Application of steric exclusion chromatography on monoliths for separation and purification of RNA molecules

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**A B S T R A C T**

Steric exclusion chromatography (SXC) is a method for separation of large target solutes based on their association with a hydrophilic stationary phase through mutual steric exclusion of polyethylene glycol (PEG). Selectivity in SXC is determined by the size or shape (or both) of the solutes alongside the size and concentration of PEG molecules. Elution is achieved by decreasing the PEG concentration. In this study, SXC applicability for the separation and purification of single-stranded (ss) and double-stranded (ds) RNA molecules was evaluated for the first time. The retention of ssRNA and dsRNA molecules of different lengths on convective interaction media (CIM) monolithic columns was systematically studied under variable PEG-6000 and NaCl concentrations. We determined that over 90% of long ssRNAs (700–6374 nucleotides) and long dsRNAs (500–6374 base pairs) are retained on the stationary phase in 15% PEG-6000 and ≥0.4 M NaCl. dsDNA and dsRNA molecules of the same length were partially separated by SXC. Separation of RNA molecules below 100 nucleotides from longer RNA species is easily achieved by SXC. Furthermore, SXC has the potential to separate dsRNAs from ssRNAs of the same length. We also demonstrated that SXC is suitable for the enrichment of ssRNA (PRR1 bacteriophage) and dsRNA (Phi6 bacteriophage) viral genomes from contaminating cellular RNA species. In summary, SXC on CIM monolithic columns is an appropriate tool for rapid RNA separation and concentration.

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1. Introduction

Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) are macromolecules built from nucleotides, which consist of a heterocyclic nitrogenous base, a phosphate, and a pentose sugar. In aqueous solutions, duplex DNA is primarily in B-conformation, while RNA is mostly a single-stranded molecule adopting complex three-dimensional structures. When RNA duplexes are observed, they are predominantly in an A-form [1]. DNA and RNA differ in one of four bases; DNA contains thymine, while RNA contains uracil, a demethylated derivative of thymine. Furthermore, the pentose sugar in RNA is ribose but deoxyribose in DNA, which is derived from ribose by the loss of a 2' oxygen atom [2]. This loss provides DNA with the stability necessary to fulfill its main biological function as a keeper of genetic information [3]. One of the numerous functions of RNA molecules is to serve as a mediator in the flow of genetic information from DNA to proteins. Furthermore, RNA itself carries genetic information in many viruses either as a single-stranded (ssRNA) or a double-stranded (dsRNA) molecule. Biochemical and structural studies of viral RNA biosynthesis require large amounts of pure RNA molecules. For some applications, ssRNA and dsRNA can be simply synthesized in vitro [4] and purified from the reaction components. However, viral RNAs are heavily modified [5]. Accordingly, to study the effect of these modifications RNA species must be isolated directly from host cells with subsequent removal of cellular RNA, separation of viral ssRNA from dsRNA, and size-separation of RNA molecules.

There are a number of biochemical methods and their modifications developed for RNA isolation, purification, and separation [6]. However, all of these methods have certain limitations. While isopycnic centrifugation yields RNA molecules of high purity [7], the method is laborious, time-consuming, requires expensive equipment, and hence is rarely used. The most commonly used laboratory method for RNA isolation is acid guanidinium thiocyanate-phenol-chloroform extraction [8,9], which is unsurpassed in terms of pure RNA yields and provides excellent protection from RNases due to protein denaturation by guanidinium thiocyanate. However, the method has serious disadvantages as it is time-consuming and requires noxious reagents. Although fast and easy, solid phase extraction by immobilizing RNA on a specific support (silica, glass, magnetic beads, polystyrene-late) in the presence of chaotropic salts generally results in low
yields of RNA and requires DNase treatment [6]. Recently, two alternative methods of RNA isolation have been proposed, namely RNA snap [10] and RNAswift [11]. Both methods do not involve toxic reagents and total RNA can be recovered by isopropanol precipitation after a single round of differential centrifugation of cell lysates. Furthermore, amino-acid based affinity chromatography (histidine affinity chromatography or arginine affinity chromatography) can be successfully applied to isolate total RNA or certain fractions of small RNAs [12–14].

All the methods listed above lead to isolation of total RNA, which is a subject of further fractionation. Preparative polyacrylamide gel electrophoresis (PAGE) allows RNA separation with single-base resolution for ssRNA molecules up to approximately 1000 nt in denaturing conditions [15]. However, the method is extremely lengthy and provides only low yields of RNA molecules with acrylamide contaminants, which are difficult to remove from the sample. The RNA purified under denaturing conditions must be refolded, which is not always possible or might result in unproductive conformations with the loss of biological activity [16,17]. LiCl fractionation can selectively separate long and short RNA as well as ssRNA and dsRNA molecules from impurities [18]. However, the procedure is not very efficient and might require additional steps to obtain RNA of required purity and to remove LiCl, which might interfere with downstream applications. A separation of dsRNA from ssRNA and DNA can be achieved on CF11 cellulose using ethanol in the elution buffer [19]. This chromatographic method is commonly used to isolate viral dsRNA from plants and fungi. Nonetheless, the procedure is laborious, lengthy, and requires considerable amounts of initial sample. Furthermore, it does not provide separation of viral dsRNA segments of different lengths.

Fast processing and superior resolution of RNA molecules of different lengths can be achieved by ion-pair reversed-phase chromatography [20,21]. In this chromatography mode an ion-pairing reagent forms ion pairs with phosphates of nucleic acid. The resulting complex is strongly retained in the column via hydrophobic interactions [22]. However, to achieve a good separation of different RNA species, this technique requires denaturing conditions that can result in irreversible loss of RNA secondary structure. While ion-pair reversed-phase chromatography is an excellent analytical tool, its application for preparative purposes is limited as some ion-pairing reagents and toxic solvents (acetoniitrole) used in the procedure are difficult to remove from the purified sample. Furthermore, resins have only moderate loading capacity. Anion exchange chromatography (AEX) is based primarily on reversible electrostatic interactions between negatively charged phosphates of nucleic acids and the positively charged stationary phase. Stationary phases are also designed to have some hydrophobicity, which contribute to the separation. AEX is a robust and widely applied preparative method to obtain pure RNA molecules [23]. In our laboratory, we have developed a non-denaturing AEX protocol to purify enzymatically produced siRNA molecules on a monolithic strong anion exchange QA column [24]. According to our observations [24], it was not possible to resolve RNA molecules longer than 1000 base pairs (bp) by AEX. Since viral RNA genomes range in size from 1700 nucleotides (nt; human hepatitis D virus [25]) to 32 000 nt (coronaviruses [26]), we sought alternative methods to separate RNA molecules larger than 1000 nt under non-denaturing conditions.

A novel mode of steric exclusion chromatography (SXC) was recently described as a tool to separate and purify large biomolecules [27]. It should be noted that the term “SXC” was previously used to refer to size exclusion chromatography (SEC) [28]. Although both SXC and SEC do not rely on any direct chemical interactions and the separation of sample components is based on differences in their size or shape (or both), the SXC method developed by Lee et al. in 2012 substantially differs from SEC since it is suitable only for large solutes whose retention strongly depends on the mobile phase composition and is feasible only in hydrophilic stationary phase. In this approach, a sample is mixed with a specific size and concentration of polyethylene glycol (PEG) and is immediately loaded onto a hydroxyl-functionalized monolithic column. The mutual steric exclusion of PEG from both the large target solutes (proteins, virus particles) and the hydrophilic stationary phase results in their association without direct chemical interactions, while lower molecular weight molecules are washed away. The elution is achieved by reducing PEG concentration [27]. This method is mechanistically similar to the size fractionation of nucleic acids [29] and proteins [30] with PEG, in which the effectiveness of PEG increases along with the size of the polymer and larger molecules precipitate at lower PEG concentrations. The phenomenon is based on the steric exclusion of chemically non-reactive solutes [31,32]. According to this theory, PEG and biological solutes are sterically excluded from each other, which results in the formation of a PEG-deficient zone around a solute and creates discontinuity between the PEG-deficient zone and high-PEG bulk solution. This discontinuity results in an unfavorable increase in free energy. Grouping of the solutes followed by their precipitation leads to reduction in the contact area between PEG-deficient and high-PEG bulk solvents, and thus decreases the discontinuity and free energy of the system [27,31,32]. In the case of SXC, biomolecules accrete to the inert hydrophilic support instead of forming precipitates.

It has been demonstrated that monolithic columns can be successfully applied for SXC since their performance is minimally affected by viscosity (due to the large size of the interconnected pores) and convective mass transfer [33]. The described mode of SXC was applied for the purification of immunoglobulin M [27] and immunoglobulin G monoclonal antibodies [34], virus particles [27,35], and for the separation of serum proteins [36]. However, the potential of SXC to separate nucleic acid molecules has not been investigated.

In this study, we evaluated the binding and elution behavior of nucleic acid molecules during SXC with a focus on RNA, we determined the conditions suitable for separation of ssRNA and dsRNA molecules of different lengths, and purified viral RNA genomes.

2. Material and methods

2.1. Steric exclusion chromatography

A Convective Interaction Media (CIM) OH 1 ml tube monolithic column in polypropylene housing was obtained from BIA Separations (Ajdovščina, Slovenia). This polyglycidyl methacrylate-co-ethylene dimethacrylate-based monolith has an average pore size of 1.3 µm, outer diameter 18.6 mm, inner diameter 6.7 mm, length 4.2 mm, and bed volume 1.0 ml. The matrix is highly hydrophilic due to the high density of hydroxyl groups that originate from hydrolysis of epoxy ligands.

All chromatography experiments were performed at room temperature using an ÄKTA Purifier 10 UPC liquid chromatography system (GE Healthcare) operated by Unicorn 5.2 software (GE Healthcare). The chromatography system consisted of pump P-900, mixer M-925, monitor UPC-900, and fraction collector Frac-920. The absorbance at 260 nm was monitored during chromatography and 0.5-ml fractions corresponding to peak areas were collected automatically.

All reagents used in this study were of analytical grade. PEG-4000 and PEG-8000 were from Fluka and PEG-6000 was purchased from Ubichem. We observed that to obtain reproducible results and minimize data variability between different experiments, it was required to use PEG from the same manufacturer. Trizma base,
Tris–HCl, and NaCl were purchased from Sigma-Aldrich. Freshly prepared autoclaved Milli-Q (Millipore) water was used for buffer preparation. This water was determined to be nuclease-free by our internal laboratory tests.

The buffer A used for chromatography was composed of 50 mM Tris–HCl (pH 8.0). Buffer B contained different concentrations of PEG and NaCl in 50 mM Tris–HCl (pH 8.0). All buffers were vacuum filtered through 0.22-μm bottle-top filters (ThermoFisher Scientific) and degassed by sonication for 18 min prior to use. Fresh buffers were prepared every week and used within 4–5 days.

A basic chromatographic procedure used previously [27] was adapted as described below. (1) System equilibration. The standard setup of the ÄKTA Purifier was changed to make it suitable for SXC so that only buffer A flowed to the injector valve, while buffer B bypassed it and went directly to the mixer (Fig. 1). The column was placed in-line and equilibrated with 20% buffer A: 80% buffer B. Unless otherwise indicated, the buffer A:buffer B ratio was kept the same for simplicity, while the concentrations of PEG and NaCl in buffer B were optimized to achieve sufficient retention of nucleic acids. Further in the text, only the final concentrations of two chemicals are indicated (i.e., those in a mixture of 20% buffer A and 80% buffer B). To calculate the initial concentrations of PEG and NaCl in buffer B, the target final concentrations were divided by 0.8.

(2) Sample loading. Samples for chromatography were diluted with buffer A and centrifuged (11,000 × g, 10 min) to remove particulate matter. The injection volume was 0.25 ml and flow rate was 2.5 ml/min or 3 ml/min depending on the sample amount and PEG concentration in the system. Flow rates below 2.5 ml/min resulted in pre-column sample precipitation and chromatography failure.

(3) Washing. The system was washed until the UV absorbance of column effluent reached baseline. (4) Elution. The buffer A:buffer B ratio was reduced stepwise or linearly. (5) Cleaning in place (CIP). Removal of residual RNA from the column matrix was achieved by washing with 1 M NaOH solution followed by 1 M NaCl in 50 mM Tris–HCl (pH 8.0). CIP was performed after each chromatography experiment to avoid cross-contamination of subsequent analyses. Each chromatography experiment was repeated two to six times.

To calculate the retention of nucleic acids on the CIM-OH matrix, the molecules were precipitated from the fractions and flow through (see 2.5 for details). Concentration was measured with a NanoDrop 2000c (Thermo Fisher Scientific) and the percentage of RNA or DNA eluted by the gradient was then calculated using the formula: NA, % = $\frac{b}{a+b} \times 100$, where NA is the fraction of nucleic acid retained in the column (%); a is the amount of unbound sample in the flow-through solution (μg), and b is the amount of the sample eluted under gradient conditions (μg). The recovery of nucleic acids was calculated according to the formula: Recovery, % = $\frac{S_{l}}{S_{o}} \times 100$, where Sl is the amount of loaded sample (μg) and So is the amount of eluted sample (μg). The average recovery ± standard deviation (S.D.) was calculated in Excel based on the data from multiple experiments.

2.2. Preparation of nucleic acid molecules for chromatography

Bacteriophage λ genomic DNA (48 502 bp) was obtained from Fermentas and dissolved in nuclease-free Milli-Q water. DNA molecules ranging in length from 88 to 1800 bps were prepared by PCR amplification using Phusion HF DNA polymerase (Finnzymes), deoxynucleotide triphosphates (Thermo Fisher Scientific), and pLM659 plasmid containing a complementary DNA copy of Pseudomonas phase Phil6 genome segment S [37]. The forward primers for PCR amplification contained the T7 polymerase promoter sequence (5'-TAATACGACTCACTATAGGG-3') followed by a sequence of 17 to 21 nt complementary to the Phil6 S-segment at positions 80, 100, 200, 500, 700, or 1800 nt as counted from the 3' end. The reverse primer was complementary to the very 3' end of the Phil6 S-segment (5'-GGAAAAAGACAGACAGACCCCGAAGG-3') and contained the Phi6 polymerase promoter sequence at the 5' end (underlined). A list of primer sequences has been published [38]. Plasmids pLM659 [37], pLM656 [39], and pLM687 [40] were used as templates for the production of full-length complementary DNA copies of phase Phil6 S (2948 bp), M (4065 bp), and L (6374 bp) genome segments, respectively. The reverse primer was the same as described above and the forward primer contained the T7 polymerase promoter sequence followed by a homologous sequence (5'-GGAAAAAGACTTATATA-3') present at the 5'-end of all three genome segments of Phil6 [41]. PCR products of the correct size were excised from the gel and purified with NucleoSpin ExtractII kit (Macherey-Nagel) according to the manufacturer’s instructions. Pure DNA molecules were eluted in 30 μl Milli-Q water. The PCR-generated DNA molecules were used as templates for RNA synthesis. ssRNA molecules were prepared by in vitro transcription with T7 RNA polymerase (Thermo Fisher Scientific) as previously described [42]. dsRNA molecules were also generated in vitro using a single-tube transcription and replication reaction catalyzed by the T7 and Phi6 RNA polymerases [42,44]. The recombinant Phi6 polymerase was expressed and purified as described [42]. Nucleoside 5'-triphosphates were obtained from Thermo Fisher Scientific. Enzymatically synthesized ssRNA and dsRNA molecules were isolated with TRIZure reagent (Bioline) and chloroform (Merck) according to the manufacturer’s instructions, followed by precipitation of T7-generated transcripts in 3 M sodium acetate (Merck) or stepwise fractionation of dsRNA with LiCl (Merck) (see below). All RNAs were washed with 70% ethanol and dissolved in sterile nuclease-free water.

2.3. LiCl precipitation

Contaminating ssRNA molecules were removed from the dsRNA synthesis reactions by LiCl precipitation. ssRNA molecules were first precipitated by incubation at −20 °C for 30 min in 2 M LiCl followed by 20 min centrifugation at 13,000 × g at 4 °C. dsRNA was collected from the resulting supernatant by repeating the procedure in 4 M LiCl. The dsRNA pellet was washed twice with 70% ethanol and dissolved in sterile nuclease-free water. In case of unsatisfactory separation of dsRNAs from ssRNAs, the procedure described above was repeated.

2.4. Preparation of bacteriophage genomes for chromatography

Pseudomonas phase Phil6 [43] was propagated and purified as previously described [44]. The same protocol was used to recover Pseudomonas phase PR1 [45]. Briefly, virions were collected from the lysates of infected Pseudomonas syringae HB10Y or Pseudomonas aeruginosa PA01 by precipitation with 10% PEG-6000 and 0.5 M NaCl. To open the viral capsids, half of the precipitate was treated with proteinase K (Thermo Fisher Scientific) at a concentration of 1 mg/ml in 10 mM Tris–HCl (pH 7.5) buffer containing 5 mM EDTA (Merck) and 1% sodium dodecyl sulfate (SDS, Merck) at 50 °C for 1 h. From the remainder of the precipitate, total RNA was extracted using TRIZure reagent (Bioline) according to the manufacturer’s instructions.
2.5. Analysis of dsRNA integrity and purity

RNA was recovered from the collected 0.5-ml chromatography fractions corresponding to the peak areas on chromatograms by overnight precipitation at −20 °C in 0.3 M sodium acetate (pH 6.5) and 67% ethanol, followed by centrifugation at 10,000 × g for 30 min. The RNA pellets were washed with 70% ethanol, air dried, and dissolved in 10 μl of sterile nuclease-free water. A 5-μl sample was mixed with 2 × U loading buffer (10 mM EDTA [pH 8.0], 0.2% SDS, 0.05% bromphenol blue, 0.05% xylene cyanol, 6% [v/v] glycerol, 8 M urea). RNA molecules were separated in an 8% agarose gel by electrophoresis in Tris-borate-EDTA (TBE) buffer (6.6 g Trizma base, 3.9 g boric acid, 0.93 g EDTA). For electrophoresis, an EPS 301 electrophoresis power supply (GE Healthcare) and an Owl EasyCast B2 mini gel electrophoresis system (Thermo Fisher Scientific) were used. A detailed protocol has been described previously [46].

2.6. Treatment of the chromatography fractions with RNase or DNase

The fractions collected after the separation of DNA and dsRNA on CIM-OH column (Section 2.1) were precipitated as described (Section 2.5). 5.5 μl from the combined fractions 1 and 2; 6 μl from the combined fractions 3 and 4; 5 μl from the combined fractions 5 and 6; and 6.5 μl from the combined fractions 7 to 9 were subjected to RNase A treatment, DNase treatment, or no treatment (control). The reaction volume was 20 μl. For RNase treatment, 1 μg of nucleic acid was incubated with 0.1 μg of RNase A (Fermentas) in 0.1 × SSC buffer (3 M sodium chloride, 0.3 M sodium citrate [pH 7.0]) for 15 min at room temperature. DNase treatment was performed using RQ1 DNase (Promega; 1 unit/μg nucleic acid) at 37 °C for 30 min. All samples (including controls) were desalted with Illustra MicroSpin G25 columns according to the manufacturer’s instructions (GE Healthcare) and resolved with agarose gel electrophoresis in TBE buffer (see Section 2.5).

3. Results and discussion

3.1. Setup for steric exclusion chromatography for nucleic acid molecules

The monolithic chromatographic support is characterized by very high porosity, exceptional chemical stability, and flow characteristics, which makes it a valuable tool for separation or purification of large biomolecules [47]. CIM monolithic columns, a trademark of BIA Separations (Slovenia), are available in different formats with the same structure of the monolith and ligand density, which ensure scalability. In our work, we used a CIM-OH column with a column volume of 1 ml. A chromatographic procedure used previously for protein purification [27] was adopted here for separation of nucleic acid molecules.

To keep the pressure below 1.8 MPa (the pressure limit for the CIM-OH 1 ml column), we applied a maximum flow rate of 3 ml/min and the final concentration of PEG did not typically exceed 15%. To minimize PEG-induced precipitation of nucleic acids before entrance into the column, we used the in-line dilution technique where sample was injected in buffer A followed by mixing with a PEG-containing buffer B directly in the M-925 mixer (Fig. 1). The gradient delay volume (the volume between the mixer and the column) was also kept as low as possible (here 29 μl). Thus, at a 3 ml/min flow rate it took only 34.8 s for the sample to enter the column.

3.2. Suitability of CIM-OH column for nucleic acid separation

High-capacity binding of nucleic acids under SXC conditions has not been observed under the conditions used previously [27] and suitability of this chromatography mode for nucleic acid purification remained unclear. Therefore, the initial experiments were performed with dsDNA molecules, which are more robust and easier to prepare than RNA molecules. We used three different dsDNA species, namely 500 bp, 1800 bp, and 48 502 bp in length (see 2.2 for the origin of dsDNAs). We established that retention of nucleic acids on the column matrix required the presence of at least 0.4 M NaCl (Fig. 51) and was negligible (0.5–3%) when buffer B contained only PEG-6000 (up to 20% PEG-6000 was tested; data not shown). This observation is consistent with the PEG fractionation method, in which 15% PEG6000 at 0.55 M NaCl precipitates essentially all DNA in the size range of 100 to 46 500 bp [29]. The shorter dsDNA molecules (500 bp and 1800 bp) were eluted almost completely from the column at reduced PEG-6000 and NaCl concentrations. However, about 25% of the 48 502-bp long bacteriophage λ DNA was irreversibly retained on the column.

The requirement of NaCl for efficient retention of nucleic acids under SXC conditions is likely connected to the modification of steric effects by the repulsive forces between negatively charged chemical groups of the nucleic acid molecules [31,48,49]. Although PEG changes electrostatic interactions in solutions and the molar conductivity of NaCl-containing buffer decreases with increases in PEG concentration, all nucleic acid molecules become hydrated. In this aqueous phase, NaCl ionizes and forms ion pairs with phosphates of nucleic acids. Therefore, to neutralize the negative charge and to decrease the electrostatic repulsion forces between nucleic acid molecules, we used NaCl-containing buffers in all subsequent experiments. This resulted in substantially better binding of nucleic acids to the column matrix (see below). The requirement to neutralize charges for optimal SXC performance was also observed previously for protein samples; the best results were achieved when proteins were near their isoelectric point [27].

We also initially tested PEG molecules of different molecular weights (4000, 6000, and 8000 g · mol−1). PEG-6000 performed better in terms of RNA binding and elution and in generated back-pressure. Therefore, in our subsequent experiments we used only PEG-6000.

3.3. Optimizing SXC for ssRNA and dsRNA molecules

3.3.1. Influence of NaCl concentration on RNA retention in CIM-OH column matrix

After successful experiments with dsDNA, we tested the possibility to use SXC for the separation of RNA molecules and the influence of NaCl and PEG-6000 concentrations on RNA retention and subsequent elution. We used 300-bp, 1800-bp or 6374-bp dsRNA molecules and initially kept the PEG-6000 concentration constant (15%) while systematically varying the salt content of the buffers (Fig. 2A). Small fractions of the applied RNAs were retained on the CIM-OH column matrix in the presence of 0.2 M NaCl. However, about 75% of the 300-bp dsRNA and 50% of the 1800-bp and 6374-bp dsRNAs eluted in the flow-through (Fig. 2A). After increasing the NaCl concentration to 0.4 M or above, no RNA was detected by agarose gel electrophoresis in the flow-through samples of the 1800-bp and 6374-bp dsRNAs. For the 300-bp dsRNA, an even higher salt concentration (0.8–1 M) was required for efficient binding to the column matrix. At higher salt concentrations, PEG molecules are known to adopt a more compact, coiled structure [50]. This decreases the hydrodynamic radius of PEG and consequently its steric exclusion effects, which should lead to reduced retention of nucleic acids on the column matrix [50]. In protein SXC [27], retention of IgM diminished rapidly to zero when the NaCl
concentration exceeded certain threshold values (0.4 M NaCl at 8% PEG-6000 or 0.9 M NaCl at 10% PEG-6000). However, in our experiments a higher PEG-6000 concentration and up to 1 M NaCl did not show a substantial negative effect on the nucleic acid retention.

### 3.3.2. Influence of PEG concentration on ssRNA and dsRNA retention in CIM-OH column matrix

In the next series of experiments, we kept the NaCl concentration constant (0.8 M) and followed how changes in PEG-6000 concentration affected the binding and elution behavior of ssRNA and dsRNA molecules (Fig. 2B and 2C). Irrespective of the ssRNA or dsRNA length, no binding was achieved when the PEG-6000 concentration was 7% or less. Since the volume of the PEG-deficient zone must be proportional to the size of the target biomolecules [31], larger species of ssRNA molecules bound at lower PEG concentrations (Fig. 2B). Accordingly, 35% of 6374-nt, 20% of 4065-nt, and 7% of 2948-nt ssRNAs were retained on the column in 8% PEG-6000. None of these RNA species were detected in the flow-through in 11% or 12% PEG-6000. ssRNAs of 1800 nt and 700 nt required at least 10% PEG-6000 for binding to the column matrix. About 95% of these ssRNA species were retained in 15% PEG-6000. Some retention of the shorter ssRNA molecules used in the study (≤500 nt) was observed in 11% PEG-6000. However, even in 15% PEG-6000, less than 80% of ssRNAs of 500 to 100 nt was retained.

Small percentage of dsRNA molecules in the range of 700 – 6374 bp accreted to the column matrix already in 8% PEG-6000, while retention of 86% – 100% was achieved in 12% PEG-6000 (Fig. 2C). dsRNA molecules of 500 bp started to accrete to the column matrix in 10% PEG-6000; full retention was accomplished in 15% PEG-6000. Complete retention of dsRNA shorter than 500 bp was not achieved under the conditions tested (Fig. 2C).

In the case of longer dsRNA molecules corresponding to phage Phi6 genome segments, we did not observe a clear relationship between molecule size and the PEG concentration required for binding. We calculated the lengths of these dsRNA molecules, assuming that 1 bp of dsRNA corresponds to 0.29 nm [51]. Thus, the estimated length of Phi6 L-, M-, and S-segments (6374, 4065, and 2948 bp, respectively) were approximately 1.8 µm, 1.2 µm, and 854 nm, respectively. The structure of dsRNA molecules is more rigid compared to ssRNAs, and dsRNAs might not condense to the same extent as ssRNAs of the same length in PEG/NaCl solution. This could hamper the migration of the long dsRNA molecules through the pores of the column matrix, which are only 1.3 µm in diameter. Alternatively, cryogel monoliths [36] with superporous structures (10–100 µm) could be tested for the separation of the longer dsRNA molecules.

The dynamic binding capacity for RNA samples could not be determined, as upon loading >250 µg of dsRNA in 13% PEG-6000 the backpressure increased beyond acceptable limits. The RNA recovery under optimal gradient conditions was 80 ± 9%. Our preliminary experiments demonstrated that for effective elution of RNA species, a high-amplitude rapid change in PEG concentration is required. Conversely with long linear gradients (>18 CV), RNA molecules were spread in numerous fractions, which resulted in low ultimate RNA recovery and poor separation (Fig. S2). Thus, to achieve satisfactory results a short linear or a stepwise gradient should preferably be applied (see Figs. 3–6 for the examples of optimized gradient conditions).

### 3.4. Separation of DNA and dsRNA molecules

dsDNA and dsRNA species of the same length could be partially resolved by SXC on a CIM-OH column (Fig. 3). dsDNA was eluted first while the majority of dsRNA molecules were retained longer and eluted in the second peak. Similar results were obtained for nucleic acid molecules of 1800 bp and 500 bp. Data on the separation of 1800-bp dsDNA and dsRNA are shown in Fig. 3. In addition to the size of the nucleic acid molecule (Fig. 2), differences in their configuration and chemistry might play an important role in sample retention. Compared to the B-form of dsDNA, the A-form of dsRNA duplex is shorter and wider with a deeper major groove. Monovalent and divalent cations penetrate into the major grooves of dsRNA, which results in more efficient shielding of dsRNA charge compared with dsDNA [52]. Intermolecular and intramolecular repulsion vanishes at a lower cation concentration and, therefore, dsRNA might associate with each other and the column matrix more efficiently than dsDNA.

### 3.5. Separation of RNA molecules of different sizes

We did not achieve any satisfactory separation of RNA molecules of different sizes (either ssRNA or dsRNA) despite significant (2 times or greater) differences in size (data not shown). Only separation of RNA molecules shorter than 100 nt from longer RNA species was easily achieved by SXC (Fig. S3). This is because the short molecules are not retained on the column matrix, whereas the longer ones are retained under the conditions applied (Fig. 2).
3.6. Separation of dsRNA and ssRNA molecules of the same length

We have developed a method for in vitro production of dsRNA molecules of different lengths and sequences [4]. For subsequent biochemical applications, the dsRNA molecules must be purified from contaminating ssRNAs of the same length, abortive transcripts, NTPs, and enzymes. A stepwise LiCl precipitation can be applied for routine use. However, this method does not provide efficient removal of ssRNAs. When ssRNA molecules interfere with a subsequent application, an AEX can be used to purify dsRNA [24]. We applied SXC as an alternative means to separate dsRNA from contaminating ssRNA. Since the difference in electrophoretic mobility between ssRNA and dsRNA species is substantial only for long molecules (in our study ≥ 700 nt), we tested RNA molecules of 700 nt, 1800 nt, and 4063 nt (Fig. 4) to verify the efficiency of SXC separation using non-denaturing agarose gel electrophoresis analysis of the samples.

Generally, the separation of ssRNA and dsRNA molecules by SXC improved as the size of the molecules increased; the best separation and highest purity was obtained for dsRNAs of 1800 bp and 4063 bp, whereas after SXC dsRNA of 700 bp contained a substantial amount of ssRNA of 700 nt. In all experiments, ssRNA molecules were retained on the column matrix more strongly than dsRNAs of equal length (Fig. 4). Thus, on the basis of agarose gel electrophoresis analysis of the elution fractions, a pure fraction of dsRNA was attainable from a mixture of dsRNA and ssRNA using SXC. However, subsequent fractions always contained dsRNA molecules in addition to ssRNA. This might be due to the presence of dsRNA in the interstices of the densely packed ssRNA precipitates on the column stationary phase surface.

The observed separation efficiency of SXC is comparable to that obtained by AEX [24]. Moreover, the whole process took only 30 min (20 min for system preparation and 10 min for sample injection and elution). Achievement of similar resolution with AEX requires the use of substantially longer gradients (100 CV) [24]. Accordingly, for the separation of long dsRNA molecules from a mixture of ssRNA and dsRNA, SXC is an effective and efficient alternative to AEX.

3.7. Purification of ssRNA and dsRNA virus genomes by SXC

3.7.1. Purification of phage PRR1 genome from host cell lysate

We applied both linear and stepwise gradients to separate the 3574 nt-long genomic ssRNA of Pseudomonas phage PRR1 directly from lysate of infected bacterial cells (Fig. 5A and B). Short bacterial ssRNA molecules (<300 nt) did not bind to the column and were recovered in the flow-through. A decrease in PEG concentration resulted in the elution of the phage genome together with contaminating host RNAs. Host plasmid DNA was also detected in some of the fractions containing the phage genome (Fig. 5A and B). Moreover, some proteins co-eluted with the viral RNA (Fig. 5A), which was expected since the separation is size-dependent and large impurities can co-precipitate with the target molecules. To increase the purity of the viral genome, we extracted the total RNA from the bacterial lysate by TRIZure reagent to remove proteins and cellular DNA molecules from the sample (see 2.4). Using a stepwise gradient of PEG-6000 we obtained 85% pure phage PRR1 genome, as determined by agarose gel electrophoresis analysis (Fig. 5C).

The CIM-OH column concentrated viral RNA molecules so that even minor RNA species could be detected in some of the fractions by agarose gel electrophoresis (Fig. 5A). This confirmed previous observations [27] that unlike PEG precipitation, binding efficiency during SXC is unaffected by low target concentration. Thus, SXC could potentially be used as an analytical tool for characterization of complex RNA mixtures.

3.7.2. Purification of phage Phi6 genome from host cell lysate

A Pseudomonas phage Phi6-infected bacterial lysate was used to evaluate the possibility to purify a viral dsRNA genome from infected cells and to separate the individual genome segments. We first applied the lysate from a Phi6-infected bacterial culture onto a CIM-OH column after proteinase K and SDS treatment (see 2.4). In this case, the best separation was achieved with a stepwise gradient (Fig. 6A). However, bacterial plasmid DNA co-eluted with both dsRNA and ssRNA species. With this approach it was possible to obtain fractions significantly enriched with Phi6 genomic dsRNA directly from the host lysate without major protein contamin-

ants (Fig. 5A). However, we were unable to separate contaminating ssRNA molecules from the viral genome. We were also unable to separate the three viral genome segments from each other.

To improve the SXC-based purification of the dsRNA genome of bacteriophage Phi6, we isolated the total RNA from the lysate of Phi6-infected bacteria using TRizure reagent. A linear gradient (12% PEG-6000, 0.6 M NaCl to 0% PEG-6000 in 10 CV) provided good separation of the dsRNA genome from contaminating ssRNA molecules (Fig. 6B). Pure dsRNA genome was eluted at 7.5% PEG-6000 and 0.38 M NaCl.

The recovery and efficiency of dsRNA purification with a CIM-OH column was compared with the LiCl fractionation routinely used in our laboratory. Six identical samples of total RNA after phenol-chloroform extraction from P. syringae lysates were prepared. Three samples were applied onto a CIM-OH column and the
Chromatographic separation of dsRNA from contaminating ssRNA of the same size using a CIM-OH column and a linear or stepwise PEG-6000 gradient. Products of an in vitro dsRNA synthesis reaction containing 30 µg of dsRNA and ssRNA molecules of 700 nt (A, B), 1800 nt (C, D), or 4068 nt (E, F) in 150 µl volume were loaded onto the CIM-OH column pre-equilibrated with 75% buffer B (50 mM Tris-HCl [pH 8.0], 17% PEG-6000, 1.07 M NaCl) to obtain a final concentration of 13% (w/v) PEG-6000 and 0.8 M NaCl. Elution was performed using a linear gradient of 75% to 0% buffer B in 13 (A, E) or 18 (C, D) CV or a two-step gradient of 50% buffer B in 7 CV and 0% buffer B in 5 CV (B), 50% buffer B and 0% buffer B, both in 5 CV (D), or 48% buffer B and 0% buffer B, both in 5 CV (D). The flow rate was 3 ml/min. (Left panel) Absorbance profile (260 nm; black line) and conductivity (gray line) of the eluate. (Right panel) Agarose gel electrophoresis analysis of selected elution fractions. The mobility of the dsRNA and ssRNA molecules is indicated on the right. L – DNA ladder, S – sample, FT – flow-through, P – peak, Fr. – fractions, kbp – kilo base pairs.
remaining samples were precipitated with LiCl. After LiCl precipitation, 7.05 ± 1% of the total RNA was precipitated as dsRNA. While slightly less RNA (5.4 ± 1.6%) was recovered in the dsRNA-enriched fractions eluted from the column, the purity significantly surpassed that obtained using a single cycle of LiCl fractionation (Fig. 6B).

4. Conclusions

We evaluated the suitability of SXC for the separation and purification of ssRNA and dsRNA molecules of different lengths. We determined the conditions under which efficient retention and elution of nucleic acids (both DNA and RNA) could be achieved (Fig. 2, Fig. S1). Retention of nucleic acids required up to 1 M NaCl depending on the molecule length and above 7% PEG-6000. We demonstrated that SXC on CIM monolithic columns can be applied to separate dsRNA from ssRNA and that the resolution is better for longer (>700 bp) dsRNA molecules (Fig. 4). Nevertheless, the use of SXC for the separation of RNAs of different lengths is limited, and only short RNA molecules (<100 nts) can be easily resolved from longer RNA species (Fig. S3). SXC on a CIM-OH column has the potential to separate dsRNA and dsRNA molecules of the same length (Fig. 3) due to the structural differences between these molecules.

Although separation of viral genome segments was not achieved, SXC could separate and purify whole viral ssRNA and dsRNA genomes from contaminating cellular RNAs (Fig. 5 and 6). In terms of recovery, SXC surpassed AEX on CIM-QA, CIM-DEAE, and Gen-Pak FAX columns by at least 25%. Furthermore, SXC is of general utility for concentrating RNA virus genomes. This is especially useful for low-abundance RNA species, such as viral replicative forms and mutualistic viruses.
Fig. 6. Separation of Pseudomonas phage Phi6 dsRNA genome from contaminating cellular nucleic acid molecules. (A) Bacterial lysate containing Phi6 virions was treated with proteinase K and SDS and applied to the CIM-HP column equilibrated with 80% Buffer B to provide an initial concentration of 13% (w/v) PEG-6000 and 0.64 M NaCl (Buffer A, 50 mM Tris-HCl [pH 8.0]; buffer B, 50 mM Tris-HCl [pH 8.0], 16% [w/v] PEG-6000, 0.8 M NaCl). The elution was performed using a stepwise gradient of 45% Buffer B in 5 CV and 0% Buffer B in 5 CV at flow rate 2.5 ml/min. (B) Total RNA extracted from the bacterial lysate with TRExol/chloroform extraction (120 µg) in 350 µl of 50 mM Tris-HCl (pH 8.0) was loaded onto the column pre-equilibrated with 75% Buffer B to obtain a final concentration of 12% (w/v) PEG-6000 and 0.6 M NaCl. A linear gradient of 75% to 0% Buffer B in 10 CV was applied for elution at flow rate 3 ml/min. An identically prepared RNA sample was used for stepwise LiCl precipitation. (Left panel) Absorbance profile (260 nm; black line) and conductivity (gray line) of the eluate. (Right panel) Agarose gel electrophoresis analysis of selected elution fractions and LiCl-precipitated ssRNA and dsRNA species. The position of the Phi6 genome, composed of three dsRNA molecules, is indicated on the right.

Declarations of interest

None.

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Appendix A. Supplementary data

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References


