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Analysis of the Effects of Different Synthesis and Processing Methods on Circular RNA Integrity, Protein Expression, and Removal of Immunogenic Impurities

Shao Wang[1,2]*, Tzuen Yih Saw[1], Zebin Hong[3], Kuo Chieh Liao[1], Haiwei Song[3], Yue Wan[1]

[1]Genome Institute of Singapore, Agency for Science, Technology and Research, 138672 Singapore, Singapore
[2]Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC V6T 1Z3, Canada
[3]Institute of Molecular and Cell Biology, Agency for Science, Technology and Research, 138673 Singapore, Singapore

***Corresponding author:** Shao Wang, wsteve20@student.ubc.ca

Abstract: Circular RNAs (circRNAs) are emerging as a promising alternative to messenger RNAs (mRNAs) in gene delivery applications due to their enhanced stability and translation. Developing circRNA-based therapeutic platforms requires efficient manufacturing of circRNA with broad scalability. However, the permuted intron-exon (PIE)-based circRNA production commonly used to date involves complex RNA synthesis, circularization, precursor RNA digestion, and impurity removal steps that have limited practical applications. While co-transcriptional circularization could effectively streamline circRNA production, and both cellulose/phosphatase treatment and high-performance liquid chromatography (HPLC) have demonstrated their reliability in mRNA manufacturing, their potential effects on the quality, translation, and reactogenicity of circRNA remained to be fully investigated. Here, using circRNAs systematically manufactured through three independent workflows, we comprehensively examined the utilities of these RNA synthesis and processing methods in circRNA production by comparing the integrity, translation, and immunogenicity of their circRNA products. We began by manufacturing a mNeonGreen (mNG)-encoding circRNA through these workflows and subsequently assessed circRNA integrity via E-gel EX electrophoresis. Protein expression was then monitored in HEK 293T, A549, and DC2.4 cells at 72 hours post-transfection. Finally, we evaluated the immunogenicity of these circRNAs by measuring their interferon beta (IFN-β) induction in A549 cells at 4 hours post-transfection. Using HPLC purification over cellulose and phosphatase treatment resulted in 10–14% higher circRNA enrichment by reducing nicking associated with processing conditions. Protein expression remained consistent across circRNAs from different workflows ($P > 0.05$), demonstrating that co-transcriptional circularization produces circRNA with translation levels comparable to those obtained from the conventional PIE method. Moreover, both cellulose/phosphatase treatment and HPLC purification effectively minimized IFN-β induction of the purified circRNAs, confirming their reliability in removing immunogenic impurities introduced during *in vitro* transcription and their compatibility with the co-transcriptional circularization strategy. Collectively, our results provide valuable insights for improving the production efficiency and scalability of circRNA manufacturing that are crucial for addressing key bottlenecks in the development of circRNA-based therapeutic applications.

Keywords: Nucleic acid therapeutics; RNA manufacturing; Circular RNA; Gene delivery

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1. Introduction

The unique capabilities of messenger RNA (mRNA) therapeutics in the repaid design, production, and distribution of therapeutic mRNA have demonstrated substantial potential for the treatment of various diseases and applications in preventative medicine [1-3]. However, the efficacy of mRNA in sustaining stable translation over time is considerably limited by its poor intracellular stability [4,5]. Circular RNAs (circRNAs), characterized by their 5' and 3' ends being covalently linked together [6], represent a promising alternative. Recent advancements in circRNA engineering have significantly enhanced their ability in translation, potentially providing 2–5 times greater stability and 3–5 times higher protein production over an extended period compared to mRNA [7-9]. Furthermore, the optimization of the permuted intron-exon (PIE) RNA circularization strategy has significantly improved the manufacturability of long circRNAs with varied coding regions [8]. To date, the development of a circRNA-based therapeutic strategy requires reliable production of circRNA with robust translation and minimal immunogenicity, both of which are crucial for the efficacy and safety of administration [10]. However, achieving these requirements through the manufacturing of high-quality circRNA via conventional PIE strategy necessitates complex RNA synthesis and processing steps, which have hindered its utility in therapeutic applications.

Conventional manufacturing of circRNA via the PIE RNA circularization strategy comprises of four steps: RNA synthesis, circularization, digestion of uncircularized RNA precursor, and the removal of immunogenic impurities (**Table 1**). A precursor linear RNA is first synthesized via *in vitro* transcription to encode an internal ribosome entry site (IRES) and a coding region flanked by an engineered split *Anabaena* pre-tRNA group I intron [8]. This precursor RNA undergoes heating in the presence of GTP and Mg^{2+} to induce splicing of group I intron and circularization [8]. Crude circRNA is subsequently treated with RNase R, which selectively degrades linear RNA species through 3'→5' exoribonuclease activity [11,12]. Finally, a combination of cellulose and phosphatase treatment (i.e. Quick CIP) or RNA size selection via high-performance liquid chromatography (HPLC) is employed [13,14] to remove any immunogenic impurities introduced during *in vitro* transcription, including short double-stranded RNA (dsRNA) [15] and RNA with 5' triphosphate motif present due to incomplete RNase R digestion [14]. Additionally, the RNA synthesis and circularization could be combined into a single-step reaction using heat-stable T7 RNA polymerase (New England Biolabs) by performing *in vitro* transcription at 50ºC instead of the conventional 37ºC, allowing for the co-transcriptional splicing of the permuted group I intron and circRNA formation. Despite the importance of impurities removal in minimizing innate immune response associated with interferon (IFN) signaling [16], it remains unclear whether the use of cellulose and phosphatase treatment or HPLC purification would affect circRNA integrity, translation, and immunogenicity. Furthermore, the effects of co-transcriptional circularization on these aspects of circRNA have yet to be fully investigated.

In this present work, we aim to comprehensively assess the impact of different synthesis and processing methods available for circRNA manufacturing on the RNA integrity, translation, and immunogenicity, including co-transcriptional circularization and removal of immunogenic impurities via cellulose/phosphatase (Quick CIP, New England Biolabs) treatment or HPLC purification. We began by manufacturing a circRNA reporter encoding mNeonGreen (mNG) via three different workflows involving these different synthesis and processing

approaches as detailed in **Table 1**. We then assessed circRNA integrity using E-gel EX electrophoresis and monitored protein expression of these circRNAs in HEK 293T, A549, and DC2.4 cells at 72 hours post-transfection. Finally, we measured the levels of interferon beta (IFN-β) induced by these circRNAs in A549 cells at 4 hours post-transfection. Our results provide valuable insights for enhancing the production efficiency and scalability of circRNA manufacturing, which are crucial for overcoming key bottlenecks in the development of circRNA-based gene delivery platforms.

Table 1. Overview of circRNA manufacturing workflows

Workflow no.	RNA synthesis and circularization	Uncircularized precursor RNA removal	Immunogenic impurities removal
1	IVT + circularization	pA + RR	Cellulose/CIP treatment
2	IVT + circularization	pA + RR	HPLC purification
3	Co-transcriptional circularization	RR	HPLC purification

Abbreviations: IVT: *In vitro* transcription; pA: poly(A) tailing; RR: RNase R digestion

2. Material and methods

2.1. Conventional circRNA synthesis and processing

CircRNA precursor was synthesized via *in vitro* transcription reaction from linearized plasmid using T7 High Yield RNA Synthesis Kit (New England Biolabs). After *in vitro* transcription, the DNA template was removed with TURBO DNase (Invitrogen) treatment for 20 mins, and the RNA was column purified using Monarch RNA Cleanup Kit (New England Biolabs). RNA circularization was carried out via permuted intron-exon (PIE) reaction as described previously [8]: 500–750 ng/μL RNA in reaction buffer (2 mM GTP, 10 mM $MgCl_2$, 50 mM Tris-HCl, pH = 7.0) was incubated at 55ºC and 300 rpm for 15 mins and then column purified. Subsequently, uncircularized RNA and spliced intron byproducts were poly-adenylated using *E. coli* Poly(A) Polymerase (New England Biolabs) according to the manufacturer's instructions and were column purified. To enrich circRNA, 500 ng/μL processed RNA in RNase R reaction buffer (100 mM KCl, 1 mM $MgCl_2$, 20 mM Tris-HCl, pH = 7.5) was combined with RNase R in storage buffer (50% (v/v) Glycerol, 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 50 mM Tris-HCl, pH = 7.5) added to a ratio of 0.024 μg RNase R per μg of RNA. The reaction was incubated at 37ºC and 300 rpm for 30 mins and the RNA was column purified. DsRNA removal using cellulose-based purification was subsequently performed as described previously [13]. Finally, to remove 5' triphosphate motifs, the RNA was treated with Quick CIP (New England Biolabs) for 20 mins according to the manufacturer's instructions and was column purified.

2.2. E-gel EX electrophoresis

100 ng RNA samples and 2 μL ssRNA ladder in 10 μL were first mixed with 10 μL 2X RNA loading dye (New England Biolabs) and incubated at 65ºC for 5 mins. Each mixture was then loaded onto 2% E-Gel EX Gel and was run on the E-Gel Powersnap Electrophoresis System (Invitrogen) with program E-gel EX 1–2% for 12 mins. After electrophoresis, gels were cooled on ice for 10 mins and images were captured using ChemiDoc XRS+ System (Bio-Rad) with program SYBR-Gold.

2.3. HPLC purification

RNase R-treated RNA in either RNase R reaction buffer or nuclease-free water was first prepared to a final

concentration of 0.1 M TEAA pH = 7.0. The RNA was then injected into AKTA pure 25 M (Cytiva) with column PLRP-S 4000A (Agilent). Separation of circRNA was carried out via gradient elution starting from 38% buffer A (0.1 M TEAA pH = 7.0) and 62% buffer B (0.1 M TEAA, 25% acetonitrile pH = 7.0) at the flow rate of 10 mL/min and was monitored via UV absorbance at 260 nm. The fraction with enriched mNG circRNA was eluted at 59% buffer B and the RNA was subsequently pre-concentrated in nuclease-free water via tangential flow filtration (TFF) (μPulse, formulatrix).

2.4. Co-transcriptional RNA circularization and circRNA processing

For co-transcriptional RNA circularization, *in vitro* transcription and PIE splicing were carried out in a single-step reaction using Hi-T7 RNA Polymerase and Ribonucleotide Solution Mix (New England Biolabs). The reaction was set up according to the manufacturer's instructions and was incubated at 50ºC for 2 hours. Template DNA was removed using TURBO DNase as described above, and the RNA was purified via TFF. Processed RNA was subsequently treated with RNase R and purified via HPLC as described above.

2.5. Tissue culture and transfection

HEK 293T, A549, DC2.4 cells (ATCC) were maintained at 37ºC and 5% v/v CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% v/v penicillin-streptomycin (Gibco). Cells were tested negative for mycoplasma and were passaged every 3–4 days. For fluorescence assay, 4×10^5 HEK 293T, 1.5×10^5 A549, and 3×10^5 DC2.4 cells were seeded per well in a 12-well plate 24 hours before transfection. For immunogenicity assay, 2×10^5 A549 cells were seeded as described above. Each well was transfected with 600 ng RNA using Lipofectamine MessengerMAX (Invitrogen) according to the manufacturer's instructions.

2.6. Protein expression analysis

Expression of mNG was determined via fluorescence measurement. After 72 hours post-transfection, each plate was loaded onto the TECAN microplate reader and the fluorescence signal was captured at an excitation wavelength of 485 nm and emission of 535 nm with an integration time of 40 μs.

2.7. RNA extraction, cDNA synthesis, qPCR

Total RNA was isolated at 4 hours post-transfection using TRIzol (Invitrogen) according to the manufacturer's instructions. Genomic DNA was subsequently removed using TURBO DNase and the reaction was column purified. After DNase treatment, cDNA synthesis was carried out using iScript cDNA Synthesis Kit (Bio-Rad) from 1 μg isolated total RNA. To determine IFN-beta level, IFNB1 (p1: CTCCTGTTGTGCTTCTCCACT, p2: GGCAGTATTCAAGCCTCCCA) and SDHA (p1: TGGGAACAAGAGGGCATCTG, p2: CCACCACTGCATCAAATTCATG) were respectively amplified using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), and signal was captured using QuantStudio Real-Time PCR System (Thermofisher). IFNB1 signal was normalized to that of SDHA.

2.8. Data analysis

RNA gels were processed using Image Lab (Bio-Rad, version 5.2) and band intensity was determined using Gel Analyzer (GelAnalyzer.com, version 19.1). Data was visualized in Prism (GraphPad, version 10.1.1) and was

presented as means ± standard deviation (SD*). P* < 0.05 was considered statistically significant.

3. Results

3.1. HPLC purification enhances circRNA integrity compared to cellulose/CIP treatment by minimizing RNA nicking post-RNase R digestion

Given that circRNA is susceptible to nicking caused by divalent ions (i.e. Mg^{2+}) and elevated temperatures during the manufacturing process [14], we first evaluated the effects of different RNA synthesis and processing methods on circRNA integrity. E-gel EX electrophoresis, which separates circRNA from linear RNAs of similar size due to their differential migration [17], was employed to visualize the circRNAs and their production intermediates from the three tested workflows. High-quality mNG circRNA was successfully obtained from all three workflows (**Figure 1A**). Notably, circRNA from workflow 2 and 3 exhibited a 10–14% higher circRNA content compared to workflow 1 (**Figure 1B**), suggesting that HPLC purification could provide improved circRNA integrity than cellulose and CIP treatment for the removal of immunogenic impurities. The improvement is likely due to the minimization of circRNA nicking after RNase R digestion, as the cellulose-based purification involves vigorous shaking and CIP treatment exposes RNA to Mg^{2+} at 37ºC for a prolonged time, Additionally, circRNA from workflow 3 displayed consistent to higher circRNA content compared to workflow 2 (**Figure 1B**), indicating that usage of co-transcriptional circularization does not adversely affect the circRNA integrity. These results demonstrate that HPLC purification is a more effective method for preserving circRNA integrity during manufacturing and is compatible with both conventional and co-transcriptional RNA circularization strategies.

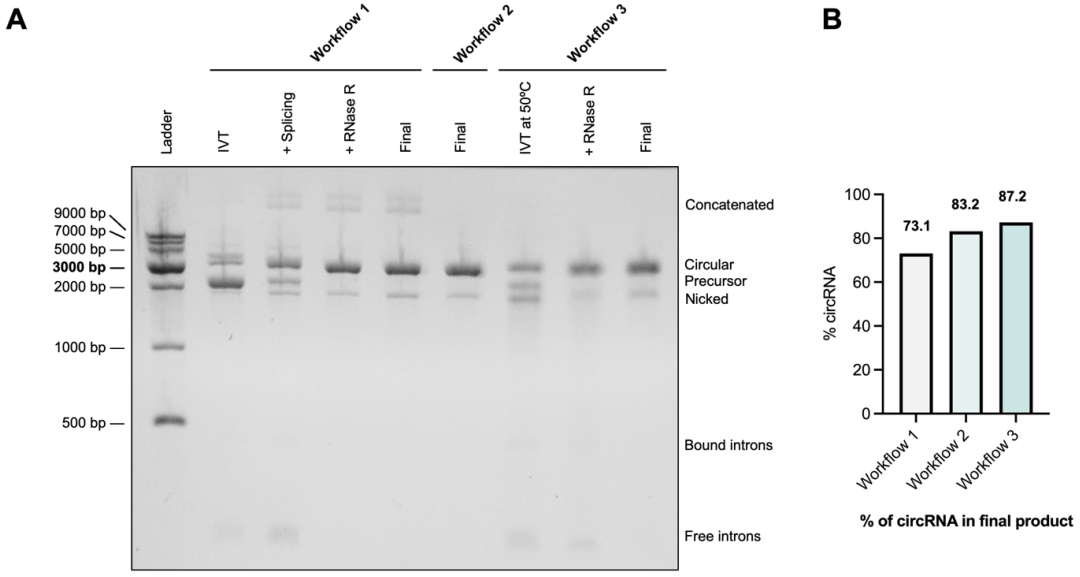

Figure 1. Assessing the effects of different RNA synthesis and processing methods on circRNA integrity. (A) E-gel EX electrophoresis analyzing the integrity of circRNAs from the three tested workflows. For each workflow, the lanes display *in vitro* transcribed (IVT) precursor RNA, RNA after splicing reaction, RNase R-treated RNA, and final circRNA products. Ladder: ssRNA Ladder (New England Biolabs). (B) Quantification of circRNA integrity in the final RNA products from the three workflows, as determined by densitometry analysis of the gel image (GelAnalyzer.com, version 19.1) The circRNA content for each workflow indicates the amount of intact circRNA relative to all RNA species in the final products.

3.2. Consistent protein expression of circRNA across varied synthesis and processing methods

To assess the effects of different circRNA manufacturing strategies on circRNA translation, we transfected the circRNA from the three tested workflows into HEK 293T, A549, and DC2.4 cells and measured their mNG expression at 72 hours post-transfection. Expression in A549 cells was monitored due to their potent innate immune response against RNA impurities [14,16,18,19], while DC2.4 cells were used for evaluating circRNA protein expression in dendritic cells, which are crucial for antigen presentation [20]. CircRNA from the three workflows exhibited consistent levels of mNG expression in all cell lines (**Figure 2**). Although workflow 3 showed moderately lower translation in DC2.4 cells, the difference was not statistically significant ($P > 0.05$) (**Figure 2**). These results suggested that the use of co-transcriptional RNA circularization and impurities removal via either HPLC purification or cellulose/CIP treatment does not significantly affect circRNA protein expression.

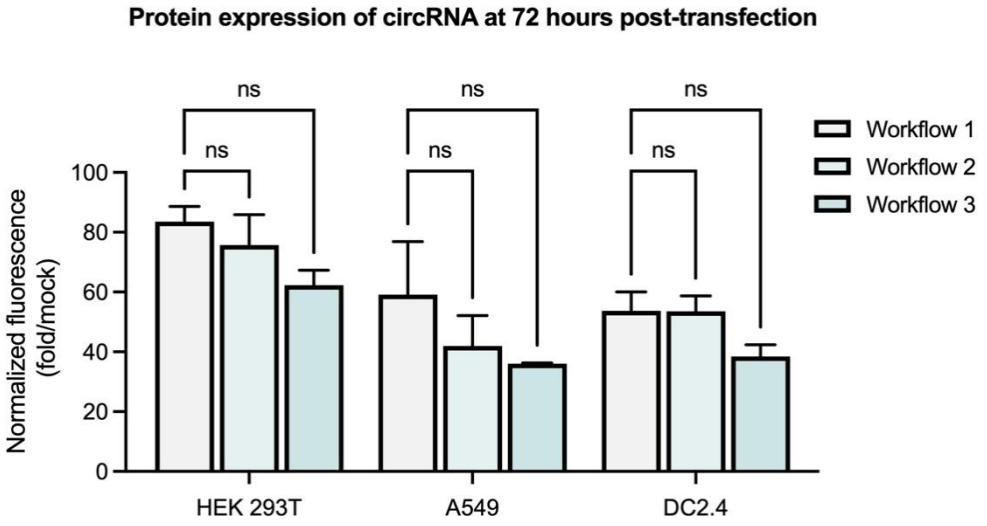

Figure 2. mNG expression of circRNA from the three tested workflows in HEK 293T, A549, and DC2.4 cells at 72 hours post-transfection. Protein expression of circRNA is represented as the level of mNG normalized to mock transfection. Normalized mNG expression from different workflows was compared using ordinary 2-way ANOVA and Tukey's multiple comparison test (with single pooled variance) in Prism 10 (GraphPad, version 10.1.1). The error bars represent standard deviation ($n = 2$). "ns" indicates non-significant differences between groups.

3.3. Cellulose/CIP treatment and HPLC purification are both effective methods for removing immunogenic impurities

Finally, we evaluated the impact of co-transcriptional circularization, HPLC purification, and cellulose/CIP treatment on the removal of dsRNA and RNA with 5' triphosphate motif, contaminants introduced during *in vitro* transcription. Using circRNA treated with RNase R only as a control, which does not completely eliminate these contaminants [14], we monitored mNG expression at 72 hours post-transfection and IFN-β level at 4 hours post-transfection in A549 cells. Due to the presence of immunogenic impurities, RNase R-treated circRNA synthesized from both conventional (workflow 1) and co-transcriptional RNA circularization strategies (workflow 3) exhibited poor translation and a high level of IFN-β expression. In contrast, the removal of impurities through either cellulose/CIP treatment or HPLC purification resulted in a 5-fold increase in translation and a 500-fold decrease in IFN response (**Figure 3A, 3B**). CircRNA from all three tested workflows displayed consistent levels of low immunogenicity, with workflow 1 archiving the lowest level of

IFN-β expression (**Figure 3B**). This suggests that while both HPLC purification and cellulose/CIP treatment are reliable methods for removing immunogenic impurities, usage of the cellulose/CIP treatment may slightly improve the efficacy of eliminating these impurities during RNA manufacturing. Additionally, the consistent IFN-β expression levels observed between circRNA from workflow 2 and workflow 3 indicate that co-transcriptional circularization does not negatively affect the downstream removal of immunogenic impurities required for robust expression and low immunogenicity.

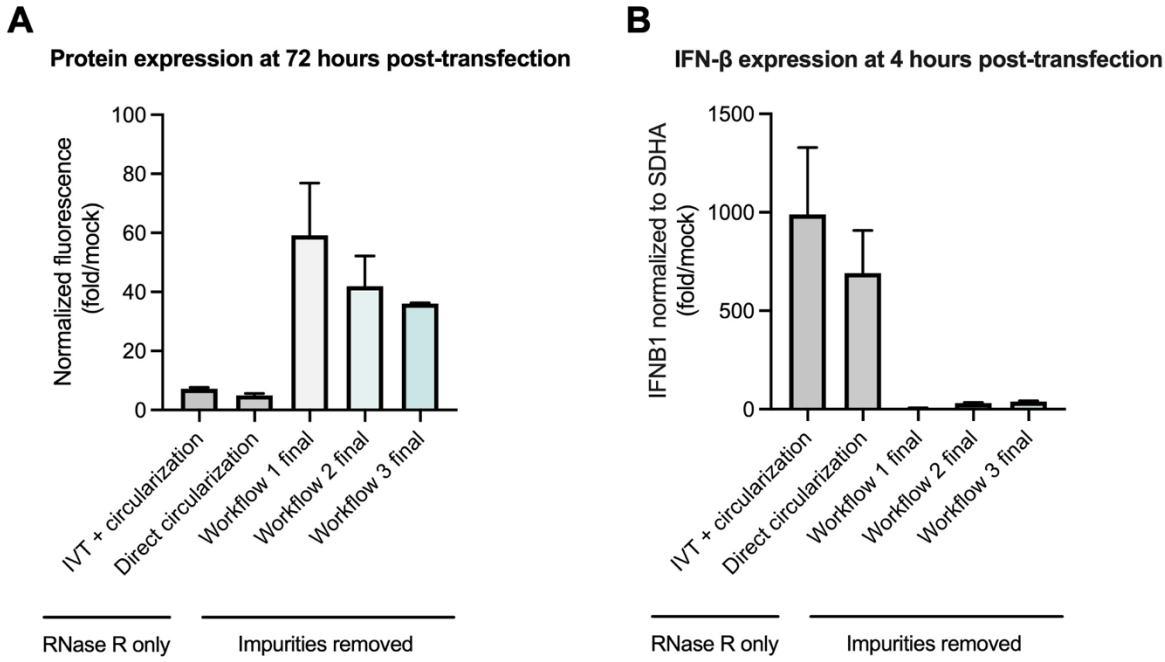

Figure 3. mNG expression at 72 hours post-transfection and IFN-β level at 4 hours post-transfection in A549 cells of the final circRNA and RNase R treated production intermediates from the three tested workflows. (A) mNG expression of circRNA at 72 hours post-transfection (n = 2). RNase R-treated production intermediates from workflow 1 and workflow 3 were used as controls to assess the expression of circRNA from conventional and co-transcriptional circularization strategies in the presence of immunogenic impurities. (B) IFN-β level of circRNA at 4 hours post-transfection (n = 2). Expression of IFN-β was determined via RT-qPCR using gene-specific primers against IFNB1 and normalized to that of SDHA.

4. Discussion

With the recent advancements in permuted group I intron engineering and protein expression optimization, circRNAs have emerged as a promising novel gene delivery platform that could provide more potent and longer-lasting therapeutic effects compared to mRNA [8,9,21]. However, the intricacy of circRNA manufacturing, which involves complex synthesis and processing steps to circularize the RNA and remove linear RNA byproducts, has significantly hampered its therapeutic applications [8]. While co-transcriptional circularization can streamline circRNA production by combining RNA synthesis and circularization, and both cellulose/CIP treatment and HPLC purification have demonstrated reliability in mRNA manufacturing [13,22], their effects on circRNA integrity, translation, and immunogenicity remained to be evaluated. In the present work, we performed a comprehensive assessment of circRNA integrity, protein expression, and immunogenicity across multiple manufacturing workflows to determine the impact of different synthesis and processing methods

on circRNA quality. Our results indicated that co-transcriptional circularization provides an efficient method for producing circRNA with quality comparable to that obtained from the established PIE circularization method. Additionally, both cellulose/CIP treatment and HPLC purification are capable of effectively removing immunogenic impurities, which provides flexibility in workflow customization based on production demands. The consistent circRNA quality across varied synthesis and processing methods is crucial for the reliable manufacturing of circRNA with enhanced production efficiency and scalability.

The development of circRNA-based therapeutic applications relies on reliable large-scale manufacturing of circRNA with potent expression and low reactogenicity. In addition to ensuring circRNA manufacturability, further characterization of the various elements of the circRNA vector, including 5' UTR, IRES, and 3'UTR, remains crucial to fully assess their potential in maximizing translation and reducing immunogenicity [23,24]. Addressing these bottlenecks in developing a circRNA-based gene delivery platform requires efficient and cost-effective circRNA production across different scales. The consistency between HPLC purification and cellulose/CIP treatment in removing immunogenic impurities enables broader scalability for circRNA production. Specifically, HPLC purification is particularly useful in large-scale manufacturing of circRNA for its direct therapeutic applications, while the cellulose/CIP treatment is ideally suited for small-scale circRNA production, facilitating optimization of the circRNA platform due to its flexibility in carrying out multiple reactions in parallel. Despite their effectiveness in eliminating immunogenic impurities, we observed an 8–63-fold IFN-β induction among the purified circRNAs compared to mock transfection (**Figure 3B**). This elevated IFN-β level is likely associated with the lack of nucleotide modifications and prevalence of structured regions present on the circRNA, which activate innate immune response sensors upon circRNA delivery, including TLR3, MDA5, and ADAR1 [23,25]. Due to the abolishment of group I intron ribozyme activity and IRES-mediated translation, circRNA is not compatible with N1-methylpseudouridine (m1Ψ) modification commonly used for minimizing mRNA reactogenicity [14,26,27]. Therefore, the identification of a novel modified nucleotide may further reduce its immunogenicity and potentially enhance protein expression.

CircRNA synthesized via the PIE RNA circularization strategy is prone to nicking that results in breakage of the circularized RNA during the manufacturing process [8], which could potentially affect its stability and proper translation. Consistent with previous studies [8,17], we observed that RNase R treatment did not completely eliminate nicked RNA (**Figure 1A**). This suggests that circRNA nicking may occur during the digestion of the uncircularized precursor RNA and spliced introns. Although exposure to Mg^{2+} and elevated temperature has been known to contribute to circRNA nicking through induction of the intrinsic 2'OH ribozyme activity [28], the specific sites of nicking and the potential involvement of the group I intron ribozyme remain unclear. Understanding the underlying mechanisms of circRNA nicking could further enhance the intracellular stability of circRNA by reducing subsequent degradation by mRNA decay machinery [4]. Additionally, developing novel ligase-based RNA circularization methods or improving the ligation efficiency of the established circRNA synthesis approaches via T4 RNA ligase [29,30] could enhance the utility of circRNA manufacturing by eliminating the incorporation of intronic scars, which are potentially immunogenic [23] and may be undesirable in certain circRNA-based applications.

5. Conclusion

In summary, by systematically comparing the quality of circRNA from varied manufacturing workflows, our

work confirms the reliability of the different synthesis and processing methods for circRNA production via the PIE RNA circularization strategy. The comprehensive profile of these circRNA manufacturing approaches provides valuable insights for broadening the scalability of circRNA production and addressing key bottlenecks in the development of circRNA-based therapeutic applications.

Disclosure statement

The authors declare no conflict of interest.

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