one obstacle; we need to maintain a holistic view. To accelerate this field, we must focus on three core areas: scientific/biological problems, regulatory challenges, and pricing/commercialization. The key lies in understanding the precise connection between these three areas, rather than addressing them in isolation.

A decade into the cell therapy commercial era, the market has matured through its initial challenges, yet it remains a young and rapidly expanding field. DNA-based nanotechnologies, however, are still in their 'teenager' phase. Maturation will take another ten years, and while excitement is high, 2021–2023 taught us that simply throwing money at this space will not solve everything. Time, data, and clinical trials are essential, non-negotiable steps.

The scientific hurdle is complex, often oversimplified as 'solving delivery.' As drug developers, we need to keep a more nuanced view, breaking delivery down into three

critical barriers: reaching the correct organ or tissue, entering the cell and nucleus, and accessing the genome.

For tissue and cell delivery, viruses were a solid first step, but we now need non-viral solutions. Targeted delivery solutions for nucleic acids, such as those being developed by MilliporeSigma and Samyang Biopharm, are key to achieving low toxicity and redosability. Once inside the cell, durability is the next challenge. For DNA-based therapies, this means actively engineering passage into the nucleus, especially in non-dividing cells where passive diffusion is not an option. Finally, accessing the genome involves navigating the high complexity of tools such as CRISPR, serine recombinases, and transposases – it is a massive categorization challenge to determine the best tool for the right job.

As these complex medicines come together, they hit regulatory and commercial roadblocks. Regulators face a confusing landscape: 20–30 different systems claiming to enable site-specific genome editing, but with no clear rules for regulating them. Without these clear regulatory parameters, addressing the pricing narrative is impossible.

This brings us to the commercial challenge: what is the value of a cure? The existing insurance systems, built decades ago, struggle to understand how to price and deliver a one-time cure. Ultimately, discomfort around advanced medicines blocks their adoption. Pricing uncertainty at the end of the pipeline slows early R&D. While founders can demonstrate patient impact, much larger forces must decide whether this truly represents a global shift in medicine.

Q Lastly, what motivated you to join the *Nucleic Acid Insights* Editorial Advisory Board?

FE The future of this field is being shaped by the decisions we make right now. Many people are uncertain about nucleic acid therapies. The reason I was motivated to join *Nucleic Acid Insights* ties directly into this: we have a major communication challenge, a perception problem, surrounding new medical technologies.

We have to acknowledge the public's deep-seated skepticism toward the pharmaceutical industry, often driven by high pricing and a lack of transparency about how billions of dollars are spent.

When we try to communicate science, we typically fail by falling into one of two extremes. On one side, you have the often inaccessible, dense papers in journals like Nature or Science. On the other hand, you have oversimplified, high-level summaries by general news outlets. This leaves a massive gap: either you get something only a specialist understands, or something so basic that experts dismiss it as inaccurate.

This is where *Nucleic Acid Insights* excels and why I believe in its mission. It focuses on that crucial middle ground: creating stories vital for translational work, written clearly enough for anyone in the field – regardless of their specific background – to truly grasp and apply the information. I believe this has a massive, transformative impact on the entire market. It enables us, as professionals, to understand each other's work without introducing distortion or oversimplification, which is the foundational step toward building better products and earning the public's trust. Moving forward, I am committed to seeing this platform continue to empower the entire community to communicate effectively and accelerate the development of life-changing therapies.

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INTERVIEW

Meet the *Nucleic Acid Insights* Editorial Board

Andreas Kuhn has worked with RNA for almost thirty years. This started with his diploma and PhD theses on the structure and function of small non-coding RNAs using biochemical and molecular biology methods. In his post-doctoral work, Andreas studied RNA-protein interactions in the spliceosome in yeast and later worked on small molecules to affect pre-mRNA splicing. His work on mRNA-based immunotherapies began in 2007 in the academic group of Ugur Sahin at the University Clinic Mainz, and Andreas joined BioNTech SE shortly after its founding in 2008. In his current role as Senior Vice President RNA Biochemistry & CMC Development the main focus is expanding proprietary technologies to increase the efficacy of mRNA-based therapies and to develop and optimize GMP-compatible manufacturing processes for RNA.

INTERVIEW

Andreas Kuhn, BioNTech SE, Senior Vice President RNA Biochemistry & CMC Development

Jokūbas Leikauskas, Editor, *Nucleic Acid Insights*, welcomes **Andreas Kuhn**, Senior Vice President, RNA Biochemistry & CMC Development, BioNTech SE, as a Senior Editor of *Nucleic Acid Insights*. In this article, they discuss major recent developments in the mRNA therapeutics field, including personalized cancer immunotherapy and advances in gene editing, as well as the key obstacles that must be overcome to reach broader patient populations.

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Q What inspired your journey into the nucleic acid therapeutics field?

AK During my Chemistry studies, I became fascinated by RNA as a versatile molecule that can perform multiple functions within a cell. It can encode genetic information as mRNA, play structural roles, and have enzymatic functions, among others. After more than a decade of academic research on the structure and function of different RNA classes, I had the opportunity to apply my expertise and experience in biomedical research and to use the knowledge I gained over the years to hopefully, ultimately help people by developing RNA-based therapeutics.

Q From your background and current role, what aspects of nucleic acid therapeutics excite you the most today?

AK I am excited about the individualized cancer immunotherapy approach, in which a tumor sample is taken, sequenced, and tumor-specific antigens called neoepitopes are identified. Then, a tailor-made mRNA-based therapeutic is made for each individual patient, encoding the selected neoepitopes like pearls on a string. mRNA is ideally suited for this approach because its platform-like manufacturing process enables developers to encode any sequence and easily produce it. There have been very exciting results in recent years from this approach across different companies, which clearly demonstrate it is a worthwhile approach to pursue.

In the broader context, I am also excited about various gene-editing efforts that are now gaining traction. Again, mRNA is ideally suited to encode the various nucleases or base editors currently in trial. I think this will deliver strong results, as we have already seen in the Baby KJ case, which was in the news about six months ago and involved a personalized *in vivo* gene editing therapy [1].

Q What has been the most pivotal development in the mRNA-based therapeutics space in the past decade?

AK While the Baby KJ event would be at the upper end of that list, I think the real game-changer was the COVID-19 pandemic. People had long discussed the promise of mRNA, and there had been significant progress. Then it was put to the test in a direct way: several companies decided to go 'all in', and an mRNA-based coronavirus vaccine was developed in record time.

That clearly brought us into the bright light of the industry and the whole world at that time, demonstrating the power of mRNA. Of course, expectations were very high, and it was seen as the 'next big thing'. Overall, I think the development of mRNA-based COVID-19 vaccines really changed the field.

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Q In your view, what are the biggest obstacles the field must overcome to reach its full potential – whether scientific, regulatory, manufacturing, or delivery-related? How can these hurdles be addressed?

AK Firstly, one of the biggest hurdles is delivery. Having the right formulation and delivery system for your approach is probably going to make or break companies. Of course, we have seen the success of lipid nanoparticle formulation in prophylactic vaccines. However, for many of these other approaches, improved or entirely new formulations will likely be necessary. Currently, the liver is relatively easy to target, but treating diseases in other organs or delivering mRNA to tumors requires developing targeted delivery approaches. Some researchers have already started addressing this by incorporating antibody fragments or aptamers into their formulations to help ensure that the mRNA reaches the intended target cells.

Secondly, regulatory obstacles still exist. While there is now a handful of initial or draft guidelines available, I think bringing greater clarity to the field on expectations here and on those of regulatory agencies will make a massive difference.

Thirdly, another bottleneck is the availability of analytical tools. I think we need more and better technologies to characterize mRNA products and, of course, the formulated nanoparticles. This will help shape manufacturing processes, as the more we understand our molecule using these technologies, the better we can refine it.

Lastly, further improving the mRNA molecule itself is also important. It has been a valuable tool in vaccine development, where you need only a small amount of your antigen of interest, and the immune system acts as a powerful accelerator, amplifying the response. However, when the goal is to express functional proteins, further improvements may be needed. One way to achieve that might be to make therapeutic mRNA more closely resemble endogenous mRNA.

Q Lastly, what motivated you to join the *Nucleic Acid Insights* Editorial Advisory Board?

AK One part of my job that I have always enjoyed is sharing news and know-how about the science I work on, both by presenting at conferences and by writing research papers and reviews. Recently, I had the opportunity to co-edit, together with Ana Margarida Azevedo, Rok Sekirnik, and Zoltán Kis, a special issue on the *in vitro* transcription reaction – the process by which mRNA is synthesized – in the journal *Frontiers in Molecular Biosciences*. This allowed me to help raise awareness of mRNA research. This is also the reason for my engagement with the Alliance of mRNA Medicines. Similarly, as a member of the Editorial Advisory Board of *Nucleic Acid Insights*, I would like to help strengthen the visibility of the field of mRNA-based therapeutics and vaccines.

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