Facile Preconcentration of Cell-free DNA in Human Plasma by Ion-specific Poly-ionic Sorbents Featuring an Anion Exchange Mechanism

Derek R. Eitzmann and Jared L. Anderson*

Department of Chemistry, Iowa State University, Ames, Iowa 50011, United States

***Corresponding Author:**
Jared L. Anderson
Department of Chemistry
Iowa State University
1605 Gilman Hall
Ames, IA 50011
Email: andersoj@iastate.edu

Abstract

The expanding horizon of diagnostic and therapeutic applications involving nucleic acids (NA) requires novel tools for purification, including minimal sample preparation. In this work, thin film microextraction (TFME) devices featuring five poly-ionic sorbents were examined as anion-exchange extraction phases for the rapid purification of NAs. Each sorbent is composed of a non-ionic crosslinker and a methacrylate monomer containing a core tetra-alkyl ammonium moiety with an alkyl, anionic, or cationic residue. Extraction devices were produced through application of the pre-polymer sorbent mixture onto a functionalized nitinol metal support followed by photo-induced free-radical polymerization. The miniaturized extraction devices (10 mm x 3.5 mm) were directly immersed into aqueous samples to isolate NAs via electrostatic interactions with the poly-cation. The ammonium methacrylate (AMA) monomer containing a propyl trimethyl ammonium group (AMA-$C_3N(CH_3)_3$) exhibited the highest affinity for DNA, with 80 ± 10 % of DNA being isolated. Recovery of DNA from the sorbents required introduction of ions in aqueous solution to exchange the anionic biopolymer from the poly-cationic moiety. An investigation of three anion species revealed that the AMA-$C_3N(CH_3)_3$ sorbent showed the highest recoveries, with the perchlorate anion producing a preconcentration factor of 4.36 ± 0.86 while requiring only 250 mM $NaClO_4$. A directly compatible quantitative polymerase chain reaction (qPCR) assay was developed to quantify the recovery of spiked DNA with lengths of 830, 204, and 98 base pairs in heat-treated human plasma. The AMA-$C_3N(CH_3)_3$ sorbent was uninhibited by the complex human plasma matrix and enabled high preconcentration factors for the spiked DNA at a biologically-relevant concentration of 10 pg/mL. While Qiagen's circulating cell free (ccf) DNA MinElute extraction kit enabled higher preconcentration of all analytes, the methodology described in this work requires fewer steps, less user intervention, and minimal equipment requirements to isolate DNA making it more amenable for high-throughput and low resource applications.

Introduction

Nucleic acid (NA) analysis is pivotal in advancing the frontiers of personal medicine,[1] therapeutics,[2] and pathogen detection.[3] The determination of explicit genetic coding is routinely conducted with various sequencing methodologies[4-6] to determine predispositions and discover novel biological pathways for diseases.[7] Amplification-based enzymatic assays enable quantification or detection of specific genetic code segments at concentration levels as small as 10 copies/μL. Regardless of the downstream methodology, an essential consideration of NA samples is the purity and concentration of inputs. Targeted NAs are generally low in abundance compared to cellular debris and matrix components of cell lysates, oral fluids, blood, or plasma. Sample preparation techniques dedicated to NAs facilitate their analysis by removing unwanted components and enabling preconcentration for enhanced downstream sensitivity, robustness, and reliability.

Conventional approaches such as solid phase extraction (SPE) kits employ high concentrations of chaotropic salts to force NA adsorption to a silica spin column.[8] More recently, silica-coated magnetic beads have been commercialized to reduce user intervention and increase throughput by utilizing agitation to facilitate extraction and magnetic racks to collect the beads, circumventing the requirement of centrifugation. Adoption of silica-based methodologies is widespread because of its robustness and ability to recover NAs in pure water, which is compatible with virtually all downstream analyses. However, principal challenges remain as both SPE modalities utilize a non-specific binding mechanism and a large volume of extraction phase leading to the extraction of matrix components along with the desired NAs. Removal of these co-extracted components is performed through multiple washings with organic solvents and is generally the most time-consuming and labor-intensive facet of silica-based SPE methodologies.

An attractive alternative technique must feature a selective extraction phase with high affinity for NAs to reduce washing requirements and facilitate more rapid analysis to meet current demands.

Anion-exchange materials were first featured by Cohn[9,10] to separate nucleotides and perform purification of larger NAs. Current commercial materials consist of cationic extraction phases composed of tertiary amines at high pH or quaternary ammonium residues that bind the anionic NA biopolymer through electrostatic interactions. The cationic material's affinity for NAs is high and enables simple purification by washing with water to elute proteins, sugars, or other hydrophilic species. Recovery of bound NA can only be achieved by introducing high ionic strength solutions for the anions in aqueous solution to exchange with the anionic NA. For example, a NaCl concentration of 1.5 M is required to facilitate DNA recovery from Qiagen's anion exchange resin.[11] These concentrations are exceptionally inhibitory to most downstream analyses, in contrast to the aforementioned silica SPE technologies that elute NAs in pure water. Additionally, anion exchange materials have lower binding capacity than silica-based SPE due to the requirement of specific chemical interactions and the small diffusion coefficient of large NAs.[12,13] These factors have largely confined deployment of this technique to preparative scale separations for plasmids,[12] oligos,[14] and RNA.[15]

An alternative platform is solid-phase microextraction (SPME), which features sorbents attached to a support with a much lower sorbent-to-sample ratio than SPE.[16] As a result, SPME devices generally extract only a small portion of the total analyte but enable substantial preconcentration through desorption into a much smaller volume. Isolation of analytes in microextraction approaches is often diffusion-controlled, enabling greater discrimination based on each component's affinity to the sorbent.[17] Sorbent materials possessing higher affinity for targeted analytes than matrix components can significantly improve workflows by isolating larger amounts

of analyte(s) or reducing the presence of downstream matrix components. The geometry of the support is also a critical parameter, as it can be manipulated to modulate the surface area and affect overall mass transfer. Lower surface area geometries, such as fibers, enable capture of analytes with small diffusion coefficients, while higher surface area geometries such as stir bars[18] or blades[19] are more advantageous for higher molecular weight analytes with smaller diffusion coefficients to reduce the time required to reach equilibration.

Anion exchange microextraction devices were recently developed as a simple and rapid methodology for the isolation and purification of NAs. These devices enabled detection of the cancerous BRAF V600E mutation,[20] SARS-CoV-2,[21] and fecal indicator bacteria[22] from plasma, artificial saliva, and environmental water, respectively. The thin film microextraction (TFME) blade geometry was demonstrated to be superior to the fiber geometry by enhanced extraction rates and higher sensitivity due to higher amounts of DNA being extracted.[21] Nacham and coworkers identified the optimal polymeric ionic liquid (PIL) sorbent containing vinyl imidazolium moieties and exchangeable halide anions.[23] However, the sorbent exhibited a significant decrease in DNA isolation from human plasma and required 1.00 M NaCl or KCl for DNA recovery.[20-22] Additionally, vinyl imidazolium-based polymers have been shown to possess lower polymerization efficiencies due to the lower reactivity of the vinyl group[24,25] and the close proximity of the imidazolium cation.[24,26,27] Methacrylate poly-ions feature larger spatial separation of the polymer backbone and charged moiety enabling theoretically more accessible charged moieties and larger molecular weight products to be obtained.[26] Application of poly-ionic methacrylate-based microextraction sorbents will enable elucidation of NA extraction and specific anion exchange interactions in relation to sorbent chemistry to alleviate the aforementioned bottlenecks.

The mechanism of anion exchange and anion-cation interactions relies on a more complicated phenomenon than can be attributed only to electrostatic charges. Current theory, as described by Collins, describes that an ion's water affinity drives ion-ion interactions in the aqueous environment.[28] An ion's affinity for water is measured by the hydration enthalpy, or the enthalpy increase following the solvation of one mole of gaseous ions.[29] The hydration enthalpy is directly related to the charge density of the ion and, for an isoelectronic series, is inversely related to the ionic radius. Aqueous ion-ion interactions are governed by Colin's theory of matching water affinities and indicates that cation-anion pairs are preferentially formed based on similarities of their hydration enthalpy. Underlying this phenomenon is that the resultant ion-ion pair will interact less strongly with water compared to the sum of the individual ions. Additionally, the selectivity of anion exchange materials has been characterized based on the preference for anions with decreasing hydration enthalpy, as alkyl ammonium moieties on the resins have even lower hydration enthalpies.[30] Recent studies featuring a quartz crystal microbalance[31,32] and ellipsometry[33] have shown that poly-ions exhibit confirmational changes indicative of ion-pairing at lower concentrations of closely matching ionic species. In contrast, other ionic species must be present at much higher concentrations to produce the same effects. Understanding this phenomenon is critical for designing anion exchange sorbents for NA purification as the isolation and recovery equilibria are governed by ion-ion interactions. More fundamentally, this is determined by the hydration enthalpy and chemical nature of each cationic moiety. The isolation of NAs is mechanistically complicated due to its size and may require numerous electrostatic interactions. However, the interaction of individual poly-cationic moieties and aqueous anions is critical for the recovery of NAs from the sorbent. Identifying anions with a more similar water affinity will enable the recovery of NAs using a minimal salt concentration.

Herein, we report the synthesis of five ionic monomers containing a principle quaternary ammonium cation with varied non-ionic, cationic, and anionic substituents. These monomers feature a methacrylate polymerizable moiety that is linked to an alkyl ammonium cation formed following the nucleophilic attack of the tertiary amine. Devices featuring crosslinked polymeric sorbents of each monomer were prepared and applied on a blade geometry to form TFME devices. Each sorbent's affinity for DNA is assessed by comparing the mass of DNA extracted to understand critical monomer interactions. Following DNA isolation, recovery was systematically evaluated using five salt species to determine the poly-ion's ion-specific interactions. Promising devices were identified and evaluated in the extraction and recovery of spiked DNA in human plasma and measured by quantitative polymerase chain reaction (qPCR).

Experimental

Reagents

Ultrapure water (18.2MΩ-cm) was collected from a Millipore Simplicity purification system (Bedford, MA. USA). Deoxyribonucleic acid sodium salt from salmon testes, sodium perchlorate (≥98%), sodium acetate (≥99%), 1,4 butane sultone (≥98 %), (3-bromopropyl)trimethylammonium bromide (97%), 10-bromodecanoic acid (95%), 9-bromo-1-nonanol (95%), bromobutane (99%), 1,4-butanediol diacrylate, methanol (≥ 98.0%), and DAROCUR 1173 (> 96%) were acquired from Sigma-Aldrich (St. Louis, MO. USA). Tris(hydroxymethyl)aminomethane (98%) was purchased from P212121 (Ypsilanti, MI. USA). Ammonium chloride (≥99.5%), sodium chloride (99%), hydrochloric acid, vinyltrimethoxysilane (98%) and 30% hydrogen peroxide were obtained from Fisher Scientific (Hampton, NH. USA). Lithium chloride (≥99%) was obtained from Thermo-Scientific (Waltham, MA. USA). For

synthesis of poly-ionic monomers, 2-(dimethylamino)ethyl methacrylate (>98.5%) was purchased from TCI (Toyko, Japan).

Synthesis of ionic monomers

A simple reaction scheme was followed for the synthesis of all monomers studied in this work. A 2:1 molar excess of 2-(dimethylamino)ethyl methacrylate (11.8 mmol) and 5.9 mmol of bromobutane, 10-bromodecanoic acid, 1,4 butane sultone, 9-bromo-1-nonanol, or (3-bromopropyl)trimethylammonium bromide were added to a round bottom flask. Reaction with (3-bromopropyl)trimethylammonium bromide required 2.0 mL of dimethyl sulfoxide to solubilize the reagents. The solution was covered and stirred for seven days at room temperature. Following completion, 15 mL of ethyl acetate was added to precipitate out the product. All other reactions were covered in foil and stirred at room temperature for 48 hours. Pure, solid products were obtained following the addition of acetone to the reaction mixture and subsequent filtration with acetone. Characterization of all products was carried out using nuclear magnetic resonance (NMR) spectroscopy and ^{1}H and ^{13}C spectra can be found in the supporting information.

Fabrication of TFME devices

Segments of nitinol metal (150 mm x 50 mm x 0.5 mm) acquired from Kellogg's Research Labs (New Boston, NH) were cut into 3.5 mm x 25 mm strips prior to hydrogen peroxide treatment and reaction with vinyltrimethoxysilane, following a previously reported method.[34] To produce the sorbent coating, 20.0 mg of the solid monomer (Figure 1A), 10.0 µL of 1,4 butanediol diacrylate, 20.0 µL of MeOH, and 2.5 µL of 2-hydroxy-2-methylpropiophenone were homogenized. A 1.0 µL volume was applied to a 10.0 mm x 3.5 mm segment on the lower portion of each side of the device. The devices were placed in a Rayonet photoreactor and subjected to a fan for 30 minutes to evaporate the solvent followed by photoirradiation with RPR—2537Å

(Branford, CT, USA) lamps for an additional 30 minutes to initiate polymerization. A previously reported PIL coating composed of 3-vinylimidazolium-1-decanoic acid bromide [VimC$_9$COOH][Br] monomer and 1,12-di(3-vinylimidazolium) dodecane dibromide [(Vim)$_2$C$_{12}$]2[Br] crosslinker were prepared matching the aforementioned dimensions.[21] The devices were conditioned by placing them in water (18.2 MΩ-cm) and vigorously agitating at 2500 rpm with a vortex mixer. The devices were then transferred to a solution containing saturated NaCl to displace all counterions with chloride anions. Prior to extractions, the devices were equilibrated in a solution of 2.5 M NaCl, unless otherwise specified.

Preparation of DNA standards and general extraction procedure

A solution of salmon testes DNA (stDNA) was sheared by ultrasonication using a UP200st VialTweeter (Heilscher, Teltow, Germany) at 250 kHz and 30 W for 10 seconds and then placed on ice for 30 seconds. Ultrasonication and icing was repeated 20 times to produce a distribution of lengths ranging from 200-750 bp, as determined by gel electrophoresis (Figure S1). A 830 bp DNA fragment was synthesized by Integrated DNA Technologies (Coralville, IA). Linear DNA fragments (204 bp and 98 bp) were generated by PCR amplification, separated by gel electrophoresis, and purified with a QIA quick gel extraction kit (Qiagen, Hilden, Germany). All stock sample concentrations were quantified using a Qubit 4 fluorometer using the 1X HS DNA assay (Thermo Scientific) and subsequently stored at -20 °C. These analytes and primers can be found in Table S1 of the Supporting Information.

A schematic describing the extraction procedure is shown in Figure 1B. All extractions were performed by spiking a 1.0 mL sample with 340 ng of stDNA or 10 pg of DNA into a 1.5 mL DNA Lobind® tube. Extraction solutions consisted of either 2.00 mM Tris-HCl buffer (pH 8.00) or heat-treated human plasma. Pooled human plasma acquired from Innovative Research

(Novi, MI, USA) was heat treated at 65 °C for 15 minutes followed by centrifugation at 4,800 relative centrifugal force for 10 minutes at 4 °C. Prior to immersion in extraction solutions, the poly-ionic sorbents were conditioned in a salt-containing equilibration solution for at least 1.5 hours to ensure maximal extraction and prevent carryover, as shown in Figure S2. Individual extraction devices were reused for sixteen trials to ensure consistent extraction performance (Figure S3). For each trial, poly-ionic sorbents were removed from their equilibration solution, washed with water, and directly immersed into the sample. All extractions were carried out at ten minutes of vortex agitation at 2500 rpm. Following another brief wash, DNA recovery was carried out in a previously designed 3D-printed desorption vessel[21] containing a 45.0 µL volume of salt solution for 30 minutes. The desorption vessels were fabricated with polylactic acid (PLA) which possesses high compatibility with downstream amplification assays and effectively preserves DNA samples at high salt concentrations.[35] The salt solution is specified for each experiment and features sodium acetate, sodium chloride, sodium perchlorate, ammonium chloride, potassium chloride, or lithium chloride with concentrations ranging from 1000 mM to 62.5 mM.

Quantification of DNA using a fluorescence microplate reader assay and quantitative polymerase chain reaction (qPCR)

DNA quantification was carried out using the Qubit™ 1X dsDNA HS assay (Thermo Fisher Scientific) and a Synergy H1 microplate reader acquired from BioTek (Winooski, VT. USA). Samples consisting of 90.0 µL Qubit™ assay and 10.0 µL of aqueous DNA sample were analyzed at wavelengths of 500 nm and 528 nm for excitation and emission, respectively. Serial dilution of the DNA standard yielded a linear calibration curve with a limit of quantification of 15.6 pg/µL and limit of detection of 7.8 pg/µL, following background subtraction of the blank

(Figure S4A). No variance in the relative fluorescence unit (RFU) values was observed by comparing positive and negative control samples of 2.00 mM tris buffer or 1.00 M salt solutions (Figure S4B-C). Other ions such as guanidinium, tetramethyl ammonium, iodide, nitrate, and sulfate were tested but deviated from ideal behavior in the fluorometric assay.

Assessment of DNA preconcentration factors from heat-treated human plasma was completed by quantitative polymerase chain reaction (qPCR). A single qPCR primer set was employed to amplify a common 98 bp region contained within three DNA targets of 830 bp, 204, and 98 bp length. Samples of DNA subjected to qPCR analysis were either in 2.00 mM tris buffer, pure water, or 250 mM $NaClO_4$. Assays with inputs of tris buffer or pure water required identical optimal conditions whereas conditions for the salt containing solution featured a higher annealing temperature. Assessment of spiked initial DNA concentrations were carried out using 2.00 mM tris buffer and pure water for samples recovered by the QIAamp MinElute ccfDNA kit (Qiagen, Hilden, Germany). Recovered DNA from the PIL and poly-ionic device were in solutions of 250 mM $NaClO_4$. All qPCR reactions had a total volume of 20.0 µL and contained the following components: 10.0 µL SYBR green supermix, 8.0 µL of deionized water, 1.0 µL of a 10.0 µM solution of forward and reverse primers, and 1.0 µL of template DNA. The thermocycling protocol was 2 minutes at 95.0 °C followed by 40 cycles of 95.0 °C for 5 seconds and 60.0 °C for 30 seconds for pure water samples or 61.0 °C for 250 mM $NaClO_4$. Determination of cycle of quantification (Cq) values was completed by manually setting the threshold at 500 RFU for all experiments. Efficient qPCR calibration curves for each reaction condition are shown in Figure S5.

DNA isolation was compared between devices by calculating the percent DNA extracted from the difference in initial DNA concentration (C_o) and remaining DNA concentration (C_1) following device exposure to the extraction solution, as shown in Eq. 1:

$$Percent\ Extracted\ = \left(\frac{C_o - C_1}{C_o}\right) \times 100\ \%\quad \text{Eq. 1}$$

The preconcentration factor for each device was calculated by dividing the concentration of the recovered DNA sample (C_r) with the initial sample concentration, as represented in Eq. 2:

$$Preconcentration\ Factor = \frac{C_r}{C_o}\quad \text{Eq. 2}$$

Results and Discussion

Comparison of DNA extraction by poly-ionic sorbents and effect of salt pre-equilibration

The extraction devices described in this work feature polymeric sorbents covalently bound to a rectangular metal support for rapid isolation and recovery of DNA. The sorbents are primarily composed of one of several ionic monomers possessing a common quaternary ammonium cation with a varied substituent. The proposed mechanism for DNA interactions is illustrated in Figure 2A, and requires direct interaction of the poly-cation and the negatively charged backbone of the DNA biopolymer. Recovery is carried out by immersing the sorbent in a higher ionic strength solution where anions in the aqueous solution desorb DNA through a replacement exchange with the poly-cation. Interactions of the poly-cations with anionic analytes were studied by synthesizing monomers with residues containing linear alkyl chains, alcohols, carboxylic acids, sulfonates, and or alkyl ammonium groups (Figure 1A). The methodology used in this work (Figure 1B) features an initial regeneration step and equilibration under a high concentration of salt to eliminate carryover and enable reproducible amounts of DNA to be isolated. Extraction is performed by directly immersing the extraction device in a DNA-containing sample and expedited with vortex agitation. The device is washed and introduced into a recovery solution containing specified salt at a known concentration.

For DNA extraction to occur by the mechanism shown in Figure 2A, the associated anion must be replaced with the anionic DNA backbone. Therefore, the amount of DNA extracted could

be a function of the anion species that is initially associated with the poly-cation prior to extraction. To examine this "loading" effect, all devices were equilibrated with 1.00 M NaAcO, NaCl, or $NaClO_4$ prior to DNA extraction. The AMA-C_9COO device extracted a negligible amount (-0.1 ± 1.4 %) of the initial DNA mass when conditioned with NaAcO compared to 9.6 ± 2.6% or 9.7 ± 5.2% with 1.00 M NaCl or $NaClO_4$, respectively (Figure 2B). This device's "loading salt" specificity is unique as all other devices extracted similar amounts of DNA, regardless of the different anions in the aqueous solution. This ion-specific interaction of the AMA-C_9COO device could be a result of the acetate anion causing the monomer to take on more zwitterionic character compared to the other anions. To rationalize this, the acetate anion has the largest hydration enthalpy (Table 1) resulting in stronger interactions with water molecules compared to chloride and perchlorate. As a result, acetate comparatively forms less inner sphere ion pairs with the tetra-alkyl ammonium poly-cation during equilibration compared to the other larger anions. Additionally, the preferential hydration of the acetate anion and high concentration can reduce the number of available water molecules for polymer hydration. Both phenomena result in a higher degree of inter/intra monomer ion pair formation and depreciate the capability of DNA isolation as the anion exchange mechanism cannot occur. The AMA-C_4SO_3 device may have also experienced the same phenomenon following NaAcO equilibration as no DNA was extracted (-1.2 ± 3.9 %). The experiment was repeated using 2.50 M NaCl to determine if equilibration with a higher concentration of aqueous ions could result in more DNA extracted. The strongly cationic monomers of AMA-C_4 and AMA-$C_3N(CH_3)_3$ were the only devices that exhibited an increase in DNA extraction following equilibration with 2.50 M NaCl as opposed to 1.00 M NaCl or $NaClO_4$, while the other devices did not vary. The other cationic device, AMA-C_9OH, did not exhbit the same effects as it only extracted approximately 10 % of the total DNA, which may be attributed

to its significant hydrophobic character compared to the remaining monomers analyzed. Overall, the 2.50 M NaCl equilibration solution was selected as the optimal condition and was used for all devices and in subsequent experiments.

Ionic sorbent anion specificity and concentration to enable DNA recovery

The recovery step requires exposure of the sorbent to a higher salt concentration in order to elute DNA, as featured in Figure 2A, and is the most significant limitation of this sample preparation methodology. Elution of DNA into a high ionic strength salt solution can significantly limit downstream applications for enzymatic assays; therefore, it is critical to identify sorbents that maximize DNA recovery when exposed to desorption solutions containing low salt concentration. To understand the interplay of each sorbent for DNA recovery using various salt solutions, the devices were exposed to DNA for 10 minutes to facilitate extraction, followed by immersion in a salt solution for 30 minutes, as shown in Figure 3.

Figure 3A-C reveals a clear trend of $[ClO_4^-]$> $[Cl^-]$ > $[AcO^-]$ yielding higher and more quantifiable recoveries across the concentration levels examined for all devices. Lowering the salt concentration also decreased the ability to recover DNA from the ionic sorbent, resulting in lower enrichment factors. Closer examination allows classification of the sorbents into two groups, namely, those composed of purely cationic moieties and the other with anionic moieties. Cationic monomers were observed to be more ion-specific characterized by high preconcentration factors at lower concentrations of $NaClO_4$ compared to NaCl and NaAcO, which required increasingly higher concentrations. This effect is exemplified by the AMA-$C_3N(CH_3)_3$ sorbent which produced the highest preconcentration factor of 5.65 ± 0.39 for 0.500 M $NaClO_4$ while miniscule preconcentration factors were obtained with 0.500 M NaCl (0.046 ± 0.02) and NaAcO (0.059 ± 0.02). Solvated polymers containing cationic residues experience the “poly-electrolyte effect,”

coined in 1948 by Fuoss, manifested by decreasing polymer hydration when exposed to increasing ionic strength.[36] Under low salt, the poly-cation is extensively hydrated and an increase in anion concentration yields more direct ion pairing resulting in neutralization of the polymer's charge and a reduction in the number of water molecules that solvate the poly-cation. Ion pair formation occurs following Colin's law of matching water affinities[28] where ions having similar hydration enthalpies are more readily formed. The alkyl ammonium cation has a relatively low hydration enthalpy and is estimated to be approximately -250 kJ/mol from two reported alkyl ammonium cations found in Table 1. This value most closely matches with perchlorate, the largest anion, which has a reported hydration enthalpy of -229 kJ/mol.[37] As a result of the more similar water affinities, direct ion pairing with perchlorate is the most favorable compared to chloride (-381 kJ/mol) and acetate (-425 kJ/mol) which will increasingly favor interactions with water molecules. The increased difference in water affinities for the poly-cation and the anion required higher anion concentrations to achieve the same number of direct ion pair interactions.

Sorbents featuring an anionic moiety, such as AMA-C_9COO, exhibited significantly less salt-specificity resulting in smaller variations in DNA recovery when subjected to different salts or decreasing salt concentration. For example, preconcentration factors of the AMA-C_9COO device at 0.500 M were 0.89 ± 0.25 for $NaClO_4$, 0.86 ± 0.07 for NaCl, and 0.93 ± 0.09 for NaAcO. The results are in stark contrast to the cationic devices, and while smaller maximum enrichment factors were obtained, significantly lower salt concentrations (0.0625 M $NaClO_4$) were used to achieve quantifiable DNA recovery. The underlying mechanism for these devices is considerably more complicated due to the different charged groups, as discussed previously. Polymer systems that include cationic and anionic moieties within the same residue can demonstrate the "anti poly-electrolyte effect," characterized by higher polymer hydration with increased salt concentration.[38]

This effect results from the polymer system's ability to utilize inter/intra chain interactions, and the addition of electrolytes disrupts these interactions resulting in increased relative hydration of the polymer. Experiments have also demonstrated that the "anti poly-electrolyte effect" depends on the salt species' water affinity, as well as concentration.[32] This mechanism is partially in conflict with the DNA recovery step as the increase in polymer hydration results in decreased mass transport of DNA from the polymer to the bulk solution. It must be noted that literature reports describing the "anti poly-electrolyte effect" are confined to ammonium cations and sulfonate anions which offer a better match in water affinities compared to the carboxylate anion evaluated in this work.[39] As a result, inter/intra chain electrostatic interactions from ammonium cations and carboxylate anions are not as preferred comparatively as their water affinities are less matched.

To examine the role of cations in the recovery solution, the ammonium, sodium, and lithium chloride salts were also compared (Figure 3D). Most devices showed no variation in DNA preconcentration factor across the different salts, except for the AMA-$C_3N(CH_3)_3$ sorbent. This result follows expectations that the cation does not participate in the proposed anion exchange mechanism through any meaningful DNA-cation interactions in solution (see Figure 2A). The exception, AMA-$C_3N(CH_3)_3$, provided higher preconcentration factors with LiCl (5.08 ± 0.48) compared to NH_4Cl (3.07 ± 0.47) or NaCl (3.23 ± 0.32), which can be attributed to the greater ion sensitivity of this device, as previously discussed. The water affinity of the lithium cation is significantly larger than the other cations, as shown in Table 1, and may contribute to decreasing the number of water molecules available for hydration of the chloride ion, resulting in a greater extent of anion exchange with this polycation.

Isolation of DNA from human plasma

Extraction and isolation of DNA from human plasma is exceptionally challenging due to the complexity of the biological matrix.[40] Commercial kits for DNA extraction from human plasma, such as the QIAamp MinElute ccfDNA kit from Qiagen, enable direct sample processing by utilizing additional enzymes, surfactants, and binding reagents to deactivate nucleases and facilitate DNA adsorption to magnetic beads. Following washing of the beads, DNA is eluted prior to adsorption onto an additional spin column. The column is typically washed several times with solutions containing organic solvents before it is finally eluted in pure water, where it has undergone substantial preconcentration and is ready for use in virtually all downstream applications. However, this workflow requires substantial user intervention by trained technicians and is not amenable to complete automation. Previous work from our group has shown that DNA capture and recovery from plasma is challenging for PIL-based SPME extraction devices featuring an anion exchange mechanism that facilitate extraction and recovery. The sorbent (composed of a divinyl imidazolium crosslinker $[(Vim)_2C_{12}]2[Br]$ and a vinyl imidazolium monomer $[VimC_9COOH][Br]$) demonstrated a significant decrease in the recovery of a DNA spike in human plasma compared to 2.00 mM tris buffer.[20] Extractions performed from a solution of plasma salts led to a clear decrease in DNA recovered due to the increased ionic strength, which diminished the amount of isolated DNA. Additionally, the experiment was repeated in a solution of bovine serum albumin (BSA) to mimic plasma protein content, and a similar decrease was also due to non-specific adsorption of BSA limiting the amount of DNA extracted. More recently, a higher surface area thin film support was used for the same PIL sorbent and led to increased downstream sensitivity.[21] However, the advantages gained through enhanced kinetics of the thin film support do not mitigate unwanted chemical interactions which lead to decreased downstream sensitivity.

The dicationic monomer, AMA-$C_3N(CH_3)_3$, was selected as the most promising sorbent for isolation and purification of DNA from human plasma. As previously discussed, this monomer extracts much more DNA than other devices, and the anion specificity demonstrated in Figure 3 indicates that the sorbent's DNA affinity may be unaffected by the elevated salt concentration in plasma, as no DNA was recovered from the device even when examining concentrations as high as 250 mM NaCl. To explore this advantage, linear DNA fragments of 98, 204, and 830 bp length were spiked at 10 pg/mL into either heat-treated plasma or 2.00 mM Tris buffer followed by extractions with devices containing the AMA-$C_3N(CH_3)_3$ and PIL sorbent coatings. The previously identified optimal conditions for the AMA-$C_3N(CH_3)_3$ sorbent were also optimal for the PIL device, as shown in Figure S6-S7, and were implemented in the experiment. Across all sizes of DNA examined, the AMA-$C_3N(CH_3)_3$ sorbent was unaffected by the human plasma matrix as similar preconcentration factors were obtained for tris buffer, as shown in Figure 4. On the other hand, the PIL device recovered approximately 7 times less DNA from plasma than tris buffer for all lengths of DNA examined. Additionally, the AMA-$C_3N(CH_3)_3$ sorbent produced significantly higher preconcentration factors in human plasma (3.9-3.2) than the PIL device in tris buffer (1.6-1.1). To benchmark the anion exchange microextraction devices, the QIAamp MinElute ccfDNA kit was employed for preconcentration of the DNA spike in plasma. The kit enabled preconcentration factors that were 2-4 times higher than the AMA-$C_3N(CH_3)_3$ device, but it is important to note the drastic differences in workflows for these sample preparation methodologies. The Qiagen kit features 11 steps utilizing five separate reagent solutions, magnetic beads, and a spin column prior to the final elution step resulting in a purified DNA sample. Equipment such as magnetic racks, microcentrifuges, and a controlled heat source are also required to complete the workflow. In contrast, the ionic sorbent does not require this equipment or any reagents except for

$NaClO_4$ and NaCl solutions for binding or elution. Additionally, the complete workflow utilizes only a regeneration solution, a 10 minute extraction step, and 30 minute DNA recovery in 45.0 µL of $NaClO_4$ with momentary washes with water in between each step. It is also important to note that extraction and recovery times were maintained throughout the work for consistency and the duration of each can be reduced without decreasing downstream sensitivity.[21]

Conclusions

The dicationic sorbent developed in this work provides high DNA enrichment with no reduction in performance when used with heat-treated plasma samples.. The AMA-$C_3N(CH_3)_3$ sorbent also exhibited significant specificity for the perchlorate anion enabling high preconcentration factors to be achieved at more moderate salt concentrations. These coated devices demonstrate significant progress in developing an effective anion exchange methodology that is compatible with downstream detection while offering significant improvements to sample throughput and minimizing the cost per sample. Direct analysis of samples containing 250 mM $NaClO_4$ by qPCR demonstrated no inhibition following a minor adjustment of assay conditions. This result suggests that such "salty" samples are not destructive to many downstream analyses following minor optimization, especially for enzymatic amplification assays. Future work will explore anion exchange sorbent affinity for additional classes of NAs such as smaller oligos, RNA, and other larger DNA molecules in addition to their respective optimal recovery conditions. Tuning the poly-cation's hydrophobicity will increase selectivity for specific classes of these NAs and enable higher preconcentration to be achieved. Additionally, anion exchange microextraction devices with multiple extraction phases can greatly expand analyte coverage or offer enhanced purification through fractionalized recovery. Implementation of these devices with downstream methodologies, such as chromatography and mass spectrometry, is expected to enhance

separations and downstream sensitivity. In particular, hydrophilic interaction liquid chromatography (HILIC) is a promising technique due to the requirements of salt for optimal separation and compatibility with NA analysis.

Supporting Information

List of primers and sequences of DNA template used, agarose gel electrophoresis data of sonicated stDNA samples, comparison of DNA recovery and carryover, extraction device reusability, quantification of dsDNA by Qubit HS microplate reader assay, qPCR calibration curves for 830 bp DNA target in different sample solutions, examination of salt solution pre-equilibration for PIL devices prior to exposure to extraction solution, and recovery data for isolated DNA from PIL sorbents. NMR spectra are also provided for all synthesized monomers evaluated in this study.

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Table 1. Reported ionic radii and hydration enthalpies for ions featured in this study.

Ions	r (nm)[a]	$-\Delta H°_{hydration}$ (kJ/mol)[b]
$[CH_3COO^-]$	0.162 ± 0.014[c]	425[d]
$[Cl^-]$	0.172 ± 0.005[c], 0.186[d]	381[d]
$[ClO_4^-]$	0.240 ± 0.005[c]	229[d]
$[Li^+]$	0.042[d]	519[d]
$[Na^+]$	0.067[d]	409[d]
$[NH_4^+]$	0.137 ± 0.002[c]	307[d]
$[N(CH_3)_4^+]$	0.201 ± 0.011,[c] 0.280[f]	261.4[g]
$[N(CH_2CH_3)_4^+]$	0.337[f]	243.4[g]

[a]Ionic radii (in nanometers), as reported in other studies

[b]Hydration enthalpies of gas phase ions, as reported in other studies

[c]Values reported from reference [41]

[d]Values reported from reference [37]

[e]Values reported from reference [42]

[f]Values reported from reference [43]

[g]Values reported from reference [44]

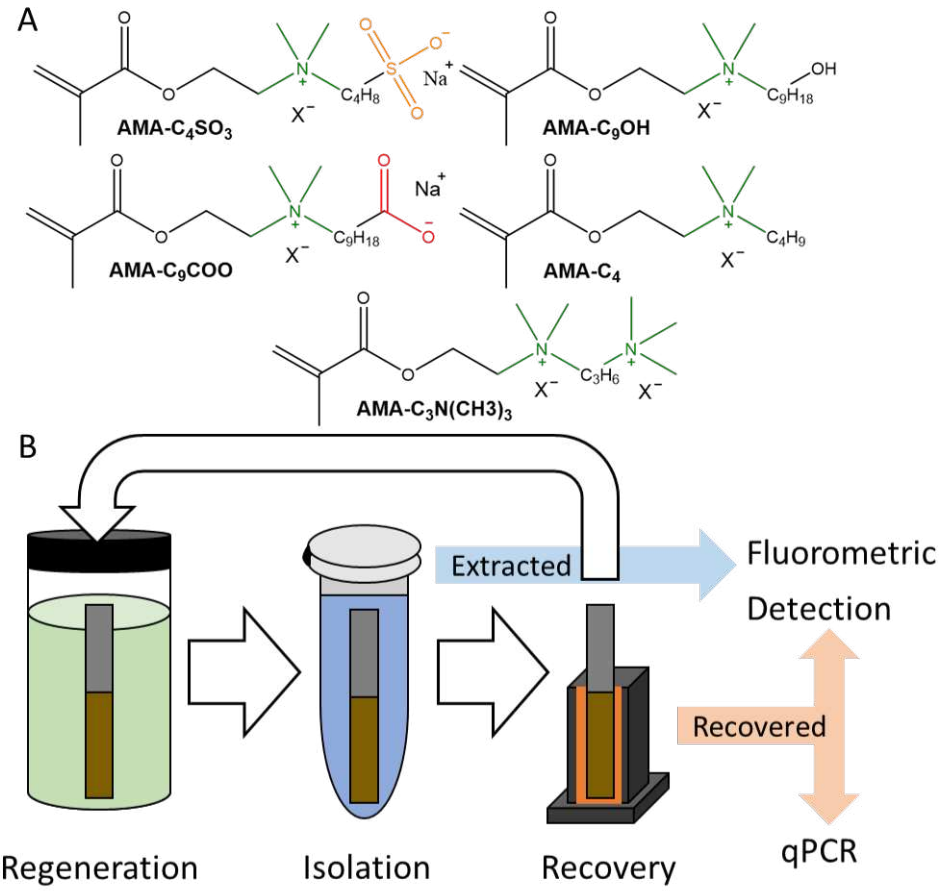

Figure 1. (A) Chemical structures of ammonium methacrylate-based ionic monomers. Structures are representative of the expected chemical state during the regeneration process of the sorbents. (B) Representative workflow featured in this work to examine the poly-ionic sorbents. Devices are removed from an initial regeneration solution for equilibration with a high concentration of salt and added into the extraction solution to isolate DNA. Isolation was carried out for 10 minutes followed by a 30-minute recovery step in a small volume of salt solution. The recovery solution is then examined by either a fluorometric assay or qPCR.

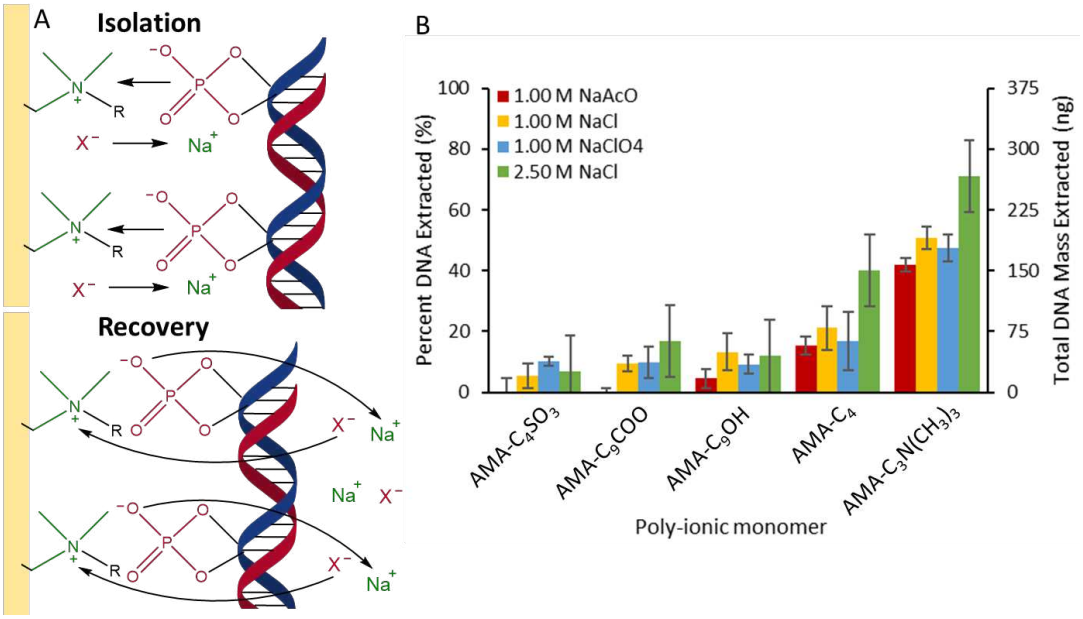

Figure 2. (A) Proposed anion-exchange mechanism which facilitates the high affinity interaction to isolate DNA through exchange of the poly-cation's counter anion for the negatively charged phosphodiester backbone. Recovery of DNA is carried out in the presence of higher concentration of anions to preferentially interact with the poly-cation resulting in release of the DNA molecule into the bulk solution. (B) Assessment of salt solution pre-equilibration on total extraction of DNA by poly-ionic monomers. Devices were equilibrated with each salt solution at a minimum of 1 hour prior to each extraction.

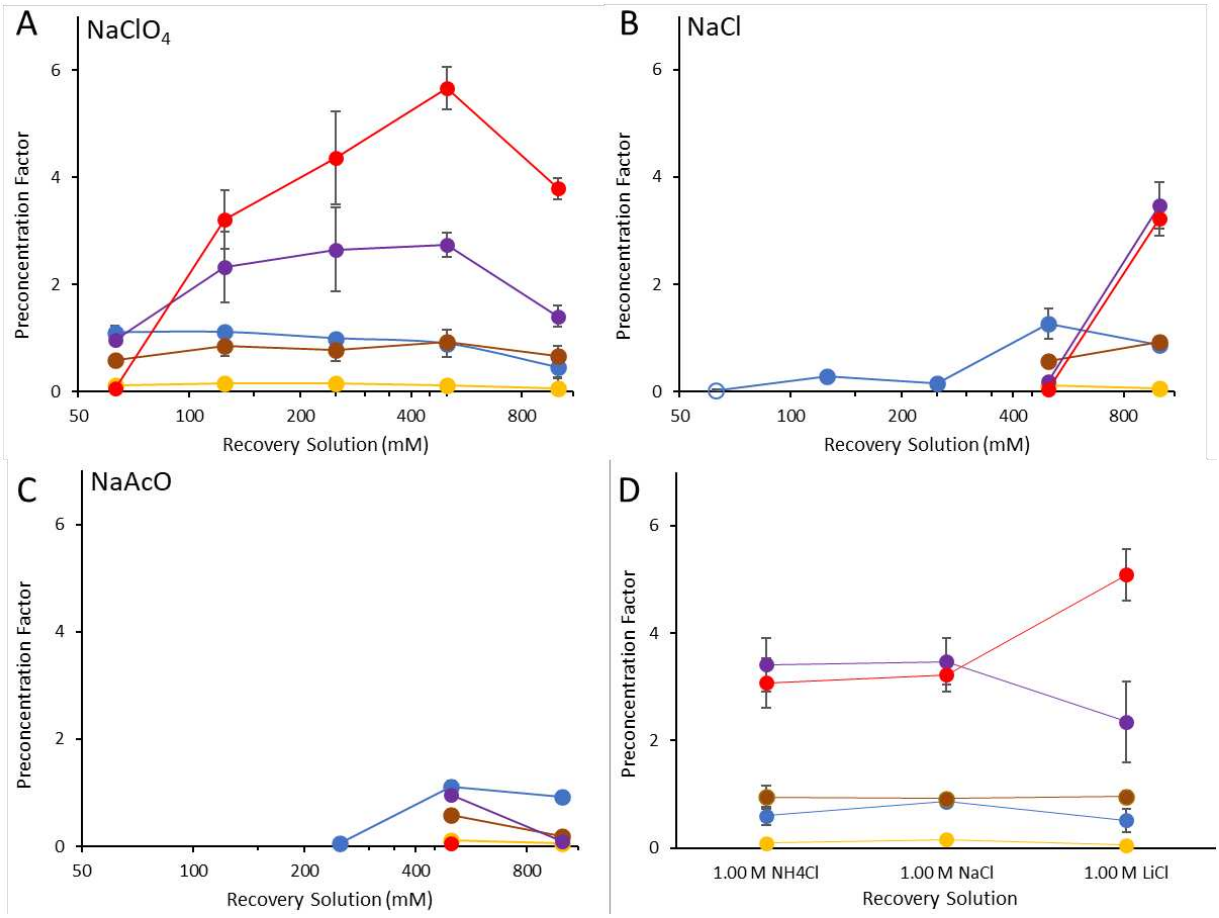

Figure 3. Recovery of isolated DNA from poly-ionic sorbents in (A) $NaClO_4$, (B) NaCl, and (C) NaAcO and (D) various chloride salt solutions (gold) AMA-C_4SO_3, (blue) AMA-C_9COO, (brown) AMA-C_9OH, (purple) AMA-C_4, (red) AMA-$C_3N(CH_3)_3$. Recoveries lower than the LOD are not shown while open circles represent data points which are below the LOQ. (D) Preconcentration factors for recoveries performed in 1.00 M NH_4Cl, NaCl, and LiCl salts to determine the influence of the cation.

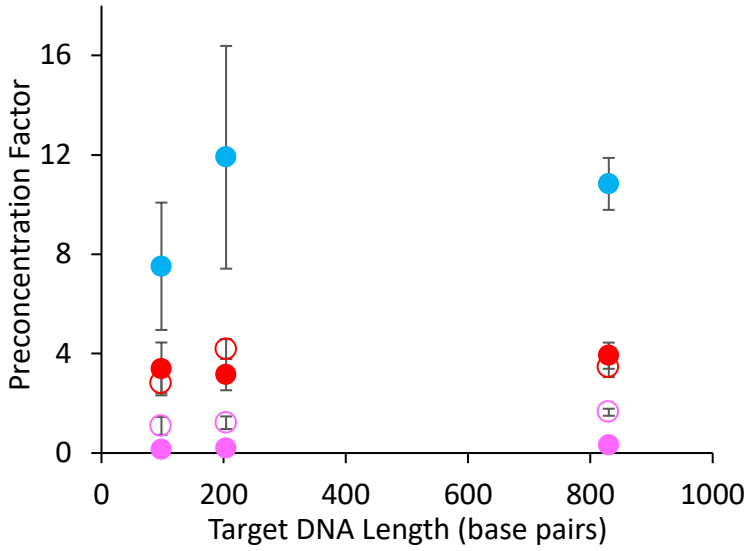

Figure 4. Extraction of a 830 bp, 204 bp, or 98 bp DNA spiked at 10 pg/mL in 1.0 mL of heat-treated human plasma (closed circles) or 2.00 mM tris buffer (open circles). Quantification of recovered DNA spikes was performed by qPCR. The Qiagen ccfDNA MinElute extraction kit (light blue) was employed per the manufacturer's instructions and eluted in a final volume of 50.0 µL. Recoveries from the AMA-$C_3N(CH_3)_3$ (red) and PIL (pink) sorbents were carried out in 45.0 µL of 250 mM $NaClO_4$. Quantification of initial DNA concentration was determined in 2.00 mM tris buffer for all preconcentration factor calculations.

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