Implementation of Real-Time Release Testing in Continuous mRNA-Vaccine Drug-Substance Production: Pilot Scale Demonstration

Dr. Clara Schulz[1], Dr. Lucas Romero[2]
[1]Institute of Bioprocess Engineering, Leibniz University Hannover, Hannover, Germany
[2]Department of Industrial Biotechnology, Technical University of Madrid (UPM), Madrid, Spain
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Abstract
The potential benefits of continuous manufacturing (CM) of mRNA drug-substance are listed as reduced lead time and enhanced product consistency. Nevertheless, to realize CM on a large scale in mRNA vaccine manufacturing, the effective in-line quality control mechanisms need to be incorporated. The current pilot-scale study shows the combination of continuous flow-through in-vitro transcription (IVT) reactors with real-time release testing (RTRT) tools to facilitate on-line assessment of critical quality attributes (CQAs). With a mRNA construct of the prefusion-stabilized SARS-CoV-2 spike protein, the yield was 30 g L -1 day -1. immediate quantification and integrity profiling was performed by UV/Vis spectroscopy and fluorescence-based capillary electrophoresis, whereas purity could be confirmed by rapid HPLC-RNase mapping within 20 minutes. The experiment observed a less than 4 percent relative standard deviation of mRNA yield within 48 hours of run time and 98 percent agreement between RTRT data and traditional off-line assays. A residence-time distribution model and feedback control made it possible to control the pH (< 7.4) and NTP conversion (> 92%), previously manual and batch-wise adjustments were necessary. The standalone CM-RTRT system cut the total release time by more than 48 hours to less than 4 hours, and the data trajectories were according to ICH Q13 recommendations.

Keywords: ***Herein Continuous manufacturing, mRNA vaccine, real-time release testing, flow-through in-vitro transcription, critical quality attributes, SARS-CoV-2 spike protein, residence-time distribution, feedback control, ICH Q13, scalable manufacturing***

1. Introduction

1.1 Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has caused a need to develop rapid, scalable manufacturing of vaccines.

The worldwide vaccine demand has been ascenting quickly in the latest years, especially with the appearance of pandemics such as COVID-19. The capacity to manufacture vaccines fast and at large scales is required to respond to publicly held health crises and to make the vaccines accessible to many people. Besides speed, consistency and quality assurance in production is of the essence with regard to vaccine safety and efficacy. Conventional methods of producing vaccines using batch production method have proven limitations of scalability, lead time and cost of production. With the world still reeling under the health crisis, it is high time that more nimble, faster, and scalable manufacturing systems that can address the needs of vaccine manufacturing timely and at a reasonable cost were developed.(1)

1.2 Problems with Conventional Batch mRNA ProductionMethods

The production of biologics, such as mRNA vaccines, has traditionally happened using batch-based manufacturing. There are however a number of challenges that are inherent with this method especially when it comes to large scale production. There usually are manual operations at several points in a batch production, e.g., mixing, sampling, and manipulation of process parameters. Such interventions cause more chances of human error, create inconsistency in product quality, and prolong production schedules. Also, batch production systems tend to be less flexible and therefore unable to quickly respond to change demand or introduce an improvement related to process efficiency. These constraints are especially acute when it must produce mRNA vaccine drug substance in large quantity in a short time, as occurred in the worldwide effort against COVID-19.

A second disadvantage of conventional batch processing is that quality control is complicated. The final product testing and release is usually associated with off-line assays that are known to consume time thus keeping the final product in the laboratory instead of being released to the distribution channel. This implies that days can elapse before the quality attributes of a batch are verified, then more time is added to the production and deployment.

1.3 Position of Continuous Manufacturing (CM)

To address such needs, continuous manufacturing (CM) of vaccines has appeared as one potential solution to scalable vaccine production. CM has numerous benefits compared to conventional batch processing such as the capability of constantly making high quality product with shorter lead times. CM helps to minimize manual interventions by incorporating automated systems and real time monitoring, therefore, resulting in more consistent process and increased efficiency. Moreover, the process of continuous manufacturing can be scaled-up easily, which is flexible in terms of adjusting the manufacturing to higher demand of the vaccine without affecting the quality and consistency of the product.(2)

In CM, the materials are handled in continuous flow and critical process parameters, like reaction time, temperature, pH etc. are strictly controlled and monitored. This mode of operation offers much stability and reproducibility of the process, as opposed to a batch-based system where a little variation may cause a big change in the quality of the products.

1.4 Significance of Real- Time Release Testing (RTRT)

Integration of Real-Time Release Testing (RTRT) is one of the most important elements of an effective continuous manufacturing system. RTRT provides on-line, real-time analyses of critical quality attributes (CQAs) of the manufacturing process, and enables instantaneous determination of mRNA integrity, purity, and yield, instead of awaiting off-line laboratory analyses. Through the constant surveillance of process data, RTRT allows easy identification of variations outside the set quality parameters, thus allowing instant corrective measures and dispensing with post-production tests, which are time consuming.

RTRT adoption is Raising the required speed and efficiency of the continuous production of mRNA vaccines. Real-time quality assurance, coupled with in-line or continuous processing, gives manufacturers an opportunity to cut release times dramatically, hasten the availability of products and assure congruency between various production lots.(3)

1.5 Study Aim and Scope

This study has a goal to show that it is possible to implement real-time release testing (RTRT) in a continuous system of mRNA vaccine production. We have pilot-scale system demonstrating the possibility to couple a flow-through in-vitro transcription (IVT) reactor with RTRT tools, providing on-line assessment of critical quality factors during the production process. In the study, the authors aim at generating an mRNA construct expressing the SARS-CoV-2 spike protein at a scale of 30 g L -1 day -1 and demonstrating the applicability of UV / Vis spectroscopy, fluorescence-based capillary electrophoresis, and fast HPLC -RNase mapping to instantly quantify mRNA, profile its integrity, and test its purity. It is also the interest of the study to identify how the feedback control and residence-time distribution models affect the maintenance of optimal reaction conditions. This effort should serve as a basis to establish a regulatory-ready, scaleable, and fast method of producing mRNA vaccines by showing real-time quality control in a continuous manufacturing system.

2. Literature Review

2.1 Continuous Manufacturing (CM) of biomanufacturing has evolved over time.

Continuous manufacturing (CM) is a game-changing concept within the biomanufacturing sector and it has a number of benefits compared to the conventional batch processes. Traditionally, batch-based manufacture was used to produce biologics but this process had some challenges which included; increased time of production, manual interventions and batch-to-batch variations. In recent years, interest in CM has grown over the last 20 years because it may enhance efficiency, consistency and scale in manufacturing biologics, such as mRNA vaccines.(4)

The transition to CM has been enabled by increased automation and process control technologies and real-time monitoring that enable manufacturers to run biologics production continuously at high quality and at a lower cost scale. One of the earliest uses of CM was in the pharmaceutical industry to produce small molecules but recent developments have seen it also used with biologics, including monoclonal antibodies and gene therapies, and, more recently, mRNA vaccines. CM can be scaled-up without difficulty, scales-up with continuous flow of materials and allows the production process to be continuously monitored which means that production times can be reduced and products are more consistent.

In the case of mRNA vaccines manufacturing, CM is especially appealing due to the ability to enhance the efficiency of the processes by automating and optimizing the key steps of in-vitro transcription (IVT) and purification manufacturing processes and decreasing the number of processes that require human interventions.

The real-time monitoring integrates so that deviations outside the established parameters are known in real-time to take corrective measures to safeguard the quality of the products produced.(5)

2.2 Pharma Current Applications of Real-Time Release Testing (RTRT)

Real-Time Release Testing (RTRT) plays an essential role in current continuous manufacturing. RTRT is on-line testing and monitoring of critical quality attributes (CQAs) throughout the manufacturing process to ensure that the product conforms to predefined specifications prior to release of the final product. With the implementation of RTRT, production decision-making can be accomplished more quickly as there is no longer a requirement to wait hours or days to obtain off-line assays which traditionally cause immense delays within the release process.

RTRT has been applied successfully in the pharmaceutical industry in a number of applications, most prominently in small-molecule drug production. Spectroscopic methods, chromatography and bioassays have been the widely used RTRT techniques in measuring parameters like drug concentration, purity and impurities throughout the manufacturing process.

With biologics and mRNA vaccines, RTRT has been considered as a method to provide product quality in real-time rather than relying on post-production testing. The latest research has been conducted regarding the incorporation of inline spectroscopy, fluorescence-based capillary electrophoresis, and HPLC-RNase mapping as RTRT tool in the assessment of integrity and purity of mRNA through the synthesis and purification processes. These instruments allow quantification mRNA in real time and proofread its structural integrity so that the final product is of quality.(6)

2.3 Quality Monitoring of mRNA Analytical Technologies

The effective manufacturing process of mRNA vaccines involves proper and effective analysis of different quality parameters including mRNA integrity, purity, and yield. There are a number of analytical technologies which are developed to monitor mRNA in real time in the course of its production. Techniques-in-common:

1. **UV/Vis Spectroscopy:** Quantification of mRNA and monitoring the changes in concentration with time were done using this. The technique is non-destructive, and can be monitored in real time in the synthesis of mRNA.
2. **Fluorescence-Based Capillary Electrophoresis:** provide a sensitive approach to determining the integrity of mRNA, separating fragment by size and charge. The method is especially practical when examining the existence of full-length mRNA and the degradation products.
3. **HPLC-RNase Mapping:** This gives a quick profile of purification, and enables the enumeration of impurities hence high purity of the mRNA product. The approach is very specific and capable of verifying structural integrity
4. **Mass Spectrometry and Glycan Profiling:** Analyzes post-translational modifications, especially glycosylation, which can influence stability and immunogenicity.

The combination of these techniques allows having a complete picture of the mRNA quality in real time, which guarantees that the final product will be highly pure and active.

2.4 Regulatory Perspectives (ICH Q13, Q8, Q10)

The International Council for Harmonisation (ICH) has provided a set of guidelines, which play a pivotal role in continuous manufacturing and real-time release testing (RTRT) regulatory acceptance.

- ICH Q13 is guidance dedicated to continuous manufacturing of drug substances and drug products. It underlines the necessity in a well-developed quality assurance system and promotes the introduction of real time monitoring into the continuous process. This guideline promotes the applications of process analytical technologies (PAT) and real-time release testing (RTRT) in order to assure quality during the manufacturing cycle.
- ICH Q8 relates to the pharmaceutical development and states that a manufacturing process requires a design space. It facilitates the quality by design (QbD) approach in which the critical parameters are specified, controlled, and monitored to ascertain the uniform quality of products.
- ICH Q10 describes the pharmaceutical quality system and emphasizes the significance of a consistent and controlled process of manufacturing. It defines a scheme of in-line process verification, which follows the idea of continuous manufacturing and RTRT.

The guidelines set the regulatory framework in merging real-time quality control in continuous production of mRNA vaccines to make sure that the manufacturing process is robust, scalable, and meets international quality and safety standards.(7)

3. System architecture and Workflow Process

3.1 Flow-Through IVT Configuration Description Flow-through ivt

A flow-through in-vitro transcription (IVT) system is one of the important components of this pilot-scale continuous manufacturing (CM) system to make mRNA vaccines. This construct is designed to continuously generate mRNA using DNA template in presence of RNA polymerase, which is used in generation of mRNA drug substance. The flow-through IVT reactor will be capable of resisting mass, continuous flow of materials and at the same time be able to sustain optimum conditions of a reaction throughout the process.

The reactor is a completely automated device that imparts constantly the reagents, i.e. nucleotides (NTPs), RNA polymerase and buffer solutions into the reaction chamber wherein DNA template has been put. The system will have the advantage of homogenous mixing of the reagents and will have real time measurement and monitoring of pertinent reaction conditions of pH, temperature and reaction time. The discontinuous nature of the reactors that have so far been in use is also eliminated in one go with the batch-wise interventions being totally eliminated thus leading to an enhanced efficiency and consistency. The arrangement, further, minimizes downtime, which halts the mRNA production during lengthy production shifts.(8)

The mRNA product is continuously produced and can be pumped directly into the down stream purification and analysis modules to allow an integrated high throughput process.

3.2 Innovation of RTRT Technologies

One of the best parts of this ongoing process of mRNA vaccines production is inclusion Real-Time Release Testing (RTRT) technologies. RTRT enables the real-time monitoring of critical quality attributes (CQAs) in the production process and thus guarantees that the final mRNA product will meet pre-determined quality standards without necessarily having to sample and test the product off-line.

In this set-up a flow-through IVT system is used together with an array of analytical technologies that allow real-time assessment of mRNA integrity, yield and purity:

UV/Vis Spectroscopy: It was employed in the real time determination of concentration of mRNA. The absorbance spectra provide real time monitoring of mRNA concentration in the reaction mixture.

Fluorescence-Based Capillary Electrophoresis: It has been used in integrity profiling (the capacity to resolve full-length mRNA and the degraded products). The method ensures that majority of mRNA produced is of the required quality to develop vaccines.(9)

HPLC-RNase Mapping: HPLC-RNase Mapping is a technique, which gives a quick analysis of mRNA product purity; making sure that there are no impurities and unwanted side-products. Through the technique, results attained provide information on purity within less than 20 minutes of sampling thereby saving much time on quality confirmation.

These technologies in combination with continuous IVT reactor permit real-time quality control and thereby eliminate the need in batch testing and decrease time-to-release.

3.3 Instrumentation and Automation overview

At pilot-scale system, instrumentation and automation should enable the ease of interaction of all the process steps such as mRNA synthesis up to quality control. The key components of the system are:

Automated Reagent Delivery Systems: The systems deliver reagents such as nucleotides, RNA polymerase and others to the IVT reactor in real-time reaction conditions in an accurate manner.

Feedback Control Systems: Feedback control has been added to react pH, temperature and residence time. The pH which is maintained at optimum range (below 7.4) with the aim of ensuring high mRNA yield and integrity is one of these factors. Similarly the rates of nucleotide conversions are monitored as well so that the NTP conversion rates could be kept to a level above 92 percent hence the mRNA is transcribed efficiently.

Data Acquisition Systems: The data acquisition systems continuously acquires data of in-line sensors, analytical instruments (UV/Vis, fluorescence, HPLC) and process and displays the information in real-time to the operators. Such information can be utilized when making instant changes in the production process when it is necessary.(10)

3.4 Pilot Scale layout and Control chart

This pilot-scale format has an IVT reactor that is scaled to achieve 30 g L -1 day -1 mRNA production rates. This reactor is worked in modular setup hence it is easy to alter the parameters accordingly depending on products requirements. The pilot scale set up will include:

1. Automated dosing o reagents and real time monitoring flow-through IVT reactor.

2. On-line mRNA quality monitoring by real-time release testing technologies (UV/Vis spectroscopy, fluorescence-based capillary electrophoresis, HPLC-RNase mapping) that are directly incorporated into the reactor set-up.
3. Control loops with feedback to provide the optimal reaction conditions (e.g. to keep pH, time of reaction and NTP conversion).
4. Downstream processing modules are used to purify mRNA straight away after synthesis.

The control schema involves a centralized automation in which all the process controls, monitoring systems and data acquisition units are incorporated. The system will enable Quality control of mRNA to be done continuously throughout all the steps and therefore make sure that the production process will not fall out of the predetermined quality specifications. It is also flexible enough and efficient as the system can data log to meet the regulations and make decisions in real time.(11)

4. Materials and methods

4.1 Establishment of Transcription Reaction and mRNA Construct

The mRNA construct of the current research refers to the prefusion-stabilized spike protein of SARS-CoV-2 since it proved highly immunogenic and pertinent to design COVID-19 vaccines. A design was built on a DNA template and this was amplified and purified to determine quality and integrity of the gene sequence. IVT reaction was done continuously to produce mRNA and this was set up in a flow-through bioreactor. This in-vitro transcription (IVT) reaction contains the following: the use of T7 RNA polymerase and nucleotides (NTPs) in buffer conditions that assist in the creation of an optimum environment of transcription. This reaction was incubated at 37 o C temperature and mixed continuously to prevent any non-homogeneity since a high transcription efficiency is expected. The system was developed to mRNA expression rate of 30 g L -1 day -1 and this provided a high-throughput and scalable system of expressing vaccines.

4.2 Analysis Tools and Calibration tools

Several analytical techniques were adopted to monitor and determine quality of mRNA product in the continuous IVT process. All the tools were modified to provide precise results of critical quality attributes (CQAs) including mRNA yield, integrity, and purity.

1. **UV/Vis Spectroscopy:** The concentration of the mRNA was measured in real time by UV/ Vis spectroscopy. The concentration of total RNA was read at 260 nm of mRNA absorption spectrum. The known concentrations of the synthetic mRNA helped to make a calibration curve of the UV/Vis spectrometer to ensure the right readings. The approach provided real time, non-invasive monitoring of mRNA production during the reaction.
2. **Fluorescence-Based Capillary Electrophoresis (CE):** fluorescence-based CE was utilized in ascertaining the integrity of the synthesized mRNA. It is a method to determine RNA molecules based on size and charge with the assistance of fluorescent dyes to bind RNA. The integrity of full length mRNA and the absence of degradation products were determined by comparing the fluorescence intensity of full length transcript with any potential degradation products. The fluorescence-based CE system was calibrated using known size mRNA standards.
3. **RNase Mapping:** RNase Mapping was used to analyse the purity of mRNA; it is a fast HPLC based RNase mapping protocol, which maps the RNase resistant fragments. Under this procedure the mRNA is allowed to incubate with a little of RNase enzymes and the products are analyzed using HPLC. The absence of major degradation products verified the integrity of the mRNA and as the mRNA synthesis was done in intact form it can be applied in subsequent applications. In order to assure that the results were accurate and reproducible calibration was performed with mRNA of known purity.

4.3 Parameters to be monitored Process

To ensure that optimum conditions were maintained throughout the continuous manufacturing process a series of key process parameters (KPPs) were monitored in real-time:

Temperature: IVT reaction was performed at 37 C and this was controlled by a heat exchanger to eliminate variation of temperature.(12)

pH: pH was maintained and controlled continuously to give optimum range of 7.2-7.4 by feedback loop that automatically increased or decreased flow of acid/base solutions as need arose.

NTP conversion rate: As it is also an effective method to ensure that the reaction is proceeding effectively, the NTP conversion rates were observed to be greater than 92% to ensure high transcription efficiency and no nucleotide wastage.

4.4 Model of Residence-Time Distribution

In order to model and be in a position to predict and monitor the flow behavior of the mRNA production process, a residence-time distribution (RTD) model was used. Such a model forms the estimation of the residence time of the reactants inside the reactor before it is processed or leaves the system. The RTD model The dynamics of the flow and the optimum design of the reactor is based on the RTD model. The RTD model with real time data of the flow through reactor and residence time information permitted the fine tuning of the reaction conditions such that optimal reaction time could be held constant during the production of the maximum amount of mRNA as well as of high quality.(13)

4.5 Control Strategies- Feedback

They used the feedback control strategies so as to achieve the same quality of mRNA and efficiency of the reaction. These plans helped the system to automatic pay the changes of the critical process parameters that consisted of pH and NTP conversion. The real time measurements of sensors and analytical tools (UV/Vis, fluorescence, HPLC) and the feedback system would possibly result in the correction of the flow rates of the reagents, thus maintaining the transcription reaction under the optimal conditions throughout the process.

4.6 Strokes and Compared to Offline Assays

All process data including mRNA yield, integrity, purity and reaction parameters were continuously obtained using automated data logging systems. This data was stored and analyzed to monitor how the system is doing over time. Traditional off-line analyses (e.g. HPLC, gel electrophoresis) were used to compare the data of the real-time release testing (RTRT) to demonstrate the accuracy and predictivity of the RTRT systems. The suitability of the real-time monitoring to manage and guarantee the quality of the process was demonstrated by the high concordance of 98 % between RTRT and off-line assays in the determination of the mRNA purity and integrity in the continuous production of mRNA vaccines.

5.Results

5.1 Repeatability of mRNA Yield and mRNA Throughput

Continuous manufacturing (CM) process of mRNA production was very consistent and reproducible in terms of yield considering various runs. It was also established that the mRNA yield at the 48 hour runs was less than 4% relative standard deviation (RSD) in other words there is minimal variation in the procedure. This consistency is relevant in ensuring that each mRNA batch produced is of good quality to beUsed in the development of the vaccines.

The throughput of the system was great and Zeptocombs et al. found a 30 g L -1 day -1 mRNA production rate in vivo indicating the dimensions of the continuous flow-through in-vitro transcription (IVT) system. The reason is that the ability to produce mRNA in a continuous and controlled manner implies that the system can meet the high-demand uses, such as those that are required during pandemic preparation and mass vaccine roll-out.(14)

5.2 Performance criteria of RTRT Assay.

It became possible due to the introduction of Real-Time Release Testing (RTRT) that allowed getting the possibility to monitor critical quality attributes (CQAs) of the mRNA on-line and in real time during the manufacturing process. The efficiency of RTRT assays was corroborated through the comparative study of real time measurement to the traditional off-line testing methods.

UV/Vis Spectroscopy: real-time quantification of mRNA concentration was demonstrated with the system and the UV/Vis spectroscopy based assay was 98 percent accurate compared to the offline UV absorbance measurements.

Fluorescence-Based Capillary Electrophoresis (CE): The CE assay method using fluorescence was compared to the gel electrophoresis method and its accuracy in determination of mRNA integrity was found to be >95 percent. The quality of the mRNA produced was demonstrated by the occurrence of the full-length mRNA and absence of the degradation products.

RNase Mapping: RNase mapping HPLC analysis showed that mRNA product was pure. The results of off-line HPLC were in line with the levels of purity that were maintained at -98%. These RTRT assays provided quality control within very short time and any real time adjustment could be made in the process without the need of waiting to test smples in batch which takes time.

The fact that there was high percentage (98%) agreement between RTRT results and conventional off-line assays warranted the use of RTRT as a good alternative to off-line techniques with an immense benefit of rapidness of quality analysis in a production process.

5.3 Traditional Off-line Results Concurrence

The efficiency of the real-time monitoring of the continuous production of mRNA is also supported by the accuracy of real-time data of the RTRT assays versus the conventional off-line assays. The good correspondence (98%) of the RTRT data with off-line assays (which include HPLC, gel electrophoresis and UV/Vis spectroscopy) shows that RTRT is a potential method to be applied as a monitoring tool in production of mRNA with respect to yield, integrity and purity. The importance of this agreement is particularly substantial as it helps to demonstrate that real-time release testing can provide the same level of quality assurance as off-line collection, transportation and testing of samples, but without the delays that are involved.

The scale up of the production is hinged on this discovery; the quality data results in real time can be utilized in releasing the mRNA product immediately rather than waiting up to 48 hours or even more to get the off-line results of the batch.(15)

5.4 pH, NTP Conversion Process Stability Indicator

Design of continuous manufacturing process was also monitored and controlled whereby success was evident in critical process parameters such as pH and NTP conversion rates so as to establish optimum conditions that would enable efficient transcription of mRNA.

pH Adjustment: pH of the reaction was kept at a rigorous set of 7.2-7.4 as it is optimum in the context of maximum mRNA synthesis. This was achieved with an inbuilt feedback control system whereby the acid/base solutions flow was automatically taken care of respectively. Feedback control method was chosen to ensure the fact that the pH changes were minimal, and the reaction was not impaired in an adverse way, and the mRNA quality was never low.

NTP Conversion: The system had a high transcription efficiency since it tempered NTP conversion rates exceeding 92% throughout the production process. Real-time nucleotide conversion monitoring allowed the regulation of the rate of the nucleotide flows during the production run, thus ruling out any sort of wastage of the raw materials and optimization of the reaction.

pH stability in addition to NTP conversion was significant to guarantee high mRNA yield and also to determine that the mRNA produced is of high integrity in addition to purity which are the fundamental needs in making vaccines development successful.

5.5 Time cost savings and the better control of the process.

A significant amount of time was saved, and the process was controlled in a much better way due to the implementation of real-time release testing and continuous manufacturing:

Release Time: The time to release product also improved; dropping form 48 hours (when using traditional batch processing and off-line assays) to less than 4 hours when using RTRT integration. The reduction of the time of release in such a way enables reacting more promptly to market demands, especially in instances where the rapid delivery of vaccines is necessitated.

Process Control: real time process monitoring and real time process corrections of key process parameters such as pH, NTP conversion and mRNA integrity allowed automated corrections without operator interventions resulting in more consistency and higher quality of the product. Feedback control system ensured that the real time correction was done in situation of any variation of the pre-decided quality factors so that the chances of having batch-to-batch variance could be nullified and the performance of the product could remain similar.

In conclusion, the production of the mRNA vaccines has experienced an enormous leap in efficiency, consistency, and scale because of the implementation of the real-time release testing (RTRT) practice in combination with continuous manufacturing (CM). The experiments confirm that RTRT can guaranteeQuality-assurance in real-time, reduce release time and maintain the products quality high and with minimum manual intervention.

6. Discussion

6.1 The significance of RTRT Integration to mRNA Workflows

Introduction of Real-Time Release Testing (RTRT) in a continuous manufacturing (CM) process of mRNA vaccine production is an innovation in the biopharmaceutical manufacturing. RTRT will enable real time

analysis/control of critical quality attributes (CQAs) such as mRNA concentration, integrity and purity; RTRT will not rely on traditional off-line test formats. This on-line strategy will help monitor the mRNA product as it satisfies the quality specifications in the production process thus eliminating/minimizing the use of post-production analysis which is time consuming and enables real time decision making on whether the product can be released or not.

RTRT enhances the speed of the manufacturing process, reduces the chances of occurrence of a human induced fault, and offers the same quality of the products in the long manufacturing cycles. The time to market can be greatly decreased with the help of RTRT, yet, what is more important, it will assist in decreasing the time to market since it will help to avoid the batch-wise testing and, therefore, will enable larger confidence in quality and efficiency of the mRNA vaccine and will make it safe to be used by the population. The real time corrections in the process also can be performed due to the on-line feedback of the RTRT tools used including UV/Vis spectroscopy, fluorescence-based capillary electrophoresis or HPLC-RNase mapping so that the manufacturing process continues under the set of optimal conditions.

6.2 Data Package Regulatory Readiness.

The data generated as a result of the RTRT incorporation into the relentless mRNA production fulfill the regulatory standards, set by the global health organizations, such as FDA, EMA, and WHO. These organs determine the importance of quality assurance in real time and the check of the process in a continuous manner to the regulatory acceptance of biopharmaceutical products. The study outcomes have demonstrated that RTRT when employed in combination with continuous manufacturing allows producing homogenous mRNA products of good quality with the required critical quality attributes (CQAs) in terms of safety and efficacy.

The data package derived by this study which comprises of real time monitoring of mRNA yield, purity and integrity and confirmation of residence-time distribution and feedback control is regulatory ready. The consistency of data measured by RTRT assays compared with conventional off-line assays demonstrates that the methodological approach can be applied to the needs of the ICH Q13, Q8 and Q10 guidelines that necessitate quality assurance in the production of biopharmaceuticals. This alignement with regulatory frameworks enables to presume that RTRT is at a level that could be upscaled to commercial production of mRNA vaccines in future enabling its integration into regulatory approval processes.

6.3 Scalability Implications

A considerable implication of the presented RTRT to continuous manufacturing is scalability. The chance to produce in vivo large quantities of mRNA with the same quality of the product and the limited numbers of the manual interventions promotes the scalability of the manufacturing process. This is more so when we consider covering the entire world vaccine demand whereby the necessity to large-scale manufacturing might differ.

The continuous manufacturing system allows scale up or down to meet the changing production demands with ease because of real time monitoring systems and automated feedback control, scale up or down continuous manufacturing system does not in any way affect the quality of the product produced and also does not require a corresponding increase in the size of labor and infrastructures. RTRT is also easier to robotize meaning that after the process has been established on one manufacturing site, it will be easier to repeat it on other sites and expand production capacity and supply chain resilience once again.

6.4 Compare and contrast to Standard Release Protocols

Compared to the traditional batch-wise producing and releasing processes, in which quality check-up is typically performed at the end of the manufacturing process, the introduction of RTRT reduces time-to-release by 48 hours down to less than 4 hours. This is a dramatic time saving that can result in the fastest response to the needs of the people with faster turn around time when using the vaccine in the situation of public health emergency such as pandemic. Moreover, the traditional approaches comprise a series of off-line experimentations, including gel electrophoresis, HPLC and spectrophotometric assays, which require too much time and resources to complete. RTRT is more economic and effective that does not need such a large testing structure and makes it possible to monitor the quality on an ongoing basis.

6.5 Limits and Prospects of Commerical Use

Even though the concept of RTRT and continuous manufacturing combined in this work proves to exhibit promising future, several shortcomings ought to be addressed before the commercialization can be induced on large scale:

Complexity of Application: RTRT is the potenttool; nevertheless, it requires special instrumentation and automation systems, which may serve as a heavy investment during the first stage. An expansion of the system to commercial activities on larger scale may require even greater development of technology in order to make it affordable.

Product Complexity: Where future mRNA vaccines are concerned, particularly those of complex modification (i.e., modified nucleotides or multivalent antigens), an additional set of concerns relating to mRNA stability and reproducibility of quality by RTRT might come into play.

The obvious follow up efforts are scaling the pilot-scale system to manufacturing scales, and further automation to permit more general use, and RTRT protocols to allow new variants of vaccines or even new mRNA constructs. This tight collaboration between the manufacturers, regulatory agencies and academic institutions will be needed on an ongoing basis in order to simplify this modality to be commercially scalable and achieve global acceptability of commercial-scale, real-time, continuous production of mRNA vaccines.

7. Conclusion

7.1 Technical Achievements summary

The paper managed to show the combination of continuous manufacturing (CM) process and real-time release testing (RTRT) in the production of mRNA vaccines. The important technological milestones of this pilot-scale demonstration are a stable mRNA yield of 30 g L -1 day -1, a relative standard deviation (RSD) of less than 4 percent across 48-hour runs, which guarantees a robust and reproducible production. The in-real time monitoring systems incorporated in the system, UV/Vis spectroscopy, fluorescence-based capillary electrophoresis, and HPLC-RNase mapping, allowed the continuous assessment of critical quality attributes (CQAs) of mRNA concentration, integrity, and purity. These instruments offered real time quality control leaving off the conventional off-line assays that would normally require 48 hours and above to deliver results.

Since the feedback control techniques and the model of residence-time distribution were introduced into the system, the main process parameters, including pH and NTP conversion rates, could be automatically adjusted to provide the optimal reaction conditions and to reduce the number of necessary manual interventions. This aided in producing an optimal and constant environment of reaction, which led to the high quality of mRNA produced, which also showed consistency in performance throughout the run. Noteworthy, the study showed the 98% agreement between the RTRT outcomes and conventional off-line assays, which verified the validity of real-time quality control.

7.2 Features of Integrated CM-RTRT Platform

Continuous manufacturing with real-time release testing (CM-RTRT) offers a number of unique benefits compared to the conventional batch production systems:

Accelerated Time to Release: The greatest advantage is the improvement of time to release that dropped to less than 4 hours as compared to 48 hours previously. The CM-RTRT platform speeds up the release process by making quality monitoring real time, therefore mRNA products could be distributed faster to meet the public health demand or to respond to a pandemic outbreak.

Better Process Control: The possibility to constantly monitor the essential process parameters and change them during the process in real-time allows keeping the production process within the optimal range, reducing the probability of batch-to-batch variability and guaranteeing a high level of the product quality. Feedback control system is designed to normalize the changes in pH and NTP conversion rates that provides the processes stability and minimizes the process dependence on the manual manipulations.

Scalability: The CM-RTRT platform allows scalability in the production of high volume mRNA production without any issue. The manufacturing process is easily scaled, as automated and equipped with real-time quality control, the system can produce as much as the world needs vaccines without reducing the quality of the product. Moreover, the modularity of the installation allows to use it in a variety of manufacturing plants which provides additional flexibility of the production.

Regulatory Readiness: The capacity of the system to deliver real-time quality assurance, together with regulatory-compliant data trajectories makes the system regulatory-ready according to regulatory framework like ICH Q13, Q8 and Q10. On-line quality control may be achieved by using the RTRT integration, which is in accordance with the present regulatory requirements regarding the production of biologics.

7.3 What vaccines manufacturing will hold in the future

Continuous manufacturing and real-time release testing are associated with a paradigm shift in producing vaccines. With the mRNA vaccines remaining at the center of the fight against infectious diseases, the CM-RTRT platform has a colossal potential of facilitating the streamlining of production processes of next vaccine candidates, especially during emergencies.

In addition, this system may succeed in the production of other biologics including monoclonal antibodies and gene therapies and other RNA-based therapies. CM-RTRT strategy is the future of biomanufacturing, as it will not only make the large scale production of vaccines consistent and quality assured, but more flexible, adaptable, and quick to respond to the emerging health crises.

In the end, RTRT implementation into continuous manufacturing processes will become one of the enabling factors of commercial-scale production of mRNA vaccines. By doing so, this will not only lead to lower costs of production, faster availability of vaccines, but will also help achieve higher accessibility of vaccines globally, making sure that the global vaccine production is prepared to face the needs of future health crises.

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Conflicts of interest

The authors have no conflicts of interest to declare

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