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RESEARCH ARTICLE

Purification of Double-Stranded RNA Impurities From In Vitro-Transcribed mRNA Using Size-Exclusion Chromatography

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ABSTRACT

dsRNA is a highly immunogenic byproduct generated during in vitro transcription of mRNA, leading to unwanted immunostimulatory responses and reduced vaccine efficacy. Removal of dsRNA impurities from mRNA is challenging, as they share similar physicochemical properties. Here, we present a lab-scale size-exclusion chromatography method that effectively separates dsRNA impurities from mRNA. This method achieves baseline resolution between dsRNA impurities and mRNA (resolution factor of 1.6) while maintaining high recovery of the target mRNA (81.8%). This work introduces a novel chromatographic strategy for the removal of dsRNA from mRNA therapeutics.

1 | Introduction

Messenger RNA (mRNA) therapeutics have become an attractive class of prophylactics due to their high efficacy and low toxicity, especially when compared to DNA therapeutics (Gote et al. 2023; Qin et al. 2022). Currently, mRNA therapeutics are being developed for respiratory syncytial virus, influenza, cancer, as well as the continual manufacturing and refinement of the SARS-CoV-2 vaccine (Al Fayez et al. 2023; Han et al. 2025). Additionally, gene-editing therapeutics are being developed using mRNA to address previously untreatable disorders (Popovitz et al. 2023).

However, a major drawback of these therapeutics is double-stranded RNA (dsRNA) impurities, which are byproducts formed during in vitro transcription (IVT) of mRNA. dsRNA impurities elicit an immunogenic response, reduce vaccine efficacy, and have been proposed as a potential contributor to rare cases of myocarditis observed following SARS-CoV-2 vaccination (Milano et al. 2022). As such, the removal of dsRNA impurities from mRNA is of utmost importance.

Purification strategies for dsRNA removal from mRNA include cellulose chromatography, low pH denaturation, or enzymatic digestion with RNase III (Baiersdörfer et al. 2019; Puc et al. 2026; Siew and Zhang 2025). Cellulose chromatography suffers with scalability and difficulty in column preparation (Baiersdörfer et al. 2019). Low pH denaturation uses $\mathrm{pH} = 3.5$ to disrupt hydrogen bonds formed in dsRNA and then performs oligo dT chromatography to remove the dsRNA byproducts; however, there is a risk of mRNA degradation due to the low pH (Puc et al. 2026). RNase III digestion can lead to mRNA degradation and requires an additional purification step to remove the enzyme (Siew and Zhang 2025). Other methods include reverse-phase ion-pairing chromatography (RP-IP-HPLC), anion-exchange chromatography, or dsRNA scavenger resins. RP-IP-HPLC requires organic/flammable solvents, high temperature for separation, and low recovery

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(≈50%) of the target mRNA (Siew and Zhang 2025; Weissman et al. 2013). Anion-exchange chromatography has poor recovery for large mRNA (Peršic et al. 2021; Siew and Zhang 2025). dsRNA scavenger resins are highly scalable and effective at removing dsRNA but may have a small degree of nonspecific binding of mRNA (Clark et al. 2025).

To address some of these limitations, we developed a size-exclusion chromatography (SEC) method that can remove dsRNA byproducts from IVT mRNA. SEC has high recovery (≈80%) with mRNA, does not require flammable or low pH buffers, and each column is multiuse (Miklavčič et al. 2023). We show that all detectable dsRNA impurities can be removed from mRNA using SEC, with a recovery of 81.8%.

2 | Experimental

2.1 | Amplification and Purification of DNA Plasmid Template

The amplification and purification of the DNA plasmid template have been described previously (Tynan et al. 2026). Succinctly, lyophilized plasmid DNA encoding firefly luciferase (FLuc) was purchased from Invitrogen GeneArt Services (Waltham, MA) and reconstituted in TE buffer (10-mmol/L Tris, pH ≈ 8.0, and 1-mmol/L EDTA) to a final concentration of 50 ng/μL. One vial of One Shot TOP10 chemically competent *E. coli* (Invitrogen, #C404010) was partially thawed on ice, and 100 ng of plasmid DNA was added. Transformation was performed according to the manufacturer's protocol with the following modifications: Heat shock was conducted for 20 s, and S.O.C. medium was warmed to 37°C.

Selected colonies from the overnight culture were used to inoculate 250-mL Luria broth cultures containing 100-μg/mL kanamycin. Cultures were then incubated overnight at 37°C and 125 rpm. Overnight liquid cultures were harvested and then centrifuged at 4000 rpm for 40 min at 4°C. The cell pellet was purified using the PureLink Quick Plasmid Miniprep Kit (Thermo Scientific, #K210010) according to the manufacturer's protocol. The DNA plasmid templates were stored at −10°C until used.

2.2 | IVT of mRNA

IVT of mRNA has been described previously (Tynan et al. 2026). Plasmid template was thawed on ice prior to use. One microgram of DNA, 5 μL of 10× NEBuffer r3.1, and 1.0 μL of BspQI (New England Biolabs, #R0712S) were added to a 1.5-mL microcentrifuge tube. DNase/RNase-free water was added to achieve a final volume of 50 μL. The plasmid-enzyme mixture was incubated at 50°C for 15 min. A Monarch Spin PCR & DNA Cleanup Kit (New England Biolabs, #T1130L) was used to purify the linearized DNA according to the manufacturer's protocol.

To synthesize mRNA, New England Biolabs' Standard RNA Synthesis Protocol for RNA > 0.3 kb (#E2040) was used, except that the reaction was incubated for 4 h. To purify the mRNA, the PureLink RNA Mini Kit (Thermo Scientific #12183025) was used, following the manufacturer's protocol for RNA cleanup from liquid samples. FLuc mRNA (2083 nucleotides, nts) was stored at −8°C until used.

2.3 | Nucleic Acid Sample Preparation

mRNA samples were thawed on ice, diluted in the running buffer to the desired concentration, and analyzed.

2.4 | SEC

SEC experiments were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC (Waltham, MA). Detection was performed with a Dionex Ultimate 3000 RS Variable Wavelength detector at 260 nm. A Biozen 3-μm dSEC-7 (Phenomenex, #H25-176122) column was used for separation. The separations were performed under isocratic conditions with a mobile phase of 50-mmol/L phosphate buffer (PB) and 100-mmol/L NaCl, pH = 6.33 or 6.44, and a flow rate of 0.2 mL/min. The mobile phase was prepared by adding 4.29 g of anhydrous sodium phosphate monobasic, 3.82 g of sodium phosphate dibasic heptahydrate, and 5.84 g of NaCl to 1 L of LC–MS grade water. HCl was used to titrate the pH to either 6.44 or 6.33. The LC system was controlled by Chromeleon 7.2.9. MATLAB R2025b was used to determine the full-width of the peak at half of the maximum peak (FWHM) and the retention time of the peak. These values were used to calculate the resolution factor (Rs). Prior to injection, the sample was diluted in the running buffer to the target concentration.

Limit of detection (LOD) and limit of quantitation (LOQ) for SEC were determined with the following equations:

$$LOD = 3.3\frac{\sigma}{S} \tag{1}$$

$$LOQ = 10\frac{\sigma}{S} \tag{2}$$

where σ is the standard deviation of the lowest quantitated value (1.0 ng), and S is the slope of the regression curve.

To calculate the weighted coefficient of determination, ${R_w}^2$, the formula:

$${R_w}^2 = \frac{\sum w_i\left(y_i - y_{p,i}\right)^2}{\sum w_i\left(y_i - y_w\right)^2} \tag{3}$$

where w_i is a weight factor ($1/x_i$), y_i is the observed value, $y_{p,i}$ is the predicted value, and y_w is the weighted mean of observation, was used.

2.5 | dsRNA ELISA Kit

dsRNA content was determined with a SCICONS anti-dsRNA ELISA kit (Exalpha, #10613005). The manufacturer's protocol was followed exactly. Bubbles were removed prior to measuring

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absorbance. Polyinosinic:polycytidylic acid (poly(I:C)) was used to develop the calibration curve for the ELISA kit. Poly(I:C) was supplied from Exalpha as a standard reagent in the SCICONS anti-dsRNA ELISA kit (Exalpha, #10613005). The LOD and LOQ for the dsRNA ELISA kit were determined with the following equations:

$$LOD = \mu_{blank} + 3\sigma_{blank} \tag{4}$$

$$LOQ = \mu_{blank} + 10\sigma_{blank} \tag{5}$$

2.6 | RP-IP-HPLC

RP-IP-HPLC experiments were performed on a Thermo Scientific Dionex Ultimate 3000 HPLC (Waltham, MA). Detection was performed using a Dionex Ultimate 3000 RS Variable Wavelength detector with an absorbance of 260 nm. An ADS Biotech RNAsep Prep-RNA purification column (Part No: RNA-99-3810, Omaha, NE) was used. The mobile phase was 0.1-M TEAA in water (A) and 0.1-M TEAA in 25% acetonitrile (B). The liquid profile was 40% to 58% B over 18 min. The flowrate was 0.5 mL/min. The column was kept at 75°C. One microgram of sample was injected into the column per run. The LC system was controlled by Chromeleon 7.2.9.

3 | Results and Discussion

SEC achieves an Rs of 1.2 between the impurity peak (≈10 min) and FLuc mRNA peak (≈11 min) (Figure 1A). Additionally, we observed other impurities at 15 and 19 min, which may correspond to short abortive transcripts (15 min) and unincorporated nts (19 min). Crude FLuc mRNA is defined as FLuc mRNA that has not undergone dsRNA purification or removal. ELISA of crude FLuc mRNA (Figure 1B) was performed. ELISA detected 1.68% dsRNA, confirming the presence of dsRNA in the crude FLuc mRNA. Tabulated values from Figure 1B are in Table 1.

The relative magnitude of the impurity peak in Figure 1A exceeds that expected from dsRNA alone, suggesting that it contains additional species. These additional species could be mRNA aggregates, run-off transcripts, or residual plasmid template DNA. We propose that the 1.68% dsRNA impurity detected by ELISA is partitioned within this impurity peak alongside additional components.

Definitive identification of dsRNA impurities within the impurity peak requires improved chromatographic resolution. We hypothesize that by lowering the pH, the mRNA will compact, resulting in a lower hydrodynamic radius (R_H), while the R_H of the impurity will be less sensitive to changes in pH, due to stability provided by the duplex. If successful, this would enable isolation of the impurity peak for direct dsRNA quantification.

A slight reduction in pH (from 6.44 to 6.33) enables baseline resolution between the impurity and target mRNA peaks (Figure 2A). To confirm that the impurity peak contains dsRNA, fractions corresponding to the impurity and target mRNA peaks were collected and analyzed by an ELISA

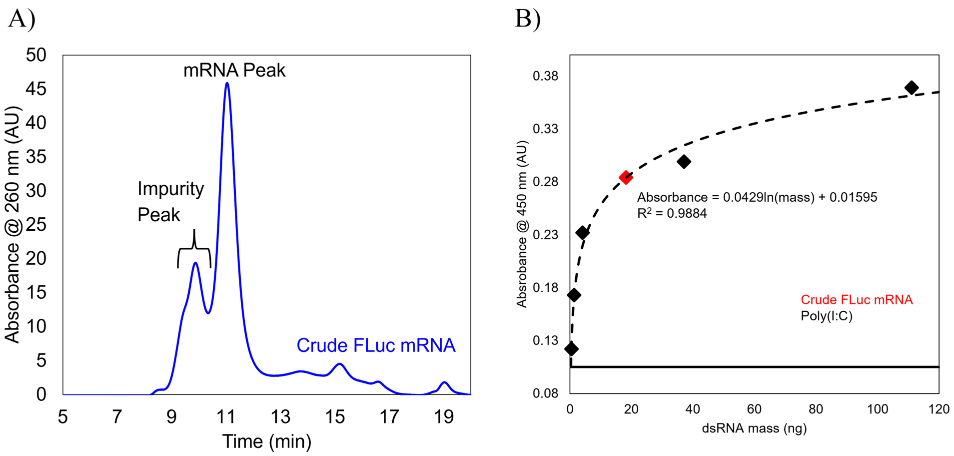

FIGURE 1 | (A) SEC chromatogram of crude FLuc mRNA (2083 nts, 1 μg). Crude FLuc mRNA is defined as FLuc mRNA that has not undergone dsRNA purification. The absorbance at 260 nm (absorbance units, AU) is plotted against elution time (min). The mobile phase was 50-mmol/L PB with 100-mmol/L NaCl, pH = 6.44. The impurity peak and mRNA peak have an Rs of 1.2. (B) ELISA to verify presence of dsRNA in the crude FLuc mRNA. The absorbance at 450 nm (AU) is shown against dsRNA mass (ng). The black diamonds are from poly(I:C) used to develop a calibration curve. The black dotted line is the calibration curve developed from poly(I:C). The red diamond is the absorbance detected and calculated dsRNA mass in the crude FLuc mRNA. The black horizontal line is the blank, which has a value of 0.104 AU. One thousand eighty-three ng of crude FLuc mRNA was tested, and 18.21 ng of dsRNA was detected, meaning the crude FLuc mRNA contains 1.68% dsRNA.

TABLE 1 | ELISA results from Figure 1B.

Sample	Tested mass (ng)	Absorbance (AU)	dsRNA mass (ng)	dsRNA % (m/m)
Crude mRNA	1083	0.284	18.21	1.68

Note: The sample tested was crude FLuc mRNA, defined as mRNA that has not undergone dsRNA purification. The LOD was 0.119 AU, the LOQ was 0.155 AU, and the blank had a value of 0.104 AU.

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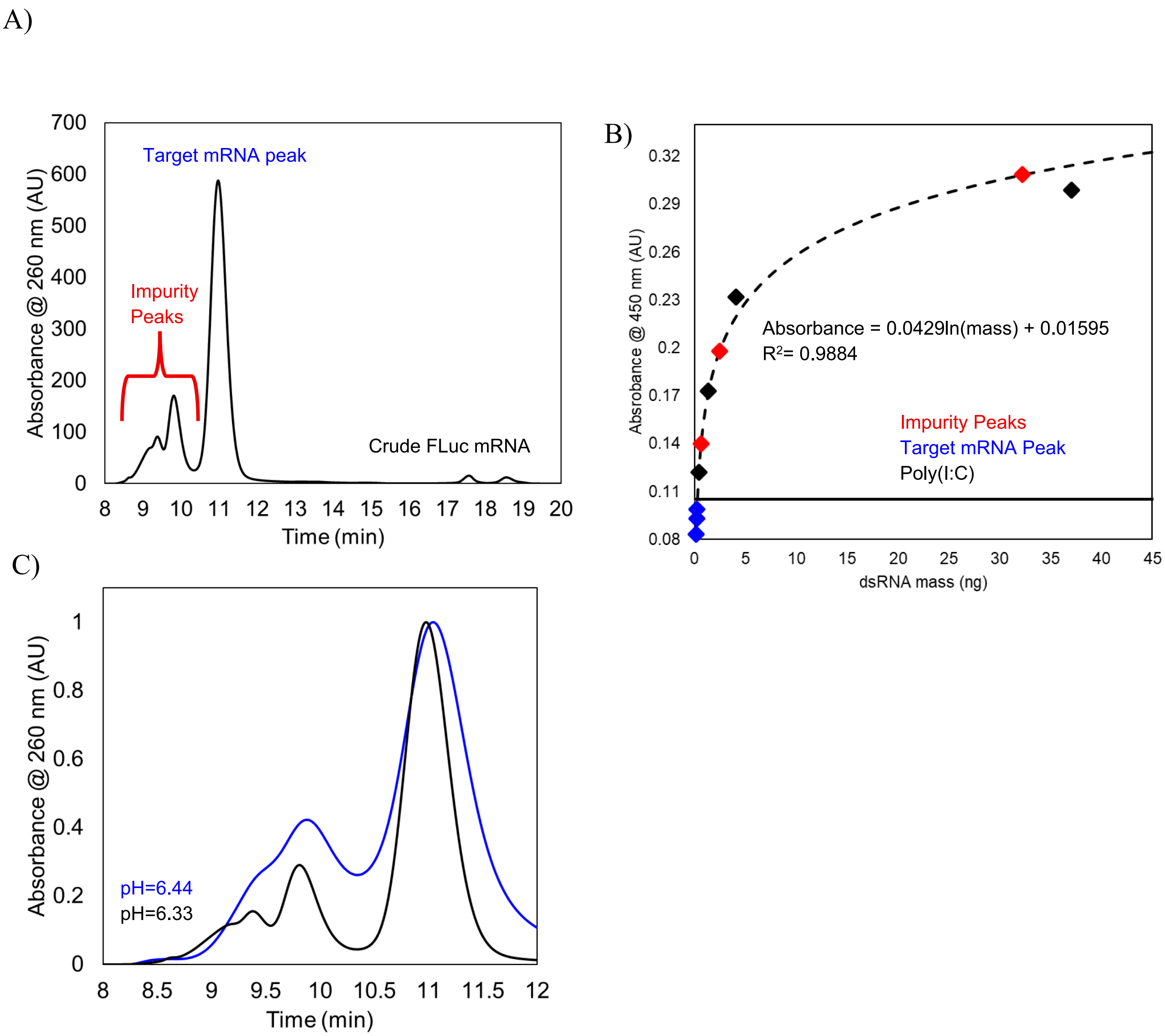

FIGURE 2 | (A) SEC chromatogram of crude FLuc mRNA (2083 nts, 5 µg). The absorbance at 260 nm (AU) is plotted against elution time (min). The mobile phase was 50-mmol/L PB with 100-mmol/L NaCl, pH = 6.33. The impurity peaks and the target mRNA peak have an Rs of 1.6. (B) ELISA of collected impurity peaks and collected target mRNA peaks. The absorbance at 450 nm (AU) is shown against dsRNA mass (ng). The black diamonds are from poly(I:C) used as a calibration standard. The black dotted line is the calibration curve developed from poly(I:C). The red diamonds are collected fractions of the impurity peaks after SEC purification. The blue diamonds are collected fractions of the target mRNA peaks after SEC purification. Three different SEC purifications were performed, resulting in three SEC-purified mRNA fractions and three SEC-purified impurity fractions. The black horizontal line is the blank from the ELISA, which has a value of 0.104 AU. The LOD and LOQ of the ELISA were 0.119 and 0.155 AU, respectively. (C) Overlaid SEC chromatograms of Figures 1A and 2A showing absorbance at 260 nm (AU) against elution time (min). The figures have been normalized to a maximum peak height of 1 AU to highlight differences in elution time.

(Figure 2B). dsRNA was detected in all impurity fractions above the LOD (0.119 AU), whereas all mRNA fractions were both below the blank (0.104 AU) and the assay LOD, indicating complete removal of detectable dsRNA impurity from the mRNA fraction. Tabulated values from Figure 2B are provided in Table 2.

Across the three impurity fractions, dsRNA content was determined to be 5.76%, 3.27%, and 4.20%. However, the absorbance for the impurity fractions containing 3.27% and 4.20% dsRNA was below the LOQ (0.155 AU), but above the LOD. dsRNA impurity is present in these samples; however, it is unlikely that these samples contained exactly 3.27% and 4.20% dsRNA. It remains that for each impurity fraction analyzed, the entire impurity peak is not composed solely of dsRNA but contains additional IVT products or reagents. We hypothesize that these additional species in the impurity peak may include mRNA aggregates, run-off transcripts, or residual plasmid template. This compositional heterogeneity would explain the discrepancy between the impurity peak (≈30% of the total chromatographic signal in Figure 1A) and the relatively low dsRNA content (1.68%) in the crude FLuc mRNA. However, the exact composition of the impurity peak in Figures 1A and 2A was not determined, and the composition remains speculative.

Overlayed chromatograms (Figures 1A and 2A), normalized to a maximum of 1 AU in Figure 2C, show a slight decrease in elution time at pH = 6.33 compared to pH = 6.44 of the target mRNA. This does not support our hypothesized mRNA

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TABLE 2 | ELISA results from Figure 2B.

Sample	Tested mass (ng)	Absorbance (AU)	dsRNA mass (ng)	dsRNA % (m/m)
Purified mRNA 1	505	0.084	X	0.00
Purified mRNA 2	345	0.093	X	0.00
Purified mRNA 3	69	0.099	X	0.00
Impurity peak 1	560	0.309	32.24	5.76
Impurity peak 2	75	0.198	2.45	3.27
Impurity peak 3	15	0.140	0.63	4.20

Note: The purified FLuc mRNA and impurity peak were collected in different fractions and tested for dsRNA content using an ELISA. Three different purifications were performed. The tested mass refers to the mass of RNA used during the ELISA. Purified mRNA 1 was from one SEC purification. Impurity peak 1 corresponds to the impurity peak found during the purification of Purified mRNA 1. The LOD was 0.119 AU and the LOQ was 0.155 AU. The blank had a value of 0.104 AU.

compaction effect, rather, it suggests an alternative mechanism is causing the separation. It could be that the increase in negatively charged ions in the slightly lower pH running buffer decreases analyte–column interactions, allowing for better resolution. Or, by lowering the pH slightly, the secondary structure of the impurity peak is being disrupted, slightly increasing the R_H, while the target mRNA's R_H remains relatively constant, thus improving resolution. However, if R_H changes, either for the impurity or mRNA, a significant shift in elution time is expected, which is not observed for either the impurity peak or the target mRNA peak. Each proposed hypotheses (mRNA compaction, reduced analyte–column interactions, or removal of the impurity peak's secondary structure causing an increase in R_H) are all unverified and remain speculative.

Overall, these results demonstrate that, with appropriate buffer conditions, SEC can effectively separate dsRNA impurities from IVT mRNA for lab-scale purification, although it is not suitable as a quantitative analytical method for dsRNA content due to the heterogeneous composition of the impurity fraction.

The SEC peak area exhibited a strong linear correlation with injected mass (Figure 3A). Figure 3A was used to quantitate the amount of target mRNA injected into each SEC run (Figure 3B). As shown in Figure 3B, 70.50% ± 0.50% of the total injected RNA mass was the target mRNA. Figure 3A was used to determine the recovery of the target mRNA compared to the injected target mRNA (Table 3). The recovery was 81.8% with a standard deviation of 13.7%. The relatively high variability is likely attributable to manual fraction collection, which is susceptible to timing and consistency errors. Implementation of an automated fraction collector would provide more reproducible fraction collection, potentially reducing variability and improving overall recovery. For therapeutic applications, further optimization of the collection process will be necessary to improve recovery consistency.

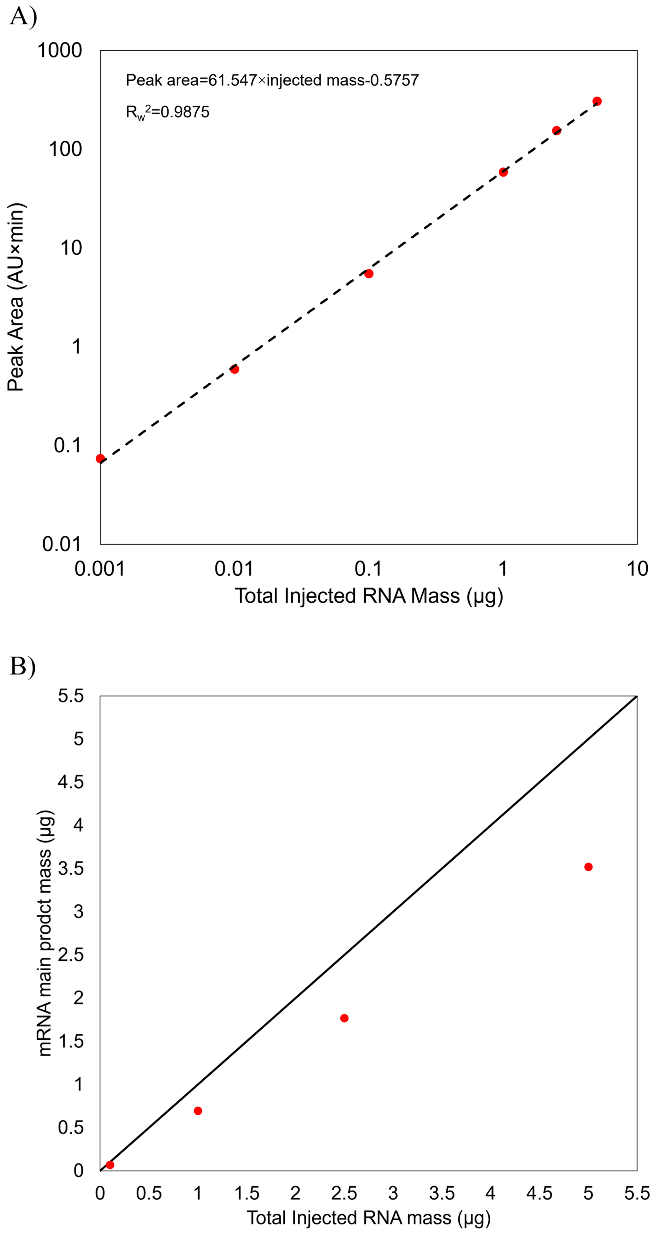

FIGURE 3 | (A) Peak area (AU × min)AU×min is plotted against injected total injected mRNA mass (μg) to develop a calibration curve of the SEC method. The LOQ is 1.92 ng. The LOD is 0.63 ng. Each test was performed three times at each mass; in most cases, the error bars are smaller than the data points. The masses injected were 1.0, 10, 100, 1000, 2500, and 5000 ng. The coefficient of variation for each mass is (respectively) as follows: 16.04%, 2.56%, 4.03%, 1.78%, 5.17%, and 0.47%. (B) Mass of main product peak (μg) in SEC compared to the total injected mRNA mass (μg) in SEC. The average total injected mRNA mass was 70.50% ± 0.50% target mRNA, with the remaining 29.50% ± 0.50% being impurity. The black diagonal line is used to highlight the difference between the total injected RNA mass and the mRNA main product mass.

Figure 4 demonstrates that the integrity of the target mRNA is preserved following SEC purification. Quantitative analysis of Figure 4 is tabulated in Table 4. Compared to the purified

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TABLE 3 | Recovery of target mRNA from SEC purification.

Total injected RNA mass (μg)	Peak area of target mRNA (AU × min)	Calculated mass of injected mRNA (μg)	Recovered mRNA (μg)	Percent recovery (%)
6.58	221.52	3.61	2.87	79.53
7.89	259.68	4.23	4.10	96.96
6.58	260.92	4.25	2.87	67.55
6.58	215.59	3.51	2.46	70.04
7.89	265.46	4.32	4.10	94.85
Total	X	X	X	81.8 ± 13.7

Note: The recovery was 81.8% with a standard deviation of 13.7%.

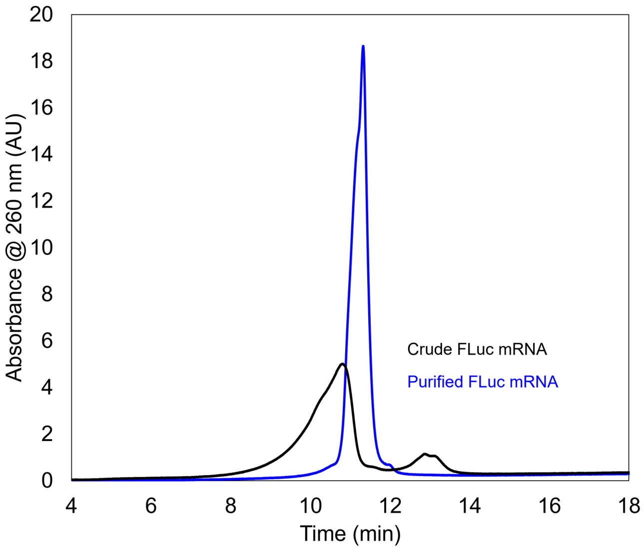

FIGURE 4 | IP-RP-HPLC chromatogram of crude FLuc mRNA (FLuc mRNA that has not been purified via SEC, 1 μg, black) and purified FLuc mRNA (FLuc mRNA that has been purified via SEC, 1 μg, blue). The absorbance at 260 nm (AU) is plotted against elution time (min).

TABLE 4 | Quantitative analysis of Figure 4.

Sample	Peak	FWHM (min)	Time of peak max (min)	Peak area (AU × min)
Crude mRNA	1	1.12	10.81	4.26
Crude mRNA	2	0.57	12.87	0.46
Crude mRNA	3	0.35	13.06	0.26
Crude mRNA	Total	X	X	4.98
Purified mRNA	1	0.44	11.32	6.18

Note: FWHM (min), time of peak max (min), and peak area (AU × min) of each observed peak in both chromatograms are shown.

sample, the crude FLuc mRNA exhibited greater band broadening of the target mRNA peak, as indicated by a larger FWHM (the crude FLuc mRNA has a FWHM of 1.12 min, while the purified FLuc mRNA has a FWHM of 0.44 min). The total peak area of the purified mRNA chromatogram (6.18 AU × min) is greater than that of the crude mRNA chromatogram (4.98 AU × min). The crude mRNA chromatogram also contains two unresolved peaks at 12.87 and 13.06 min that accounted for 14.46% of the total peak area. These peaks were absent in the purified FLuc mRNA chromatogram and likely correspond to higher molecular weight impurities that were removed during SEC purification.

A slight shift in the target peak elution time was observed between the crude and purified samples. This difference may result from interactions between the target mRNA and co-eluting impurities in the crude sample, which could alter chromatographic behavior. The reduced band broadening and increased peak area observed following purification are also consistent with the removal of contaminants that interfere with separation. Overall, similar elution times and comparable peak areas before and after purification indicate that SEC removes impurities without compromising mRNA integrity.

The crude mRNA chromatogram exhibited a pronounced pre-peak shoulder that was absent in the SEC-purified mRNA. This observation is consistent with Clark et al. (2025) who reported a similar pre-peak shoulder in crude IVT mRNA that disappeared following purification with dsRNA scavenger resins, as assessed by RP-HPLC and high-performance SEC (Clark et al. 2025). The disappearance of this feature after SEC purification further supports that dsRNA impurities have been removed.

4 | Conclusions

We have developed a lab-scale SEC purification method that removes all detectable dsRNA impurities from mRNA. However, the separation mechanism allowing for dsRNA impurity and mRNA resolution remains unclear. Possible explanations include pH-induced compaction of mRNA while dsRNA structure remains unchanged, reduced column interactions due to increased ionic strength at lower pH, or structural disruption and elongation of dsRNA under acidic conditions. Each of these hypotheses remains speculative.

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The method achieved a sample recovery of 81.8%, with the removal of all detectable dsRNA impurity, although the variability is high (standard deviation of 13.7%). This variability may be reduced, and recovery potentially improved, by using an automated fraction collector. The variability must be decreased prior to adoption into a therapeutic context. A key limitation of the approach is that while the impurity peak contains dsRNA, the exact composition is unknown, which means it is possible that desired products are being removed. Additionally, this prevents this SEC method from functioning as a standalone dsRNA quantification method, since the composition of the impurity peak is not fully characterized and may include mRNA aggregates, run-off transcripts, and residual DNA template in addition to dsRNA. It is also possible that target mRNA is present in the impurity fraction, thus lowering overall recovery.

Future work should focus on elucidating the mechanism governing dsRNA impurity–mRNA separation. Once established, chromatographic parameters such as pH, buffer composition, and pore size can be further optimized to improve resolution and potentially increase mRNA recovery. In addition, characterization of the collected impurity fractions is needed to determine their composition and to guide strategies for minimizing their formation.

Nomenclature

AU	absorbance units
DNA	deoxyribonucleic acid
dsRNA	double-stranded ribonucleic acid
ELISA	enzyme-linked immunosorbent assay
FLuc	firefly luciferase
IVT	in vitro transcription
LOD	limit of detection
LOQ	limit of quantitation
m	mass
mRNA	messenger ribonucleic acid
nts	nucleotides
PB	phosphate buffer
poly(I:C)	polyinosinic:polycytidylic acid
R^2	coefficient of determination
$\mathrm{R_H}$	hydrodynamic radius (nm)
Rs	resolution factor
RP-IP-HPLC	reverse-phase ion-pairing high performance/pressure liquid chromatography
$R_w{}^2$	weighted coefficient of determination
S	slope of the regression line of the standard curve$\left(\frac{\mathrm{AU} \times \mathrm{min}}{\mu \mathrm{g}}\right)$
SEC	size-exclusion chromatography
TE	10-mmol/L Tris, pH ≈ 8.0, and 1-mmol/L EDTA
w_i	weight factor ($\mu\mathrm{g}^{-1}$)
x_i	x-value of the standard curve (injected mass, μg)
y_i	observed peak area (AU × min)
$y_{p,i}$	predicted peak area (AU × min)
y_w	weighted mean of observation (AU × min)
μ_{blank}	average value of blank in ELISA (AU)
σ	standard deviation of the lowest quantitated value, 0.001 μg (μg)
σ_{blank}	standard deviation of blank in ELISA (AU)

Author Contributions

Kyle J. Tynan: writing – review and editing, writing – original draft, methodology, investigation, data curation, and conceptualization. **Trina Mouchahoir:** writing – review and editing, and supervision. **Mark S. Lowenthal:** writing – review and editing, and supervision. **Karen W. Phinney:** writing – review and editing, supervision, and project administration.

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Disclosure

Certain commercial equipment, instruments, materials, or software may be identified in this paper in order to specify experimental procedures and technologies adequately. Such identification is not intended to imply recommendation or endorsement by the National Institute of Standards and Technology, nor is it intended to imply that the materials or equipment identified are necessarily the best available for the purpose.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data are available on request.

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