CELL & GENE THERAPY INSIGHTS

COMMENTARY

Two new capture options for improved purification of large mRNA

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One of the barriers to development of industrial purification platforms for large mRNA has been an inadequate selection of high-performing capture-purification tools. Hybridization-affinity uses a polythymidine (Oligo dT) ligand to base-pair with the polyadenine tail of mRNA. It can be used for capture but it cannot discriminate dsRNA (double-stranded) from ssRNA (single-stranded) and it supports only brief cleaning with 100 mM sodium hydroxide. Traditional anion exchangers elute only mRNA smaller than about 500 bases unless the columns are heated to 50–70°C. Hydrophobic interaction chromatography (HIC) and reverse phase chromatography (RPC) separate ssRNA from dsRNA and short transcripts, but their sensitivity to fouling by proteins and aggregates makes them better suited for polishing than for capture. Better capture options are needed to meet the needs of large clinical trials, scale-up, and manufacture of vaccines. Beyond that, a new spectrum of gene therapy treatments await. This article introduces two new capture options that both eliminate dsRNA, DNA, and proteins in a wash step, then provide high-resolution polishing of ssRNA in an elution gradient at ambient temperature. One represents a new class of anion exchangers. The other exploits hydrogen bonding. Both support prolonged exposure to 1 M sodium hydroxide. Easy transition to either HIC or RPC provides high-resolution orthogonal polishing.

Cell & Gene Therapy Insights 2020; 6(7), 1035–1046

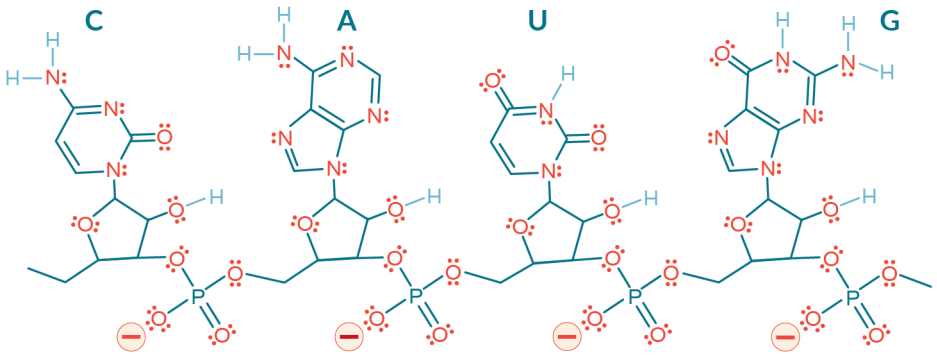
DOI: 10.18609/cgti.2020.114

INTRODUCTION

One of the barriers to development of industrial purification for large mRNA has been an inadequate selection of high-performing capture-purification tools. Hybridization-affinity uses a polythymidine (Oligo dT)

FIGURE 1

Charges, hydrogen donors, and hydrogen acceptors on RNA.

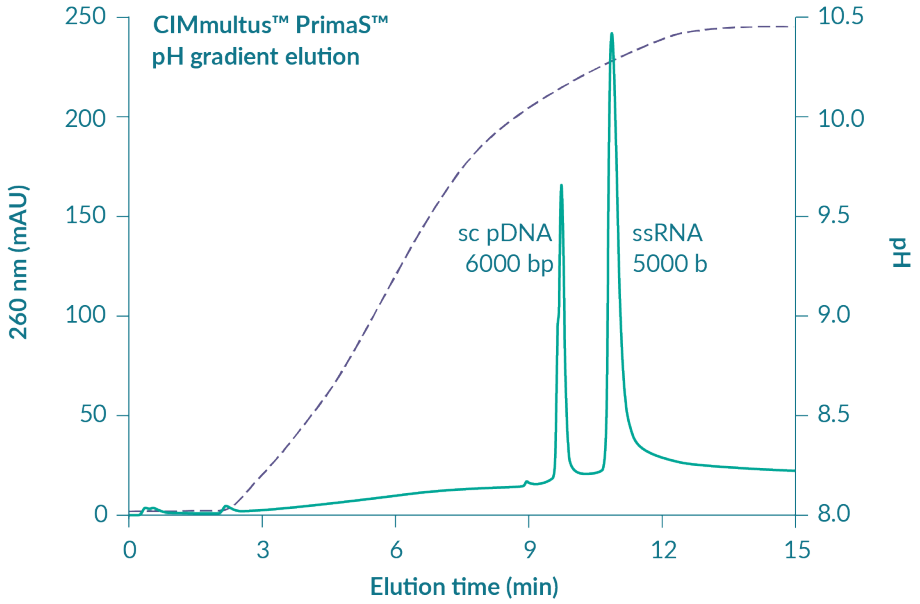


Negative charges are shown in red with a yellow halo. Each pair of red dots indicates a free lone pair of electrons that can act as a hydrogen acceptor. The term "lone pair" refers to a pair of valence electrons that are not shared with another atom in a covalent bond. Hydrogen donors are shown in blue.

ligand to base-pair with the polyadenine tail of mRNA. It can be used for capture but it cannot discriminate dsRNA (double-stranded) from ssRNA (single-stranded) and it supports only brief cleaning with 100 mM sodium hydroxide [1–3]. Ambient temperature operation of traditional anion exchangers elutes only mRNA species smaller than about 500 bases [3–5]. Elution of larger species requires elevation of operating temperature into the range of 50–70°C [6]. Hydrophobic interaction chromatography (HIC) and reverse phase chromatography (RPC) separate ssRNA from DNA, dsRNA, and short transcripts, but their sensitivity to fouling by proteins and aggregates makes

FIGURE 2

Separation of plasmid DNA and ssRNA in a pH gradient.



Buffer A: 50 mM Tris, pH 8.0. Buffer B: 125 mM glycine, pH 10.5. 50 CV linear gradient from A to B at 5 CV/min.

DOI: 10.18609/cgti.2020.114

them better suited for polishing than for capture [3,7–10].

Better capture options are needed to meet the needs of large clinical trials, scale-up, and manufacture of vaccines. Beyond that, a new spectrum of gene therapy treatments await. This article introduces two new capture options that both eliminate dsRNA, DNA, and proteins in a wash step, then provide high-resolution polishing of ssRNA in an elution gradient at ambient temperature. One represents a new class of anion exchangers. The other exploits hydrogen bonding. Both support prolonged exposure to 1 M sodium hydroxide. Easy transition to either HIC or RPC provides high-resolution orthogonal polishing.

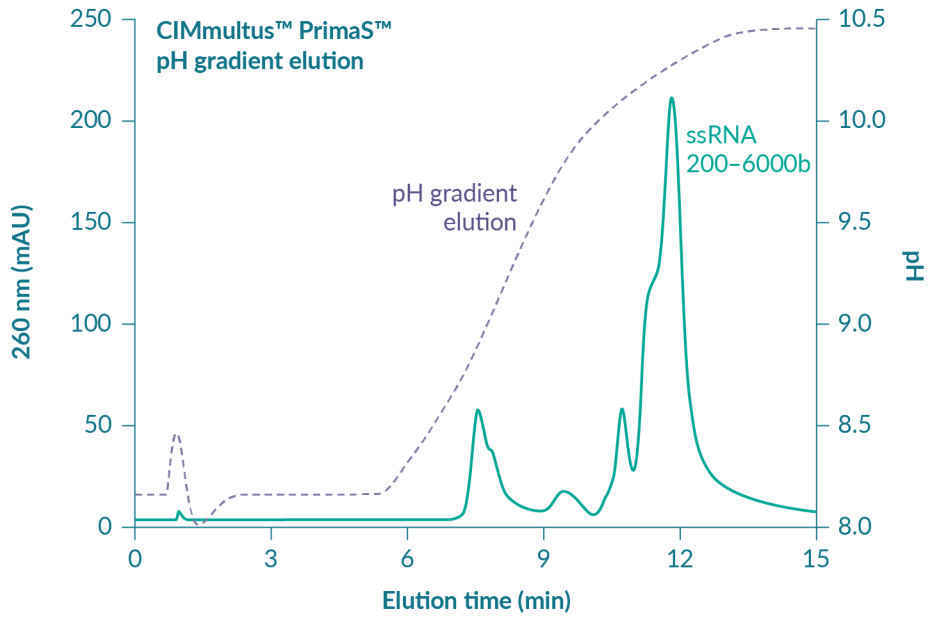
EXPERIMENTAL

CIMac™ (100 µL) or CIMmultus™ (1 mL) PrimaS™ and H-Bond™ monoliths with 2 µm channels were obtained from BIA Separations. Single-stranded and dsRNA ladders, DNA ladders, and species of single size were obtained from New England Biolabs. Analytical grade or American Chemical Society grade buffering agents and salts were obtained from Sigma-Aldrich. Buffers were prepared fresh with European Pharmacopeia grade water and filtered to 0.22 µm before use.

Purified samples of defined content were used to eliminate ambiguity of interpretation and facilitate comparison across laboratories. They were equilibrated before injection by dilution with a 10-fold volumetric excess of the column equilibration buffer. Many examples were performed with sample mixtures containing supercoiled DNA of 6000 base pairs and ssRNA of 5000 bases. Injection volumes ranged from 50 µL to 200 µL depending on the size of the column. Specific sample composition and buffer conditions are described in the Figure legends. Columns were operated at a flow rate of 5 column volumes per minute (300 CV/h). Results obtained from experiments with conditions and samples of broader scope are described in [3].

FIGURE 3

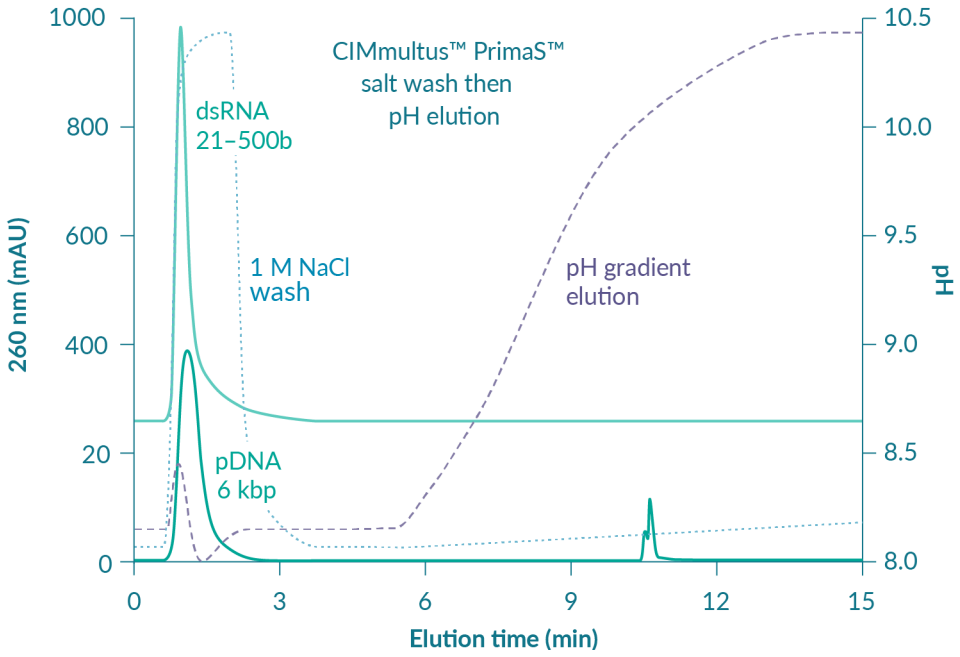
Elution of a ssRNA ladder in a pH gradient.



Same protocol as Figure 2.

FIGURE 4

Removal of DNA and dsRNA with a salt wash before pH elution.



Equilibration and elution as in Figure 2. First wash: 50 mM Tris, 1 M NaCl, 10 mM EDTA, pH 8.0. Second wash with equilibration buffer. Same elution as Figure 2. Overlay of two different chromatograms.

RNA CAPTURE BY ANION EXCHANGE CHROMATOGRAPHY, ELUTION BY PH GRADIENT

The necessity to heat traditional anion exchangers represents a burden at all stages of process development and manufacturing but it also provides a clue. The inability to elute large mRNA at ambient temperature derives from the elevated hydrogen bonding capacity of RNA [3]. The ratio of hydrogen donors and acceptors to negatively charged phosphatidic residues on the polymer backbone is more than 20:1 (Figure 1). The majority are not involved in base-pairing but they can bond with complementary features of anion exchange surfaces. An anion exchanger with reduced hydrogen bonding potential should be able to reduce the net contribution of hydrogen bonding and elute RNA at ambient temperature.

Figure 2 validates this hypothesis with ambient temperature elution of a sample mixture containing ssRNA with 5000 bases. An ascending pH gradient elutes ssRNA from CIMmultus™ PrimaS™ in a sharp peak, well separated from an earlier-eluting 6000 bp DNA plasmid. The rationale for increasing pH is that it reduces protonation and reduces the number of hydrogen bonding partners for RNA. This approach does not work with traditional anion exchangers, which tend to exhibit stronger binding with increasing pH [11,12]. Figure 3 shows pH elution of a single-stranded mRNA ladder that contains species ranging from 200 to 6000 bases. They elute in order of increasing size.

Contaminating double-stranded nucleic acids, including both DNA and dsRNA, are serious concerns from an immunological perspective [13]. Residual plasmid DNA is immunogenic [14,15] and may be present in range of degradation states at 1–2% of total RNA after transcription. Proportions of dsRNA are less well characterized but still important. Cells interpret dsRNA as a viral infection [16]. It can trigger a cytokine storm with sudden and serious health consequences. Figure 4 shows that dsRNA and DNA are both removed in a 1 M sodium chloride wash with 10 mM EDTA. Single-stranded RNA

DOI: 10.18609/cgti.2020.114

remains bound. After returning the column to equilibration conditions, DNA and dsRNA are essentially absent from the pH elution profile. The presence of salts during pH gradient elution causes ssRNA to elute at lower pH values (Figure 5).

The pH of the eluted ssRNA should be neutralized during or shortly after elution. Both methods are standard practice in the field of protein affinity chromatography where fraction collection vessels either contain a pH-titrating buffer so the product is neutralized upon collection, or the product is neutralized at the end of the run. Preliminary data indicate that brief exposure to alkaline elution conditions causes no modification of mRNA but prudence suggests avoiding prolonged exposure.

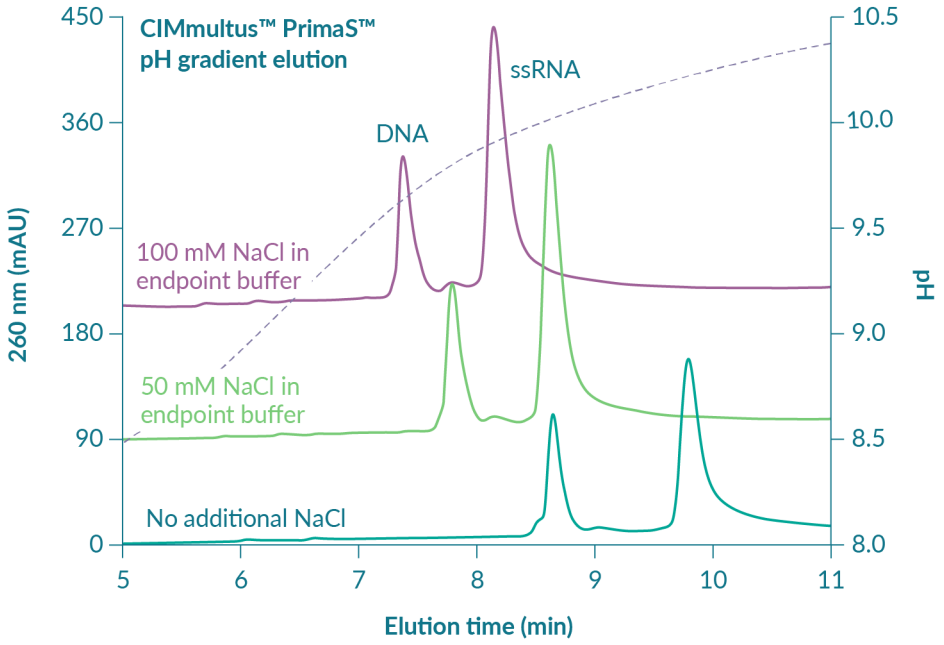
The column can be cleaned extensively with 1 M NaOH. Brief cleaning is recommended after every run since it will reveal the amount of material remaining on the column after elution. Columns loaded with large volumes of crude samples may require cleaning for 1 hour. Badly fouled columns can be restored to baseline performance by cleaning for 16–24 hours. Cleaning can be enhanced by co-formulating NaOH with 1–3 M NaCl and 10–20 mM EDTA.

RNA CAPTURE BY HYDROGEN BONDING, AFFINITY ELUTION BY DIPHOSPHATE DISPLACEMENT

Figure 6 illustrates elution of ssRNA at ambient temperature from CIMmultus™ H-Bond™ with a gradient to 100 mM pyrophosphate at neutral pH [3]. Hydrogen bonding has been exploited sporadically on conventional ion exchangers since 1960 [17–20]. The H-Bond™ ligand is more enriched with hydrogen donors and acceptors so that up to 80% or more of its binding energy comes from hydrogen bonding (Figure 7) [3]. DNA binds H-Bond™ more strongly than it binds strong anion exchangers at all pH values but binding becomes disproportionately stronger with decreasing pH. The trend becomes

FIGURE 5

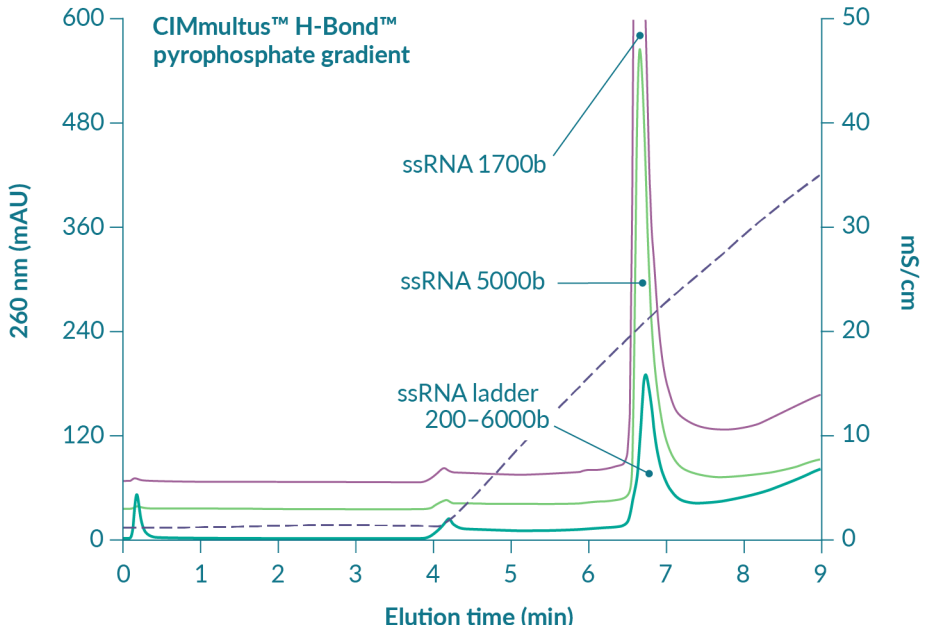
Earlier retention time and increased RNA recovery by the presence of salt during pH elution.



DNA: 6000 bp. RNA 5000 b. Protocol of Figure 2 except with the indicated concentrations of NaCl added to the gradient endpoint buffers. Overlay of 3 chromatograms.

FIGURE 6

Elution of ssRNA of different sizes in a pyrophosphate gradient.



Buffer A: 50 mM Hepes, pH 7.0. Buffer B: 50 mM Hepes, 100 mM potassium pyrophosphate. 50 CV linear gradient at 5 CV/min. Overlay of three different chromatograms.

steeper below pH 6. The differential compared to the strong anion exchanger is attributed to hydrogen bonding becoming more prevalent with increasing protonation [3].

Pyrophosphate is a diphosphate (P2O7) with up to 4 negative charges and up to 18 hydrogen donor/acceptors depending on pH (Figure 8). It represents the terminus of adenosine diphosphate (ADP) and it is a ubiquitous contaminant of phosphate buffers. All sizes of ssRNA elute at the same pyrophosphate concentration (Figure 6). Remarkably, dsRNA elutes in order of increasing size but even very-large species elute before ssRNA (Figure 9). DNA shows limited heterogeneity with respect to size, as seen in the trailing shoulder on the main peak in Figure 10, but no useful size separation overall. It elutes in advance and well separated from ssRNA.

Figure 7 suggests that reducing pH will increase RNA capacity but also predicts that elution of ssRNA will be shifted to a higher concentration of pyrophosphate [3]. It does not necessarily follow that separation between ssRNA and double-stranded contaminants will remain the same but that concern can be managed in a different way. As with anion exchange chromatography, DNA and dsRNA are eliminated at neutral pH by a wash step with 1 M NaCl and 10 mM EDTA [3]. At lower pH, increasing the salt concentration in the wash should compensate for stronger binding and leave the gradient to polish out trace-level contaminants from highly purified ssRNA.

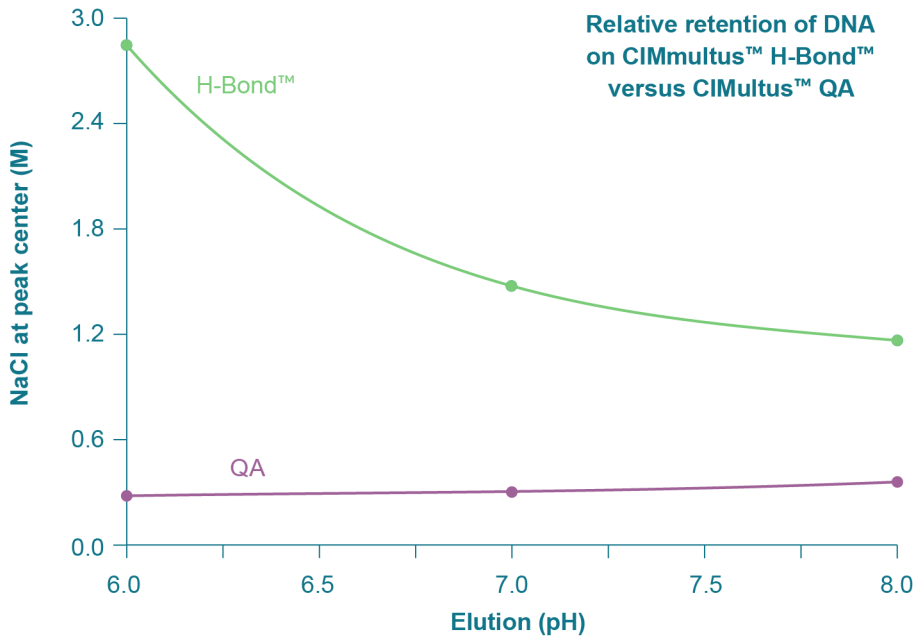
Running the gradient at alkaline pH and/or in the presence of non-pyrophosphate salts elutes ssRNA at lower pyrophosphate concentrations. Combinations of other salts and alkaline pH can elute ssRNA without pyrophosphate but only pyrophosphate elution separates dsRNA from ssRNA. Optimization parameters and ranges are discussed in detail in [3]. H-Bond supports the same robust NaOH tolerance as PrimaS™.

Pyrophosphate anions must be removed from the final product because, *in vivo*, they form precipitates with calcium that can cause adverse health consequences. Pyrophosphate has the same charge as RNA, which suggests they should repel each other. However,

DOI: 10.18609/cgti.2020.114

FIGURE 7

Elution of DNA from a hydrogen bonding column in a salt gradient at the indicated pH values.



Salmon sperm DNA from Sigma-Aldrich. Equilibration buffers: 50 mM MES, pH 6.0; Hepes, pH 7.0; or Tris, pH 8.0. Elution in a linear gradient, same buffers plus 4 M NaCl. Flow rate 10 CV/min.

their shared metal affinity creates potential for them to form stable coordination bonds via multivalent metal cations [3]. A chelating agent needs to be present at a significant concentration. Pyrophosphate removal can be performed during final formulation by diafiltration but doing it in the context of a polishing step achieves the goal earlier in the process and increases confidence that it will be absent from the final product. Sensitive pyrophosphate assays to validate clearance are available from global suppliers.

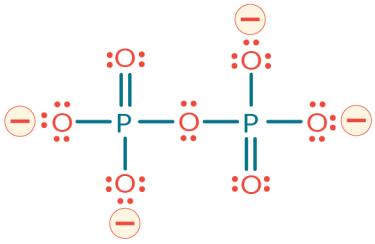
POLISHING AFTER CAPTURE

Coming out of a capture step with highly purified ssRNA, particularly lacking in double-stranded nucleic acids, contributes robustness to purification platforms using two chromatography steps. Removing the majority of DNA and dsRNA in advance allows the polishing step to accomplish what it is intended to do: polish. This is substantially preferable to the alternative of coming from capture with virtually the entire load of the most toxic contaminants at full strength, then relying on a single polishing step to fully remove them.

Otherwise, polishing may employ the same options used after capture by hybridization-affinity chromatography. HIC or RPC each provide independent orthogonal ability to separate ssRNA from dsRNA, DNA, and proteins, and each achieves a degree of size separation to remove short transcripts.

FIGURE 8

The distribution of charges and hydrogen acceptors on pyrophosphate, shown fully unprotonated.



Negative charges are shown in red with a yellow halo. Each pair of red dots indicates a free lone pair of electrons that can act as a hydrogen acceptor.

RPC gives better resolution than HIC but it requires the use of flammable solvents at elevated operating temperatures. RPC can be performed with either styrenedivinylbenzene (SDVB) or C-18 media but only SDVB is cleanable with 1 M NaOH [3,7–10].

HIC-polishing after either anion exchange or hydrogen bond chromatography enables exclusively ambient aqueous purification. In place of hazardous materials and conditions, HIC imposes a lesser logistical burden. Binding ssRNA to HIC media requires high concentrations of salts to drive retention. Those salts promote precipitation of RNA. If the RNA precipitates before it reaches the binding surfaces inside the column, those precipitates interfere with sample loading, they depress capacity, and they depress purification performance.

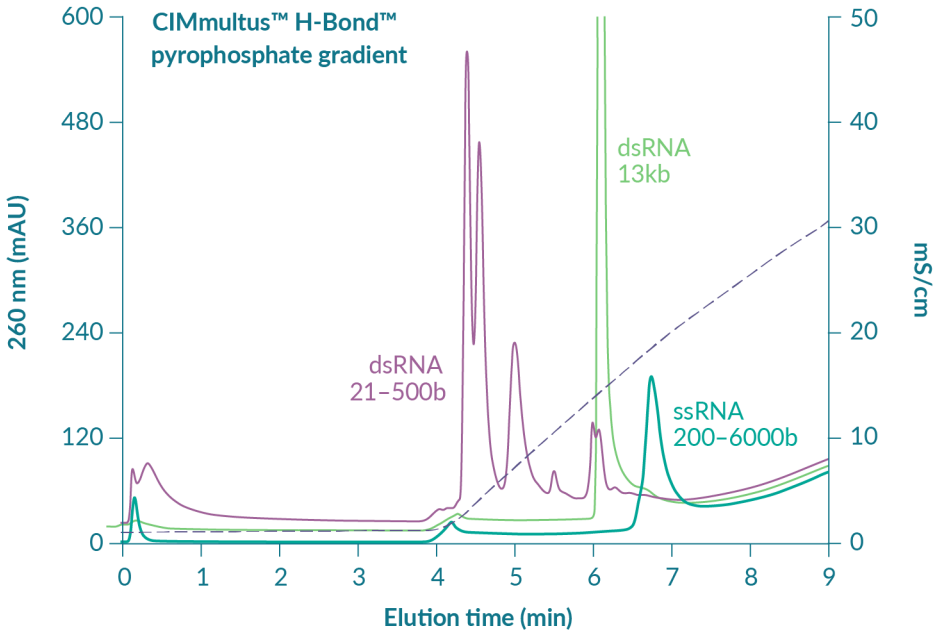
This challenge was resolved decades ago for preparative HIC purification of proteins. It requires sample loading by a technique known as in-line dilution. In-line dilution requires two input lines that meet at a mixer immediately before the column. This reduces the pre-column residence time of the ssRNA in high-salt to seconds, which prevents formation of large precipitates that would negatively affect chromatography. In addition, pre-column residence time of the sample in high salt remains uniform throughout the entire sample application phase, no matter how large the volume and how long it takes to load. Capacity and purification performance both benefit.

The first input line carries either low-salt buffer or sample, with an in-line 3-way valve to select one or the other. The second input line carries a high-salt diluent. Column equilibration is conducted with a mix of low-salt buffer with the high-salt diluent, for example 4 parts diluent to 1 part low-salt buffer. Sample application is done by switching the valve to deliver sample at the same mixing proportion. A wash step is performed by switching the valve back to low-salt buffer. Elution is performed by reproportioning the high and low salt buffers. HIC sample-loading by-in-line dilution is discussed in detail in [3].

All of these options support smooth workflow. The low salt concentration of ssRNA

FIGURE 9

Separation of dsRNA from ssRNA in a pyrophosphate gradient.



Overlay of 3 separate chromatograms, each run according the protocol of Figure 6.

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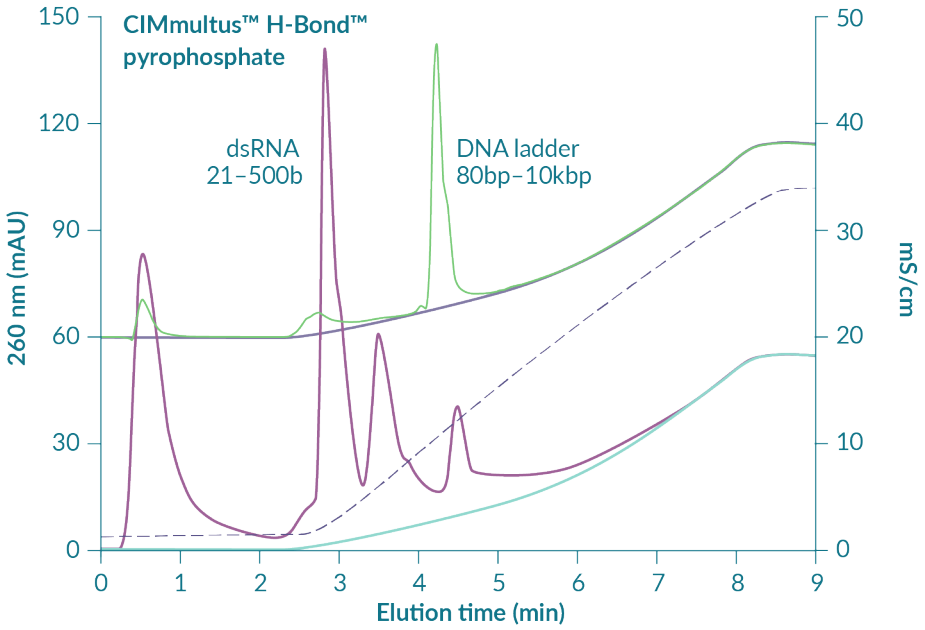
FIGURE 10

Elution behavior of DNA and dsRNA ladders in a pyrophosphate gradient.



Overlay of two chromatograms, same protocol as Figure 6.

after pH elution from PrimaS™ simplifies sample preparation going into the low-no salt method of RPC. After either anion exchange or hydrogen bonding chromatography, applying the sample to HIC simply requires adding salt. Inclusion of EDTA in the HIC binding salt helps displace residual pyrophosphates and it is also good insurance to eliminate residual metal ions carried over from any previous step.

CONCLUSIONS

Anion exchange and hydrogen bond chromatography can both be used to prepare research quality ssRNA in a single step. More importantly, they both provide an improved capture-foundation for two-step purification of clinical-quality single-stranded mRNA. Thanks to their ability to largely eliminate dsRNA and DNA with a salt wash, linear gradient elution can be converted to a step format with little or no compromise to purification of ssRNA. Both methods reduce the overall contaminant load going into polishing and they enhance robustness of the platform overall. Both methods support aggressive cleaning and sanitization with sodium hydroxide to enable multiple use, and both support a full range of scale-up options.

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DOI: 10.18609/cgti.2020.114

AUTHORSHIP & CONFLICT OF INTEREST

Contributions: *All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.*

Acknowledgements: *Some of the figures in this article were adapted from [3] with permission from the publisher.*

Disclosure and potential conflicts of interest: *The authors declare that they have no conflicts of interest.*

Funding declaration: *The authors received no financial support for the research, authorship and/or publication of this article.*

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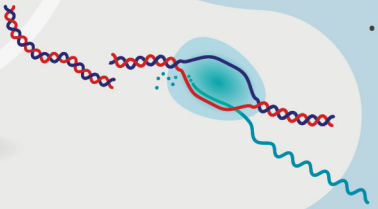

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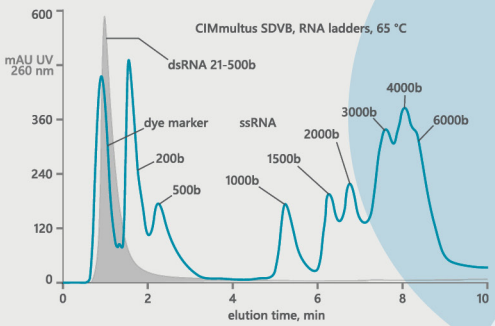

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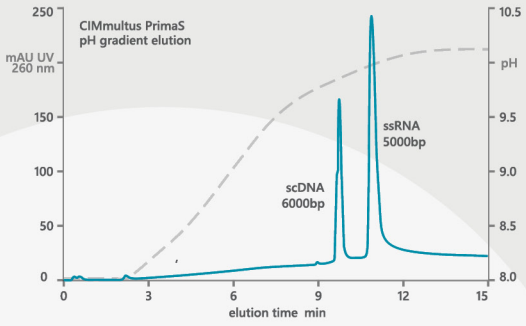

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