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Preconcentration and purification of oligonucleotides from heat-treated human plasma by anion-exchange microextraction devices coupled to high performance liquid chromatography-mass spectrometry

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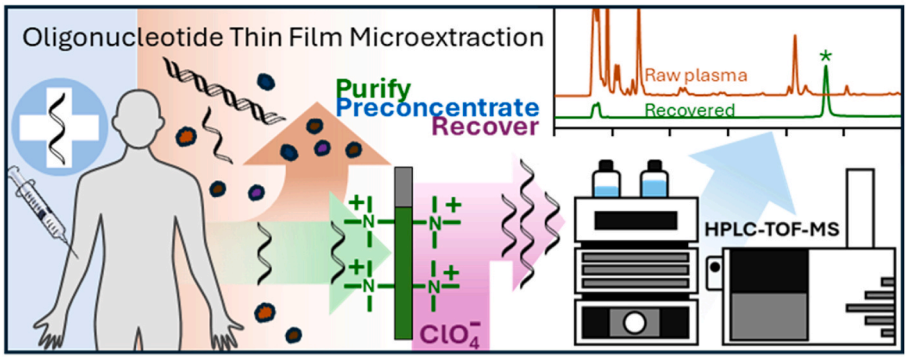
HIGHLIGHTS

- Interactions of anion-exchange sorbents with nucleic acids were examined.
- Sorbent affinities for proteins, oligos, RNA, and DNA were determined.
- Purification of an antisense oligonucleotide in human plasma was demonstrated.
- Microextraction recovery solutions were compatible TOF mass spectrometry.
- Reversed-phase and HILIC separations were coupled with sorbents for MS analysis.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: Methodologies that preconcentrate high quality nucleic acids (NAs) for downstream assays are essential for their accurate and reproducible analysis by mass spectrometry (MS). Established methods rely on solid-phase extraction that involves numerous steps and user intervention or liquid-liquid extractions that are time-consuming and employ toxic organic solvents. A promising alternative methodology involves anion-exchange microextraction sorbents that selectively isolate NAs through electrostatic interactions with the negatively charged phosphodiester backbone. The microextraction devices recover and preconcentrate NAs using a salt-containing solution, which is generally incompatible with MS analysis.

Results: Six anion-exchange microextraction sorbents featuring monomers derived from 2-aminoethyl methacrylate were synthesized and examined in this study to understand the interactions that take place between NAs and the ammonium cationic moiety. Sorbent affinities for bovine serum albumin, small single-stranded oligonucleotides, RNA, short double-stranded DNA), and 2000 bp dsDNA were determined. Recoveries of an oligonucleotide and 2000 bp dsDNA were measured and examined in salt solutions of varied concentration, anion species, and organic additives. High oligonucleotide preconcentration factors of 8.6 ± 0.2 were obtained for the sorbent featuring two cationic ammonium moieties on each monomer using 500.0 mM ammonium perchlorate. A separate sorbent composed of dimethyl ethyl ammonium moieties produced a preconcentration factor of 4.6 ± 0.2 using only 31.25 mM ammonium perchlorate. The sorbents were demonstrated in the complete workflow in

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which an analog antisense oligonucleotide was spiked into human plasma and purified, enabling successful molecular weight analysis.

Significance: This study demonstrates the compatibility of microextraction recovery solutions comprised of ammonium perchlorate with reversed-phase and hydrophilic interaction chromatographic separations and time-of-flight MS for characterization of oligonucleotides. The combination of anion-exchange microextraction sorbents and HPLC-TOF-MS enables the purification, preconcentration, and identification of a oligonucleotides from heat-treated human plasma. The compatibility of the salt-containing recovery solutions with chromatographic separation modalities highlights that anion-exchange microextraction devices are a complete and compelling sample preparation methodology for oligonucleotides.

1. Introduction

Advances in the understanding of nucleic acid (NA) function and modification have initiated revolutionary changes in the fields of diagnostics and medicine. Detecting specific NA sequences has facilitated pathogen identification and disease state determination to inform personalized treatment plans [1,2]. More recently, new classes of therapeutic treatments featuring NAs offer novel pathways for disease targeting [3]. Therapeutic NAs are biopolymers composed of the same deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) units common to all life and give rise to biological functions. The function of these drug molecules arises from the specific arrangement of these subunits, which enable regulation of protein expression. Antisense oligonucleotides (ASOs) target specific mRNA sequences through Watson-Crick base pairing and modulate downstream protein expression [4]. This approach is particularly promising for rare diseases arising from single nucleotide polymorphisms (SNPs) as mutant proteins can be explicitly targeted due to the high specificity of interactions [5]. Until now, nearly 20 oligonucleotide drug molecules have been approved by the Food and Drug Administration (FDA), with many more in the pipeline.

Oligonucleotides are often produced using solid-phase synthetic methods in a step-by-step manner to obtain the biopolymer with the correct sequence of bases and modifications. Commonly, the phosphodiester backbone is modified to phosphorothioate moieties containing sulfur atoms resulting in enhanced resistance to enzymatic degradation [3]. Drug molecules may contain only a few phosphorothioate modifications or have entire backbone replacement. Products and impurities may differ by only a single base, modification, or localization of the modification. Additionally, synthetic error is propagated exponentially to create a vast pool of side products possessing nearly identical chemical structures. The identification of oligonucleotides principally requires mass spectrometry (MS) for molecular weight analysis [6]. Upstream to MS analysis, high performance liquid chromatography (HPLC) is often employed to separate products based on their chemical structure and characteristics. Commonly, ion pair reversed-phase chromatography [7] (IP-RP) and hydrophilic interaction chromatography [8] (HILIC) are used for challenging oligonucleotide separations. Other analytical methods used for the detection of oligonucleotides include polymerase chain reaction (PCR) and enzyme-linked immunosorbent assays (ELISA), which possess significantly lower limits of detection but lack specificity [9].

The efficacy of oligonucleotide therapeutics must be demonstrated in preclinical trials through the analysis of drug molecules in complex biological samples. The isolation, purification, and preconcentration of oligonucleotides is a requirement for reliable and sensitive detection with downstream methodologies. Workflows involving commercial solid-phase extraction (SPE) kits [10–12], liquid-liquid extraction (LLE) [13–15], or both [16–18] have been demonstrated for the analysis of oligonucleotide drugs in human serum samples. SPE kits are generally expensive, laborious, and time-consuming as many steps are required for binding, washing, and elution of oligonucleotides. Methodologies using LLE rely on toxic organic solvents, such as phenol and chloroform, in order to purify oligonucleotides. An alternative method that is fast, effective, and does not require solvents is needed.

Microextractions represent a class of analytical separation methodologies which can be tailored to offer more efficient analyte enrichment than SPE with lower sorbent-to-sample ratios. Many microextraction devices are open-bed and employ selective sorbents in a geometry to control analyte sorption and enable the simultaneous isolation and purification of analytes [19]. This versatile methodology has been used for headspace [20], direct-immersion [21], and in-tissue sampling [22]. For the purification of NAs, recent work has demonstrated that sorbents with cationic moieties have high affinity for double-stranded DNA (dsDNA) through electrostatic interactions [23]. These microextraction devices alleviate the aforementioned drawbacks of SPE and LLEs with fast, solvent-free, and reusable extraction devices. Furthermore, the devices were able to achieve substantial preconcentration of dsDNA and were unaffected by direct-immersion sampling from heat-treated human plasma. The recovery of dsDNA was performed using 250 mM sodium perchlorate salt which is significantly lower than concentrations used in earlier work [24], and highlight the potential of the newly developed anion-exchange sorbents for oligonucleotide purification prior to HPLC-MS analysis.

Herein, we report for the first time the affinity of custom designed and synthesized anion-exchange microextraction sorbents for oligonucleotides, RNA, dsDNA, and proteins. The roles of anion species, salt concentration, and organic modifier in the recovery of oligonucleotides and larger dsDNA are examined. The primary focus of this study is to determine key fundamental attributes of NA affinity and recovery enabling the development of separation media for microextractions that function by an anion-exchange mechanism. In addition, coupling these devices with HILIC and reversed-phase HPLC separations is demonstrated through the analysis of oligonucleotide mixtures containing up to 350 mM ammonium salts. The combination of an anion-exchange microextraction sorbent and HPLC-TOF-MS is used in the purification, preconcentration, and identification of a spiked ASO from heat-treated human plasma.

2. Experimental

2.1. Reagents

For monomer synthesis and extraction device fabrication, 2-aminoethyl methacrylate hydrochloride (90 %), 2-(diethylamino)ethyl methacrylate (99 %), 1,4 butane sultone (≥98 %), (3-bromopropyl) trimethylammonium bromide (97 %), bromobutane (99 %), 1,4-butanediol diacrylate, methanol (≥98.0 %), and DAROCUR 1173 (>96 %) were acquired from Sigma-Aldrich (St. Louis, MO. USA). Vinyltrimethoxysilane (98 %) and 30 % hydrogen peroxide were obtained from Fisher Scientific (Hampton, NH. USA). Additionally, 2-(dimethylamino)ethyl methacrylate (>98.5 %) and bromoethane (>96 %) were purchased from TCI (Tokyo, Japan).

All solutions and mobile phases were prepared with LC/MS grade acetonitrile, methanol, and ammonium formate (LCMS grade, 10 M; CovaChem, Loves Park, IL, USA). Additionally, ammonium acetate (≥97 %; Fisher Scientific), ammonium perchlorate, sodium chloride, sodium hydroxide (98 %; Thermo Scientific), and Tris HCl (98 %; P212121, Ypsilanti, MI. USA) salts were obtained from the respective

suppliers.

2.2. Preparation of nucleic acid samples

Liquid cultures of *S. cerevisiae* (ATCC 9763) were prepared following inoculation of a solution containing 10 g/L tryptone, 5 g/L yeast extract, 0.5 g/L NaCl, and 20 g/L of sucrose. The culture was incubated at 30.0 °C in an I 24 incubator shaker (New Brunswick Scientific, Edison, NJ, USA) at 150 rpm for 12 h. The cultures were added to a 50 mL conical vial and centrifuged with an Eppendorf 5920 R bucket centrifuge (Hamburg, Germany) at 2000 rpm for 10 min at 4 °C. The supernatant was discarded, and the cell pellet subjected to TRIzol™ RNA extraction (Invitrogen, Waltham, USA) according to the manufacturer's instructions. The isolated RNA was quantified using a 2,000c Nanodrop spectrophotometer (ThermoFisher). The high-quality isolate was observed to be of high molecular weight with a wide length distribution, which is in line with other reports that used a similar RNA extraction protocol to obtain sizes ranging from 600 to 6000 base pairs (bp) [25] and an average length of approximately 1250 [26]. Aliquots were prepared and stored at −80 °C prior to use.

A standard solution containing 1000 ppm of salmon testes dsDNA (Sigma-Aldrich) was prepared and aliquoted before storage at −20 °C. Manufacturer specifications indicate an average length of approximately 2000 bp for this sample. The short 98 bp dsDNA sample was prepared following product generation by PCR, separation by gel electrophoresis, and kit purification, as previously reported [24]. Three oligonucleotides were acquired from Integrated DNA Technologies (Coralville, IA, USA). Known sequences and lengths of NAs studied in this work are provided in Table 1. Additionally, bovine serum albumin (BSA) was acquired from Sigma-Aldrich.

2.3. Synthesis of poly-ionic sorbents and fabrication of TFME devices

The monomers employed in this study are featured in Fig. 1. Synthesis of the zwitterionic (**Z**), dimethyl butyl-containing monomer (**DMB**), and the dicationic (**DC**) monomer involved using a previously reported methodology [24]. The dimethyl ethyl-containing (**DME**) monomer and triethyl (**TE**) monomers were synthesized by combining 5.9 mmol of (2-dimethylamino)ethyl methacrylate or (2-diethylamino) ethyl methacrylate with 5.9 mmol of bromoethane and stirred at room temperature for three days under darkness. The products were obtained as white solids and further purified by filtration with acetone. Characterization of the products was carried out using nuclear magnetic resonance (NMR) spectroscopy; ^{1}H and ^{13}C spectra are provided in the supporting information. The primary amine (**A**) monomer was acquired as 2-aminoethyl methacrylate hydrochloride (90 %) from Sigma-Aldrich and used without modification.

TFME device fabrication involved reducing a nitinol metal sheet acquired from Nexmetal (Sheridan, WY) into 3.5 mm × 25 mm x 0.33 mm strips. The surface of the strips was functionalized with vinyltrimethoxysilane using a previously reported method [27]. Prepolymer solutions were prepared by combining 20.0 mg of each monomer (Fig. 1), 10.0 μL of 1,4-butanediol diacrylate, 20.0 μL of methanol, and

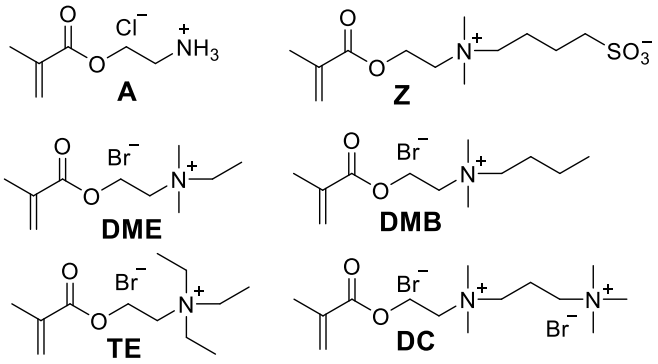

Fig. 1. Chemical structures of monomer units derived from 2-aminoethyl methacrylate that were polymerized to form the extraction sorbents examined in this study. Nomenclature featured below each structure was selected to represent the unique ionic features such as the following: primary amine (**A**), zwitterionic (**Z**), dimethyl ethyl (**DME**), dimethyl butyl (**DMB**), triethyl (**TE**), or dicationic (**DC**).

2.5 μL of photoinitiator. A 2.0 μL volume of the prepolymer solution was applied to a 20 mm × 3.5 mm area per each side and placed into a Rayonet photoreactor. The devices were dried for 30 min using a fan and then irradiated for 30 min with RPR-2537 Å (Branford, CT) lamps to achieve polymerization. A schematic of the device fabrication process and images of the produced devices can be found in Fig. S1.

2.4. Anion-exchange high performance liquid chromatography with ultraviolet detection (AE-HPLC-UV)

Separations were carried out using an Agilent Technologies 1260 Infinity II HPLC fitted with a TSKgel DNA-NPR (75 mm × 4.6 mm I.D.) strong anion-exchange column with a particle size of 2.5 μm from Tosoh Bioscience (King of Prussia, PA, USA). Mobile phase A contained 20 mM tris-HCl adjusted to pH 9.00, and mobile phase B contained the same tris-HCl buffer and 1.00 M NaCl. Injection volumes of 20.0 μL and a flow rate of 0.5 mL/min were used for all experiments. A gradient of 5 % B to 100 % B in 10 min and a detection wavelength of 280 nm was used. Similarly, NAs were separated with a gradient of 10 % B to 100 % B in 10 min and 260 nm was used for peak area determination. Example chromatograms for each analyte can be found in Fig. S2.

2.5. TFME procedures and calculations

To determine the extraction affinity of devices, 1.00 mL samples of 2.00 mM tris-HCl (pH 9.00) were spiked with either 6.00 ppm oligo1, 10.0 ppm st-DNA, 100 ppm BSA, 10 ppm yeast RNA, or 5.67 ppm amp-DNA. Samples were exposed to the extraction devices for 10 min under vortex agitation at 2500 rpm. To determine the extraction factors for each analyte, the devices were removed, and 20.0 μL of the extraction solution was subjected to AEX-HPLC-UV analysis, where the peak area of the sample was compared to the peak area of the standard peak area, as

Table 1
Sequences and names of the NAs examined in this study.

Name	Type	Sequence (5′-3′)	Length (bp)
hairpin	ssDNA	AATGCTTTTAATATTGCATT	20
oligo1	ssDNA	AAAAAAAAACAAAAAAAAAA	20
ASO	ssDNA	ACTATGCAACCTACTACCTCT	21
amp-DNA	dsDNA	TTCATGAAGACCTCACAGTAAAAATAGGTGATTTTGGTCTAGCTACAGTGAAATCTCGATGGAGTGGGTCCCATCAGTTTGAACAGTTGTCTGGATCC	98
Yeast Isolate	RNA	n/a	~1250
st-DNA	dsDNA	n/a	~2000

shown in Eq. (1):

$$Extraction\ Factor\ (EF) = \frac{Area\ sample - Area\ standard}{Area\ standard} \quad \text{Eq. 1}$$

Following each experiment, the extraction devices were immersed in 2.5 M NaCl for 1.5 h to desorb excess analyte and regenerate the anion-exchange sorbent with chloride counterions. This time-course was determined to be sufficient to prevent analyte carryover of st-dsDNA and oligo1 (data not shown). The desorption vessel used in this study was custom-designed and fabricated using an Ultimaker (Utrecht, The Netherlands) 3D printer employing fused deposition modeling with 2.85 mm transparent polylactic acid filament, as previously reported [28]. The recovery of nucleic acids was determined following the aforementioned extraction method. After extraction, devices were placed in 100 μL of 2.00 mM tris buffer pH 8.00, NH_4ClO_4 at a specific concentration, and in some cases with an organic modifier (10 % v/v) for 30 min. The desorption solution was subjected to HPLC separation, and the peak area of the recovery solution was compared to the peak area of the initial solution to determine the preconcentration factor, as described in Eq. 2

$$Preconcentration\ Factor\ (PF) = \frac{Area\ recovery}{Area\ standard} \quad \text{Eq. 2}$$

With the normalized EF and PF values, a recovery factor (RF) was calculated to serve as an estimation of the recovered fraction of extracted analyte with the volumes of the recovery solution (Vr) and standard solution (Vs) utilized, as shown in Eq. (3):

$$Recovery\ Factor\ (RF) = \frac{PF^{*}Vr}{EF^{*}Vs} \quad \text{Eq. 3}$$

The above calculations rely on assumptions that a linear relationship exists between the peak area and analyte concentration. Peak areas possessing a signal-to-noise ratio less than 10 were excluded from the calculations. Devices were not observed to exhibit variation following re-use for 20 experiments (Fig. S3); however, the re-use of devices was limited to a maximum of 16 experiments in this study.

2.6. Oligo separations by HPLC-TOF-MS

An Evosphere MAX C18/AR (100 × 3 mm I.D.) column with bioinert MAX coating and monodisperse 3 μm particles was provided as a gift by MAC-MOD analytical (Chadds Ford, PA, USA). Mobile phase A consisted of 10 % methanol (pH 7.32) and mobile phase B was comprised of 90 % methanol while both contained 30 mM ammonium formate. Injections of aqueous samples (5.0 μL) were separated at a flow rate of 0.3 mL/min increasing from 10 % methanol to 50 % after 10 min.

A Nucleoshell HILIC (100 × 3 mm I.D.) column with 2.7 μm particles was acquired from Macherey-Nagel (Dueren, Germany). Mobile phases consisting of water and acetonitrile were supplemented with 30 mM ammonium formate. Samples contained 45 % acetonitrile, and 5.0 μL was injected onto the column. Separations were carried out with flow rate of 0.3 mL/min and by increasing the concentration of water from 28.75 % to 55 % over 10 min.

Eluent from the Agilent Infinity 1260 HPLC was diverted to waste following UV analysis to prevent the injected salts from entering the time-of-flight mass spectrometer (TOF-MS). After the first 3 min, eluent was sent to the TOF-MS and the mass range of 1000–3200 *m/z* scanned. Injected samples contained 4 μM of three oligonucleotides (Table 1), 10.0 ppm guanosine and ammonium formate, acetate, or perchlorate salt. The chromatographic system was conditioned with multiple sample injections prior to all analyses.

3. Results and discussion

3.1. Measuring poly-ionic sorbent and NA interactions by AEX-HPLC-UV

As identified in a previous study, sorbents composed of cationic moieties featuring long alkyl substituents exhibited poor performance when used for the extraction of NAs compared to devices with simpler chemical structures and an overall higher degree of cationic character [24]. The chemical structures selected in this study (Fig. 1) are a series with a clear increase in aliphatic character of the ammonium cation (i.e., **A** < **DME** ≤ **DMB** < **TE**). An increase in aliphatic character also decreases the cationic charge density and water affinity, which is one of the most significant factors for electrostatic interactions. Additionally, sorbents were fabricated with zwitterionic **Z** and dicationic **DC** monomers for comparison purposes, as **Z** exhibited almost zero anion-exchange character while **DC** showed the highest. The affinity of anion-exchange microextraction devices has only been previously studied for short dsDNA and affinities for oligonucleotides, RNA, or proteins have not yet been measured. To probe these interactions, the devices were exposed to solutions containing 20 bp ssDNA oligo1, 98 bp amp-DNA, yeast RNA isolate, 2000 bp st-DNA, and BSA. Extraction factors for each analyte were calculated by comparing the peak area of the solution following device exposure to the peak area of the initial solution for each analyte following AEX-HPLC-UV analysis.

As shown in Fig. 2, the obtained extraction factors for the mono-cationic sorbents (**A**, **DME**, **DMB**, and **TE**) were similar for the "larger" NAs (i.e., amp-DNA, st-DNA, and yeast RNA), indicating that variation in hydration enthalpy of the cationic moiety does not impart significant extraction selectivity for these analytes. This result is not surprising due to their identical anionic phosphodiester backbone. No general trend was observed in the preference of any particular poly-ionic sorbent for single or double-stranded NAs. However, enrichment factors for the NAs were found to be inversely related to their length when using the mono-cationic sorbents. For example, sorbent **A** and **DME** produced the highest enrichment factors for oligo1 and the lowest for the longer 2000 bp st-DNA. This result was not observed for **DC**, which yielded the highest measured extraction factors for all NAs compared to all other

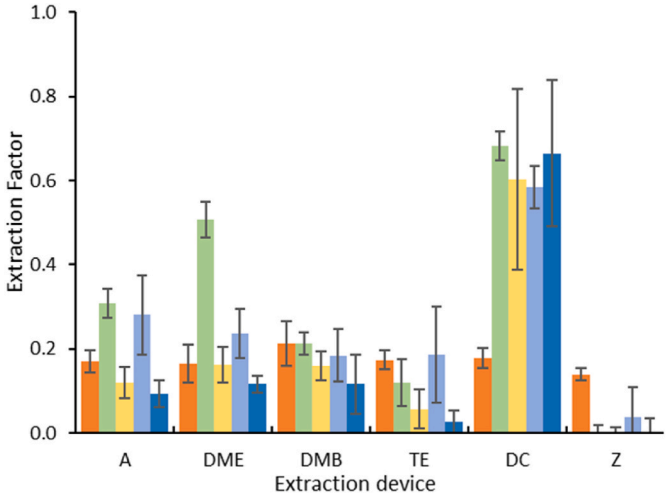

Fig. 2. Comparison of poly-ionic sorbent affinity for spiked representative nucleic acids and a common protein. The extraction factor was calculated by comparing the peak area ratio of a standard solution and extraction solution following exposure to a poly-ionic extraction device using AEX-HPLC-UV. Extraction devices were exposed to individual analytes to avoid biasing in the affinity experiment. The extraction solution was composed of 1.00 mL 2 mM Tris buffer (pH 9.00) spiked with either; st-DNA (dark blue), amp-DNA (light blue), yeast RNA (yellow), oligo1 (green), or BSA (orange). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

devices. The resulting extraction factors cannot be concluded to result only from the **DC** monomer's structure, as the cationic moieties are virtually identical to those in **DME**. It is possible that the spatial separation and proximity of the two cations within a single monomer may offer stronger interactions with the NAs, but there are many other factors related to the bulk properties of the polymeric sorbent which cannot be overlooked. Isolation of amp-DNA, yeast RNA, or st-DNA does not appear to be through the formation of a single poly-cation interaction, but rather many concurrent interactions. Therefore, it is likely that a key parameter for the high extraction factors of these analytes may be the density of surface-accessible cationic charges in the polymeric sorbent.

For the smaller oligo1, high extraction factors of 0.31 ± 0.04 and 0.51 ± 0.04 were obtained for the **A** and **DME** devices, respectively. Smaller factors were obtained with the **DMB** and **TE** devices likely due to the increase in hydrophobicity for these cations. Extraction factors for BSA were found to be virtually identical for all tested devices, including the zwitterionic device **Z**. The mechanism of BSA interaction appears to be non-specific, which aligns with literature precedence of using BSA as a sacrificial agent in preventing analyte adsorption, particularly in the field of microfluidics [29,30]. It is also important to note that negligible extraction factors were observed for **Z** in the case of all NAs due to the absence of anion-exchange under these conditions and inter/intra monomer electrostatic interactions [24,31,32].

3.2. Recovery of an oligonucleotide and 2000 bp dsDNA

Following the determination of extraction factors for the NAs, the **A**, **DME**, and **DC** devices possessing high affinity for oligo1 were selected for further analysis. A previous study demonstrated that the perchlorate anion resulted in the highest preconcentration factors for dsDNA, which was theorized to result from better water affinity matching of the poly-cationic moieties compared to chloride or acetate ions [24]. Ammonium perchlorate was selected due to its increased volatility for subsequent MS-based analysis [33] and no appreciable impact on the recovery of DNA was observed [24]. Extraction solutions containing both st-DNA and oligo1 were prepared to examine their recovery in parallel with recovery solutions of ammonium perchlorate, as shown in Fig. 3. The recovery of NAs is expressed in terms of preconcentration factors which compare the amount of analyte recovered to the amount of analyte in the initial extraction solution (Fig. 3A and B). Devices that extracted more NAs generally achieved higher preconcentration factors, but this is not indicative of the recovery solution's efficacy in desorbing the extracted NAs. For this purpose, the recovery factor (Fig. 3C) was calculated with the extraction factor and preconcentration factor to express NA recovery normalized by the amount of NA extracted, as shown in Eq (3).

The **DC** sorbent achieved the highest preconcentration factors, as shown in Fig. 3A, for oligo1 of 8.6 ± 0.2 at a concentration of 500 mM NH_4ClO_4 and was observed to plateau between 7.2 and 6.8 at concentrations of 250 mM and 125 mM, respectively. Additionally, high recovery factors of 1.09 ± 0.04 and 0.92 ± 0.04 (Fig. 3C) indicate complete recovery of oligo1 at concentrations of 500 mM and 250 mM, respectively. Examination of st-dsDNA recovery revealed both lower maximal preconcentration factors (1.91 ± 0.01) and recovery factors (0.29 ± 0.17) at the highest NH_4ClO_4 concentration of 500 mM. Additionally, a preconcentration factor of 1.91 ± 0.01 obtained for st-DNA in this study was approximately half the maximal preconcentration factor (5.65 ± 0.39) obtained for a smaller 250–750 bp dsDNA sample featured in a previous study using 500 mM $NaClO_4$ [24]. Therefore, two key trends emerge for the **DC** sorbent: (1) the recovery of NAs decrease with their increasing length and (2) recoveries of NAs drop with a decrease in salt concentration. Interestingly, sorbent **A** exhibited higher recovery factors for st-DNA compared to oligo1, which was very poorly recovered compared to **DC** and **DME**. However, recovery by the **A** device was decreased with lower salt concentration for both analytes. The **DME** device also exhibited higher recovery factors for long dsDNA (0.63 ±

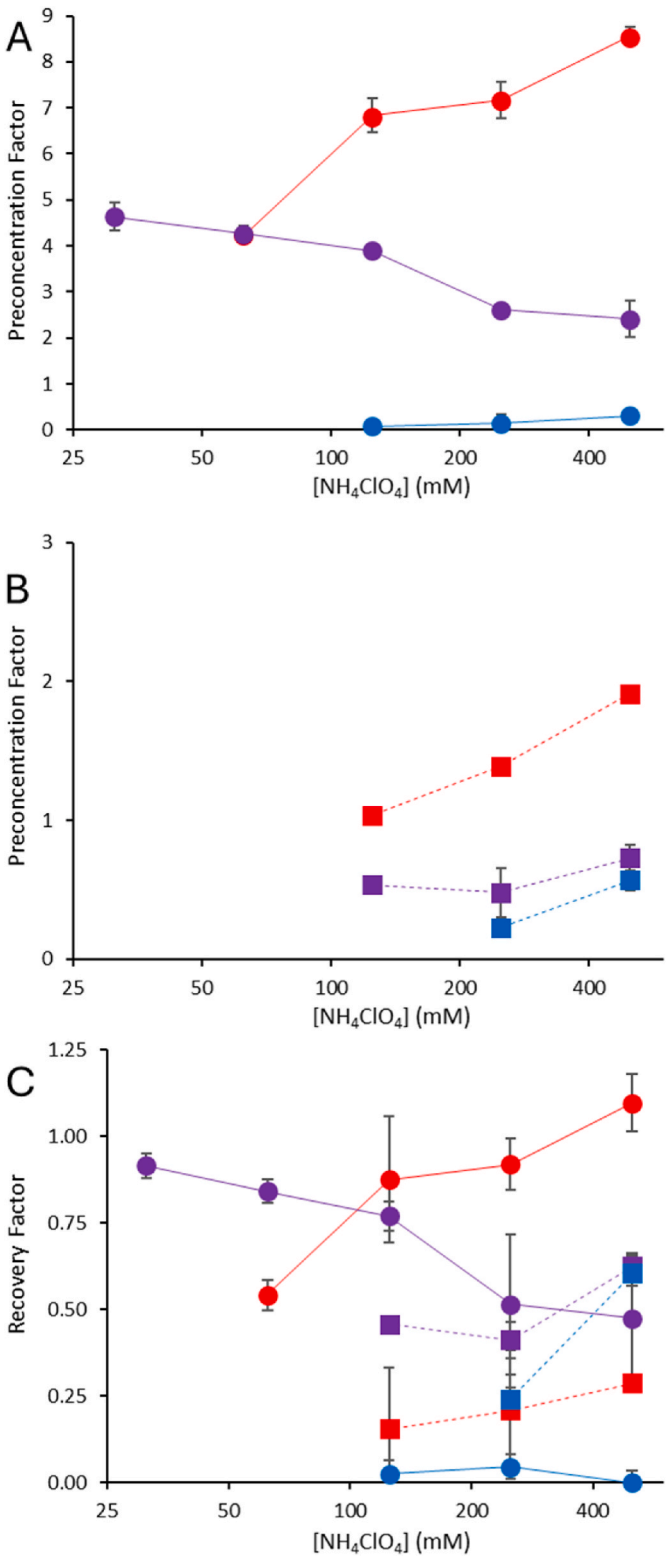

Fig. 3. Recovery of oligo1 and st-DNA from anion-exchange microextraction devices using ammonium perchlorate. Following exposure to 1.00 ppm oligo1 and 10.0 ppm st-DNA, the devices were placed into solutions containing decreasing concentrations of salt for 30 min. The red series denotes data obtained from the **DC**, **DME** (purple), and **A** (blue) devices. Calculations for preconcentration factors were performed for (A) oligo1 shown as circles and (B) st-DNA with squares. (C) Estimated recovery factors of both analytes for the microextraction devices. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

0.02) than for oligo1 (0.47 ± 0.04) at 500 mM NH_4ClO_4. With decreasing salt concentration, this result is reversed as the recovery factor for oligo1 increased continually, even at 31.25 mM where it reached nearly complete recovery (0.91 ± 0.04). These findings indicate highly favorable poly-cation and perchlorate interactions. At high salt concentrations, the most accessible moieties undergo rapid pairing leading to sorbent dehydration and may inhibit mass transfer of analytes from the sorbent. At lower salt concentrations, poly-cation pairing occurs more gradually thereby enabling higher recovery of the smaller oligo1 analyte. It is also important to note that the large dsDNA target does not exhibit this effect as it interacts mostly with the outermost cationic moieties [34].

Further studies were conducted to determine if alterations could be made to the recovery solution in order to increase NA recovery. The addition of small amounts of organic solvents is known to facilitate dehydration and ion-ion interactions resulting in increased recovery of NAs [35]. Acetonitrile and methanol were added at a concentration of 10 % (v/v) to 125 mM NH_4ClO_4 and the obtained results are shown in Fig. S4. Generally, recovery factors for oligo1 and the st-DNA were not influenced by the organic solvent for any of the devices tested. It was theorized that an anion featuring higher hydration enthalpy may be preferentially aided by the organic solvent and the experiment was carried out with 500 mM NH_4Cl. However, the combination of high salt concentration and organic solvent significantly increased the effect of NA adsorption, resulting in significant analyte loss within the sample vial (data not shown). Solutions containing anions such as nitrate, tetrafluoroborate, and thiocyanate were injected onto the AEX-HPLC column but prevented NA analysis through excessive interactions with the stationary phase (data not shown). For example, the use of tetrafluoroborate anion resulted in NAs being eluted in the dead volume while nitrate led to high retention and co-elution with st-DNA. Alternatively, fluorometric dsDNA quantification was used to measure DNA in these salt solutions. To limit inhibition of the assay, salt solutions of 125 mM were prepared and used for recovery following the extraction of 400 ng/mL st-DNA. By constructing individual calibration curves, the mass of recovered DNA was quantified for each anion, as shown in Fig. S5. The results show that the perchlorate anion was the best anion for the **DC** and **DME** devices as dramatically higher DNA recoveries could be achieved. Device **A** exhibited very low recoveries overall and the results indicate that the thiocyanate anion may produce higher recoveries with this device.

3.3. HPLC-TOF-MS analysis of oligonucleotides in recovery solutions

Identifying optimal anions for NA recovery from anion-exchange microextraction devices enables the use of significantly lower concentrations of anionic species to achieve significant recoveries for chemical measurement. Results from this work suggest that anion-exchange microextraction devices can recover oligonucleotide samples in 125 mM ammonium perchlorate for the **DC** device and 31.25 mM using the **DME** device. These concentrations have been demonstrated to be non-inhibitory in quantitative polymerase chain reaction (qPCR) and loop-mediated isothermal amplification assays [24,28,36]. However, employing these recovery solutions with chromatographic methods interfaced with MS has not yet been demonstrated. Two commercial HPLC columns utilizing HILIC and reversed-phase separation modes were used for compatibility testing of oligonucleotide separations and downstream MS compatibility.

Following optimization of the separation, samples containing 30–350 mM ammonium acetate, formate, and perchlorate salts were prepared with an oligonucleotide sample mix and subjected to separation using both columns. To prevent excessive amounts of salt from entering the MS source and interface, LC eluent was directed to waste for the first 3 min of the separation. Overall, both chromatographic systems enabled reproducible separation of oligonucleotides and detection by TOF-MS, as shown in Tables S1–S2 in the supporting information. The Nucleoshell HILIC column enabled TOF-MS detection of all oligonucleotides, while the separation produced broad peaks (Figure S6). A representative chromatogram with the Evosphere MAX C18/AR column (Fig. 4A) shows excellent separation of the analytes as well as sharp signals obtained from select extracted ion chromatograms corresponding to each oligonucleotide (Fig. 4B). It is important to note that the reversed-phased separation does not utilize ion-pairing reagents, which are normally required to achieve retention of oligonucleotides [7]. Recently, Studzinska and co-workers [37] attributed the increased retention to π electron interactions of the nucleobases with the aryl moieties of the C18/AR column chemistry. Injections of ammonium formate with increasing concentration led to an increase in retention (Fig. 4C), while ammonium acetate produced a decrease in oligo retention (Fig. 4D). Ammonium perchlorate was determined to be compatible due to no variance in retention times across all concentrations studied, as shown in Fig. 4E. Individual chromatograms with the Evosphere MAX C18/AR can be found in Figs. S7–S9 of the Supporting Information. Additionally, the increase in salt concentration did not increase the chromatographic resolution, unlike with the Nucleoshell

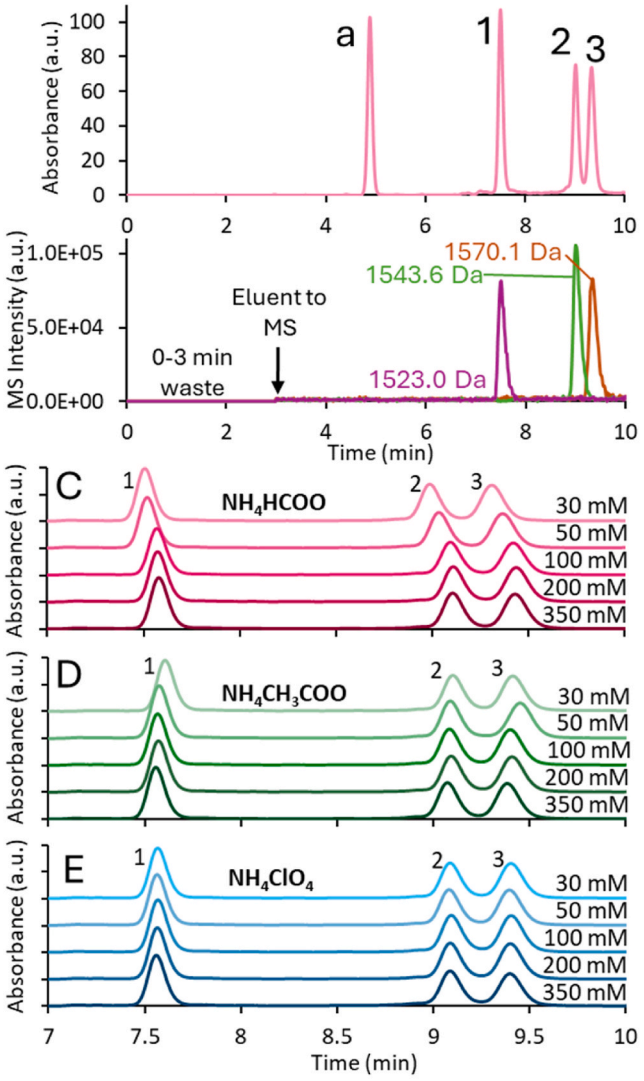

Fig. 4. Comparison of an oligo mixture and guanosine by RP-HPLC-TOF-MS, as shown by (A) UV absorption at 260 nm and (B) TOF-MS select extracted ion chromatograms. The injection solution of the test mixture was varied to contain (C) ammonium formate, (D) ammonium perchlorate, or (E) ammonium acetate salts with increasing concentration from 30 mM to 350 mM. (a) guanosine, (1) hairpin, (2) oligo1, and (3) ASO.

column (Figs. S10–12). Given these results, the Evosphere MAX C18/AR column was selected due to its strong performance and compatibility with oligonucleotide analysis using the anion-exchange microextraction devices.

3.4. Purification and preconcentration of an antisense oligonucleotide (ASO) analog from heat-treated human plasma

Most FDA approved NA therapies employ ASOs to interact with target genes through Watson-Crick base pairing with mRNA resulting in decreased target gene expression through steric blocking or promoting degradation [4]. The aforementioned experimental results from this study indicate that anion-exchange microextraction devices can serve as a successful sample preparation method for the analysis of ASOs and other nucleic acid therapeutics. To examine the purification and preconcentration capabilities of the devices, an analog for an ASO was spiked into tris buffer and heat-treated human plasma. The **DC** device was selected as the optimal device due to the highest extraction and preconcentration factors obtained for ssDNA. Initial samples and recoveries were analyzed by HPLC-TOF-MS, as shown in Fig. 5, and individual chromatograms can be found in the Supporting Information (Figs. S13–S14). Chromatograms revealed a significant preconcentration factor of 7.2 ± 0.5 using the **DC** device for ASO spiked in tris buffer, and this value is in agreement with the previously obtained value of 6.8 ± 0.4 for 125 mM NH_4ClO_4 in Fig. 3A. Mass spectra obtained from TOF-MS analysis revealed successful ASO identification by two masses corresponding to the $[M-4H]^{4-}$ and $[M-3H]^{3-}$ charge states, as shown in Fig. 5B. The analysis of the ASO in heat-treated human plasma was more challenging as the ASO was not observed in the chromatogram (Fig. 5A) or MS analysis of extraction solution (Fig. 5C). The preconcentration factor was found to decrease by approximately two-fold, but the purification and preconcentration enabled ASO detection by UV and characterization by MS. The device demonstrated low variance in the recovery of oligonucleotide from both the tris buffer (4.4 % RSD) and plasma samples (6.5 % RSD). It is important to note the high degree of purity of the recovered ASO compared to the plasma matrix by examination of the dark and light blue chromatograms in Fig. 5A. The decrease in ASO recovery from human plasma compared to tris buffer is likely a result of enzymatic degradation of the spike as well as extraction inhibition. In previous work [24], heat-treated human plasma did not reduce the recovery of dsDNA, but this study has elucidated a key difference in the interactions of ssDNA and dsDNA. Recovery of the 250–750 bp dsDNA sample in 62.5 mM $NaClO_4$ was near zero, and in this work recovery of the larger 2000 bp st-DNA sample required a minimum concentration of 125 mM NH_4ClO_4. In comparison, results from this study demonstrate that for concentrations of 62.5 mM NH_4ClO_4 significant recoveries were obtained for the oligonucleotide. This result indicates weaker ssDNA interaction strengths than dsDNA, resulting in a greater propensity for extraction inhibition, particularly by samples containing biologically-relevant salt concentrations. This phenomenon would be significantly increased for the **DMB** device, which exhibited higher preconcentration factors than **DC** for ssDNA at low salt concentrations. Electrostatic interactions can be increased with cationic moieties featuring greater water affinity than the **DC** and **DMB** devices. These new moieties would also likely be compatible with recovery salts of tetrafluoroborate or thiocyanate anions, which feature better water affinity matching.

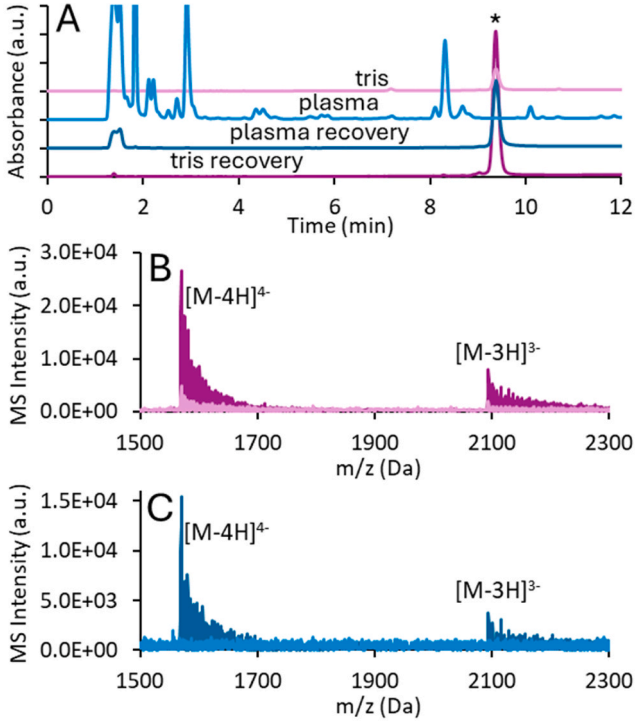

Fig. 5. (A) Chromatograms obtained by UV detection showing comparison of 0.5 μM ASO tris buffer and heat-treated human plasma with corresponding recoveries in 125 mM NH_4ClO_4. The asterisk denotes the peak around 9.5 min corresponding to the spiked oligonucleotide and selected for MS analysis. (B) Observed mass spectra of ASO in tris buffer (light pink) and preconcentrated ASO (dark pink). (C) Mass spectra of ASO in plasma (light blue) and following purification and preconcentration with the **DC** device (dark blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Conclusions

For the first time, anion-exchange microextraction devices were coupled with chromatography and mass spectrometry for the analysis of oligonucleotides. Purification and preconcentration of oligonucleotides from heat-treated human plasma enabled successful TOF-MS analysis. The compatibility of the salt-containing recovery solutions with highly relevant chromatographic separation modalities, such as reversed-phase and HILIC, highlight that anion-exchange microextraction devices are a complete and compelling sample preparation methodology. Additionally, separation modes such as hydrophobic interaction and gel filtration chromatography are likely to be compatible without modification due to the high aqueous content of their mobile phases.

The affinity of anion-exchange microextraction devices for ssDNA, RNA, and dsDNA were found to not vary significantly with respect to the chemical moiety of the cation, except for the **DC** devices. Enhanced selectivity for ssDNA was observed for more hydrophilic cationic moieties employed in the **A** and **DME** devices. Recovery of ssDNA was observed to occur more readily compared to the 2000 bp dsDNA analyte. The increased length of dsDNA sequences makes desorption more difficult for these devices. Significantly higher preconcentration factors were obtained for ssDNA using comparatively much lower salt concentrations. Additionally, poor recoveries with the more hydrophilic **A** device indicates that alternative anions, such as iodide, may enhance desorption. Future work is required to optimize and characterize the performance of these microextraction devices for the development of sensitive and quantitative oligonucleotide assays. Additionally, the efficacy of anion-exchange microextraction methodologies should be explored with other anionic analytes of interest such as phosphonothioate oligonucleotides, phosphorylated peptides, lipids, proteins, as well as small molecules. A mechanistic understanding of ion-ion interactions found in anion exchange microextraction devices enables the logical development of cation exchange sorbents and corresponding workflows. Additionally, a versatile coating platform is required to deliver consistent and higher loading of functional moieties with varying chemical substituents and charge density.

CRediT authorship contribution statement

Derek R. Eitzmann: Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **Jared L. Anderson:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no conflicts of interest in this work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2025.343624.

Data availability

Data will be made available on request.

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