

Maximizing Ion-Tagged Oligonucleotide Loading on Magnetic Ionic Liquid Supports for the Sequence-Specific Extraction of Nucleic Acids

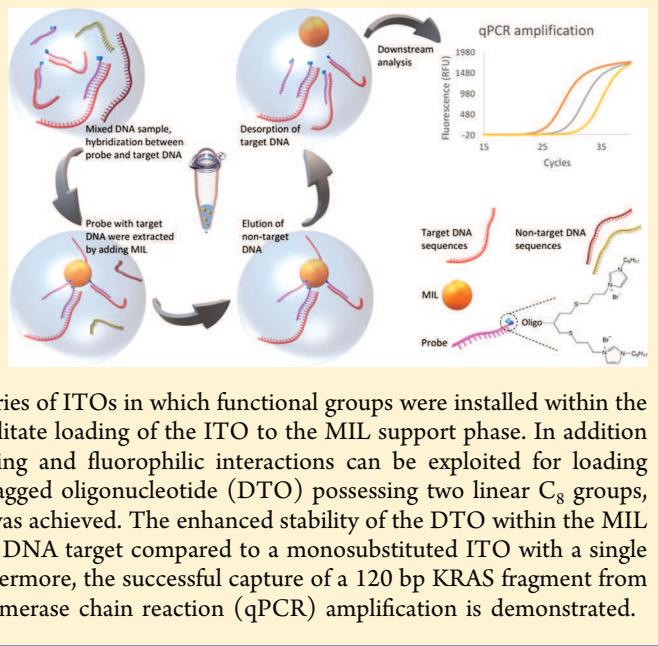
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Supporting Information

ABSTRACT: Targeted nucleic acid analysis requires the highly selective extraction of desired DNA fragments in order to minimize interferences from samples with abundant heterogeneous sequences. We previously reported a method based on functionalized oligonucleotide probes known as ion-tagged oligonucleotides (ITOs) that hybridize with complementary DNA targets for subsequent capture using a hydrophobic magnetic ionic liquid (MIL) support. Although the ITO-MIL approach enriched specific DNA sequences in quantities comparable to a commercial magnetic bead-based method, the modest affinity of the ITO for the hydrophobic MIL limited the yield of DNA targets, particularly when stringent wash conditions were applied to remove untargeted DNA.

Here, we report the synthesis and characterization of a series of ITOs in which functional groups were installed within the cation and anion components of the tag moiety in order to facilitate loading of the ITO to the MIL support phase. In addition to hydrophobic interactions, we demonstrate that π - π stacking and fluorophilic interactions can be exploited for loading oligonucleotide probes onto MILs. Using a disubstituted ion-tagged oligonucleotide (DTO) possessing two linear C₈ groups, nearly quantitative loading of the probe onto the MIL support was achieved. The enhanced stability of the DTO within the MIL solvent permitted successive wash steps without the loss of the DNA target compared to a monosubstituted ITO with a single C₈ group that was susceptible to increased loss of analyte. Furthermore, the successful capture of a 120 bp KRAS fragment from human plasma samples followed by real-time quantitative polymerase chain reaction (qPCR) amplification is demonstrated.



Despite the importance of DNA as a biological repository for genetic information, the vast majority of nucleic acid sequences are not targeted in molecular diagnostics applications. For example, mutant populations of cell-free DNA that indicate malignancy can be as low as 0.01% abundance of the wild-type sequence in human plasma.¹ Major advances in nucleic acid sequencing and detection technologies have provided a rapid means to routinely characterize thousands of samples per day, but the detection of specific DNA sequences such as single-nucleotide polymorphisms remains a significant challenge due to high levels of untargeted nucleic acids that increase the background signal in bioanalytical assays.^{2,3} In order to overcome this bottleneck in nucleic acid analysis, sample preparation methods that capture and enrich specific DNA sequences must be developed.

The most popular approaches for the enrichment of specific nucleic acid sequences are based on magnetic beads or particles coated with streptavidin. Biotinylated oligonucleotide probes that exhibit a high affinity toward streptavidin can be designed to capture DNA targets via base-pairing interactions. After a washing step to remove interfering DNA molecules, the extracted nucleic acid targets can be released for downstream analysis. Successive washes may improve the purity of the

nucleic acid recovered from the magnetic beads but often result in lower analyte yields.^{4,5} Moreover, the tendency of interferences from the sample matrix to nonspecifically adsorb to the magnetic support material often requires an additional step involving the use of bovine serum albumin (BSA) in the workflow to pacify or block the surface. Contemporary magnet-based DNA capture methods are also limited by aggregation of the solid bead/particle support material, resulting in lower capture efficiencies and the clogging of liquid handling devices.^{6–9} Such particle aggregates are particularly unwanted if downstream bioanalysis is to be performed within a microfluidic device possessing narrow channels that can be easily obstructed.

An ideal magnetic support material for DNA extraction would possess the following features: 1) mitigate or eliminate aggregation behavior, 2) exhibit low nonspecific extraction of untargeted DNA and interferences (like proteins), and 3) possess high affinity toward decorated oligonucleotide probe molecules. In contrast to conventional magnetic supports,

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Table 1. Effect of ITO and DTO Structures on Hybridization, Loading, and Target Capture Performance

Oligonucleotide (20-mer)	Structure	Melting Temp (°C)	Loading Efficiency ^a (% n=3)	Loading Capacity ^b (pmol μL ⁻¹ , n=3)	Target Capture Cq ^c
Untagged	3'-HO-DNA-5'	75	10 ± 3	7 ± 2	35.35 ± 0.6
[ABIM ⁺][Br ⁻]-ITO		75	12 ± 3	8 ± 2	36.01 ± 0.5
[ABIM ⁺][NTf ₂ ⁻]-ITO		67	40 ± 4	25 ± 2	na ^d
[ABIM ⁺][OS ⁻]-ITO		75	45 ± 2	28 ± 1	28.2 ± 1.1
[ABIM ⁺][PFBS ⁻]-ITO		70	35 ± 5	22 ± 3	na ^d
[AOIM ⁺][OS ⁻]-ITO		75	74 ± 4	46 ± 2	26.1 ± 0.3
[AOIM ⁺][Br ⁻]-ITO		72	48 ± 4	31 ± 3	25.67 ± 1.3
[ADIM ⁺][Br ⁻]-ITO		76	75 ± 5	47 ± 3	24.7 ± 2.3
[AOIM ⁺] ₂ [Br ⁻]-DTO		73	95 ± 4	57 ± 2	24.3 ± 1.1

^aConditions: concentration of ITO: 8 ng μL⁻¹; hydrophobic MIL: [P₆₆₆₁₄⁺][Mn(hfacac)₃⁻]; MIL volume: 1 μL; sample volume: 50 μL; extraction time: 10 min; quantification method: anion-exchange HPLC with UV detection at 260 nm. ^bConditions: same as a. MIL density = 1.4 mg μL⁻¹

^cConditions: amount of ITO: 1.69 pmol; amount of target: 169 fmol of 261 bp DNA target; MIL volume: 1 μL; sample volume: 50 μL; extraction time: 10 min; desorption time: 10 min at 90 °C quantification method: qPCR. ^dNot applicable. Extractions were not performed coupled with qPCR due to comparatively low loading efficiencies.

magnetic ionic liquids (MILs) are paramagnetic molten salts comprised of organic/inorganic cations and anions with melting points at or below 100 °C.^{10,11} Physicochemical properties of MILs including viscosity, hydrophobicity, magnetic susceptibility, and solvation behavior can be readily tuned by judicious selection/functionalization of the cation and anion structures.^{12–14} The liquid nature of MILs represents a key advantage over solid magnetic substrates

that suffer from particle agglomeration, making these solvents ideal for biomolecule sample preparation applications that interface with downstream bioassays. MILs based on Fe(III),¹⁵ Co(II),¹⁶ and Ni(II) anion complexes were recently shown to provide high enrichment factors for a variety of DNA fragments and, in some cases, were directly coupled to polymerase chain reaction (PCR) and real-time quantitative PCR (qPCR) for rapid detection and quantification of

extracted nucleic acids in the MIL phase.^{17,18} The particle- and aggregation-free extraction of specific DNA sequences has also been demonstrated using a Mn(II)-based hydrophobic MIL as a paramagnetic liquid support material.¹⁹ By appending amphiphilic alkylimidazolium groups to synthetic oligonucleotides, the probe molecules partitioned to the MIL support via simple hydrophobic interaction and facilitated the capture of complementary DNA sequences via Watson–Crick base pairing. Moreover, the affinity of the ion-tagged oligonucleotide (ITO) probes for the hydrophobic MIL support was highly dependent on the chemical structure of the bioconjugate where longer alkyl groups (e.g., octyl) in the ITO resulted in enhanced partitioning to the MIL phase.

Although the previously described MIL-ITO approach provided similar sequence-specific DNA extraction yields to a commercial magnetic bead-based method,¹⁹ the modest affinities (ca. 50% loading efficiency) of the ITO probe for the MIL support limited the technique in three important ways: 1) since fewer probe molecules partitioned to the MIL support, a lower capacity for target DNA was observed, 2) ITO probes with lower affinity for the MIL are more readily desorbed upon successive washing of the MIL phase to improve sample purity/selectivity for DNA targets, and 3) interferences in complex samples (e.g., cell lysate) resulted in decreased extraction yields by competing with the ITO probe for sorption to the MIL. Here, we report a series of ITOs with unique ion tag structures, elucidating the structural components of the tag moiety that result in improved affinities of the synthetic probe for the MIL support. In addition to modifying the ITO cation structure, fluorophilic anions (e.g., bis-[trifluoromethylsulfonyl]imide [NTf_2^-]) and amphiphilic anions were paired with imidazolium cations for enhanced partitioning to the MIL support. We investigated a cyclic disulfide-modified oligonucleotide starting material for the preparation of disubstituted ion-tagged oligonucleotides (DTOs) that provided loading efficiencies greater than 95% onto the MIL support, furnishing an extraction phase capable of recovering a 10-fold greater quantity of target nucleic acid compared to monosubstituted ITOs. The stability of the ITO- and DTO-MIL interactions was studied under stringent hybridization conditions, ultimately revealing that DTOs provided superior recoveries of target DNA sequences from samples containing interfering nucleotide sequences. As demonstrated herein, the structural tunability of both the cation and anion components of the ion tag greatly expands the modes of intermolecular interactions between ITO probes and MIL supports, facilitating high-efficiency DNA extraction and guiding the design of future ITOs.

EXPERIMENTAL SECTION

Reagents and Materials. Acetonitrile (99.9%), LC-MS grade acetonitrile ($\geq 99.9\%$), hexane, mixture of isomers ($\geq 98.5\%$), ethylenediaminetetraacetic acid (EDTA, 99.4% $\sim 100.06\%$), triethylamine (TEA, $\geq 99.5\%$), magnesium chloride (99.0%–102.0%), 1-bromoocetane, ammonium persulfate (APS, $\geq 98\%$), sodium octylsulfate ($\geq 95\%$), and plasma from human were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethyl ether ($\geq 99\%$) was purchased from Avantor (Center Valley, PA, USA). Allylimidazole (99%) was purchased from Alfa Aesar (Ward Hill, MA, USA). 1,1,5,5,5-Hexafluoroacetylacetone (99%) and 4,4,4-trifluoro-1-phenyl-1,3-butanedione (99%) were purchased from ACROS organics (Morris, NJ, USA). Tris(hydroxymethyl)-

aminomethane (ultra pure), tris(hydroxymethyl)-aminomethane hydrochloride ($\geq 99.0\%$), urea ($\geq 99\%$), and boric acid ($\geq 99.5\%$) were purchased from RPI (Mount Prospect, IL, USA). Tris(2-carboxyethyl)phosphine (TCEP) was purchased from Soltec Ventures (Beverly, MA, USA). Sodium hydroxide, sodium chloride, M-270 Streptavidin coated Dynabeads, SYBR Green I nucleic acid gel stain, dimethyl sulfoxide (DMSO), acetic acid, and ammonium hydroxide were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Tetramethylethylenediamine (TEMED), 40% acrylamide and bis-acrylamide solution 29:1, and SsoAdvanced Universal SYBR Green Supermix and a KRAS, human PrimePCR SYBR green assay including template and primers (120 bp amplicon) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Dithiolated, biotinylated, and unmodified oligonucleotides and primers were purchased from Integrated DNA Technologies (Coralville, IA, USA). The oligonucleotide probe sequence used for extraction of the KRAS target was 5'- TTG AAC TAG CAA TGC CTG TG -3'.

Synthesis of MILs. The trihexyl(tetradecyl)phosphonium manganese(II) hexafluoroacetylacetone ($[\text{P}_{66614}^+][\text{Mn}(\text{hfacac})_3^-]$) MIL and the trihexyl(tetradecyl)phosphonium manganese(II) 4,4,4-trifluoro-1-phenyl-1,3-butanedionate ($[\text{P}_{66614}^+][\text{Mn}(\text{PhCF}_3\text{acac})_3^-]$) MIL were synthesized using a similar approach to previously reported procedures,^{20–22} and their chemical structures are shown in Figure S1a,b. Synthesis procedures for the MILs used in this study are described in the Supporting Information. The products were characterized using ESI-TOFMS in both positive and negative ion modes (Figures S3–S6, Supporting Information).

Synthesis and Characterization of ITOs and DTOs. Allylimidazolium salts were synthesized as previously reported.^{19,23,24} The ITO and DTO structures investigated herein are shown in Table 1 and were synthesized according to published procedures.¹⁹ Briefly, 4 nmol of a 20-mer thiolated oligonucleotide (for ITO synthesis) or 2 nmol of a 20-mer oligo with a terminal cyclic disulfide (for DTO synthesis) were reduced using 40 nmol of tris(2-carboxyethyl)phosphine (TCEP). The thiolated oligo was then transferred to a UV transparent 96-well microplate along with 400 nmol of an allyl-functionalized imidazolium salt for a final solution composition of 30% (v/v) ACN. The reaction well was sealed with optically transparent tape, purged with N_2 , and placed beneath a handheld UV lamp set to 365 nm output for 1–2 h. The ITOs or DTOs were separated from unreacted starting material using denaturing polyacrylamide gel electrophoresis (PAGE). Product bands were excised, crushed, and eluted with Milli-Q water overnight. The ITOs or DTOs were characterized by reversed-phase ion-pair LC-TOFMS (see Figures S7, S8, and S12 and Supporting Information for details).

Sequence-Specific DNA Capture Using MILs and ITO or DTO Probes. For DNA extraction, ITOs or DTOs were added to a 25 mM NaCl solution containing 0.169 fmol of 261 bp DNA target at various probe:target ratios. For experiments testing probe selectivity, 0.169 fmol of a DNA sequence (complement, 1 nt, or 2 nt mismatch to the ITOs probe) was also added to solution. The probes and DNA targets were annealed by heating at 90 °C for 5 min followed by cooling of the solution to 37 °C for 5 min. A 1 μL aliquot of MIL support was then added to the solution and incubated at 37 °C for 10 min to extract the ITO- or DTO-target duplex. The sample solution was decanted, and the MIL phase was washed using 50 μL of deionized water. Extracted DNA targets were

desorbed from the MIL phase in 50 μ L of deionized water at 90 °C for 10 min. A 1 μ L aliquot of desorption solution was analyzed by qPCR amplification.

Loading Efficiency of ITO or DTO Probes onto the MIL Support. The ITO and DTO stability within the MIL phase was tested using HPLC for direct detection of the probe molecules. For these experiments, a liquid phase microextraction technique was used in which a 1 μ L aliquot of hydrophobic MIL support was immersed in a 50 μ L solution containing 60 pmol (400 ng) of ITO or DTO for 10 min at room temperature. A 20 μ L aliquot of the aqueous layer was then removed for injection onto an Agilent 1260 HPLC with a variable wavelength detector (Santa Clara, CA, USA) and separated on a 35 mm \times 4.6 mm i.d. \times 2.5 μ m TSKgel DEAE-NPR anion exchange column with a 5 mm \times 4.6 mm i.d. \times 5 μ m TSKgel DEAE-NPR guard column (Tosoh Bioscience, King of Prussia, PA). Mobile phase A consisted of 20 mM Tris-HCl (pH 8), and mobile phase B was 1 M NaCl and 20 mM Tris-HCl (pH 8). DNA was detected at 260 nm, and the amount of DNA recovered from the MIL phase was determined using an external calibration curve.

qPCR Amplification. Each qPCR reaction mix was 19 μ L which contains 4.6 μ L of deionized water, 2.6 μ L of 50 mM MgCl₂, 1 μ L of DMSO, 0.8 μ L of 10 μ M forward and reverse primers, and 10 μ L of SsoAdvanced Universal SYBR Green Supermix (2x). The forward and reverse primers for qPCR amplification of the 261 bp target sequence were 5'-CAC GCT TAC ATT CAC GCC CT -3' and 5'-CGA GCG TCC CAA AAC CTT CT -3'. For all reactions, 1 μ L of the template DNA was added to the 19 μ L of the qPCR reaction mix. For amplification of the 120 bp KRAS target, the reaction mix included 4.4 μ L of water, 10 μ L of SsoAdvanced Supermix, 1 μ L of primer mix, 2.6 μ L of 50 mM MgCl₂, 1 μ L of DMSO, and 1 μ L of template DNA. The primers from the KRAS, human PrimePCR assay kit were used without further purification. Amplification was performed using a Bio-Rad CFX96 Touch Real-Time PCR Detection System or Bio-Rad CFX Connect Real-Time PCR Detection System. The thermal program used for all qPCR assays included an initial denaturation step of 95.0 °C for 5 min followed by 40 cycles consisting of 95.0 °C for 10 s and 64.0 °C for 30 s. A five-point external calibration curve was used to determine the amplification efficiency and quantify extracted nucleic acid.

RESULTS AND DISCUSSION

Design of ITOs for Enhanced Loading Efficiency onto MIL Supports. The extraction of specific DNA sequences is highly dependent on facilitating strong interactions between the oligonucleotide probe and the support phase. Contemporary methods rely on noncovalent interactions between biotin labels and streptavidin-coated magnetic bead/particles, but these materials often suffer from agglomeration processes that diminish extraction performance. As described in our previous work, we employed a magnetic liquid support and ITO probes to circumvent the drawbacks of solid particle supports while maintaining a high yield of target DNA sequences.¹⁹ Since the studied ITOs possessed alkyl chain lengths ranging from methyl to octyl, the probes exhibited limited affinity for the hydrophobic MIL support (ca. 50% ITO loading efficiency). In order to improve the partitioning of ITOs to the MIL support, we synthesized a series of ITOs possessing one or more of the following properties: longer alkyl groups in the cation, amphiphilic anions, fluorophilic anions, and multiple ion tag

moieties. Initially, we focused on the synthesis of alkylimidazolium tags with longer carbon chains (C₁₀ and C₁₆). However, reaction mixtures containing the amphiphilic 1-allyl-3-hexadecylimidazolium bromide ([AHIM⁺][Br⁻]) salt resulted in the precipitation of the thiolated nucleic acid starting material due to the alkylimidazolium group exhibiting surfactant properties and forming aggregates with DNA.²⁵ This phenomenon was not observed when using 1-allyl-3-decylimidazolium bromide ([ADIM⁺][Br⁻]) or any other alkylimidazolium salts with shorter chain lengths.

In order to determine the influence of the longer alkyl chain on partitioning to the MIL phase, the ITO product generated from reaction with the [ADIM⁺][Br⁻] salt was subjected to liquid phase microextraction using the [P₆₆₆₁₄⁺][Mn(hfacac)₃⁻] MIL support as the extraction solvent. As shown in Table 1, a loading efficiency of 75 \pm 5% was observed for the [ADIM⁺][Br⁻]-ITO, compared to a 48 \pm 4% loading efficiency of the 1-allyl-3-octylimidazolium bromide ([AOIM⁺][Br⁻])-ITO, with an unmodified oligonucleotide probe providing 10 \pm 3% under the same conditions.¹⁹ This result is likely due to the increased hydrophobic interactions between the C₁₀ alkyl chain and the hydrophobic MIL support.

Given that dispersive interactions primarily govern the partitioning of the ITO to the MIL phase and yet the longer chain alkylimidazolium salts tended to precipitate the oligo starting material, ITOs were synthesized to include hydrophobic groups in the anion as well. Allyl-bearing imidazolium salts were prepared with octylsulfate anions via metathesis reaction between the bromide form of the imidazolium salt and sodium octylsulfate.²⁶ As shown in Table 1, a loading efficiency of 45 \pm 2% was observed for the 1-allyl-3-butylimidazolium octylsulfate ([ABIM⁺][OS⁻])-ITO. The loading efficiency was a substantial increase from the [Br⁻] analog, which exhibited a loading efficiency of 12 \pm 3%. In addition to amphiphilic anions, fluorine-rich anions were also imparted to the tag moiety of the ITO in an effort to capitalize on fluorophilic interactions with the fluorinated ligands of the MIL support (i.e., hfacac). Allylimidazolium salts with either [NTf₂⁻] or [PFBS⁻] anions were prepared by metathesis reactions and subsequently studied for their ability to partition to the hydrophobic MIL. When paired with the [ABIM⁺] cation in the ITO structure, both [NTf₂⁻] and [PFBS⁻] anions contributed to higher ITO loading efficiency (40 \pm 4% and 35 \pm 5%, respectively) compared to the [Br⁻] form of the ITO (ca. 12%). Since the [OS⁻] anion appeared to have the greatest influence on loading efficiency, an ITO with the [AOIM⁺] cation and [OS⁻] anion components was synthesized. A significantly higher affinity for the MIL phase was observed for the [AOIM⁺][OS⁻]-ITO, resulting in a loading efficiency of 74 \pm 4%. These results strongly suggest that the anion component of the ITO plays an important role in facilitating binding of the ITO probe to the MIL support and could be used for further optimization of probe-support affinity.

Because the noncovalent labeling of the ITO probe with an additional alkyl moiety (i.e., [OS⁻] anions) provided enhanced loading efficiency, the covalent attachment of another alkyl group of the same length would also be expected to improve affinity toward the MIL. A dithiolated oligonucleotide starting material was employed as a means to introduce two ion-tags with C₈ alkyl groups into the same probe structure via covalent linkage. The structure of the resulting [AOIM⁺]₂[Br⁻]-DTO is shown in Table 1. The loading efficiency was investigated under the same conditions used for the ITO probes and

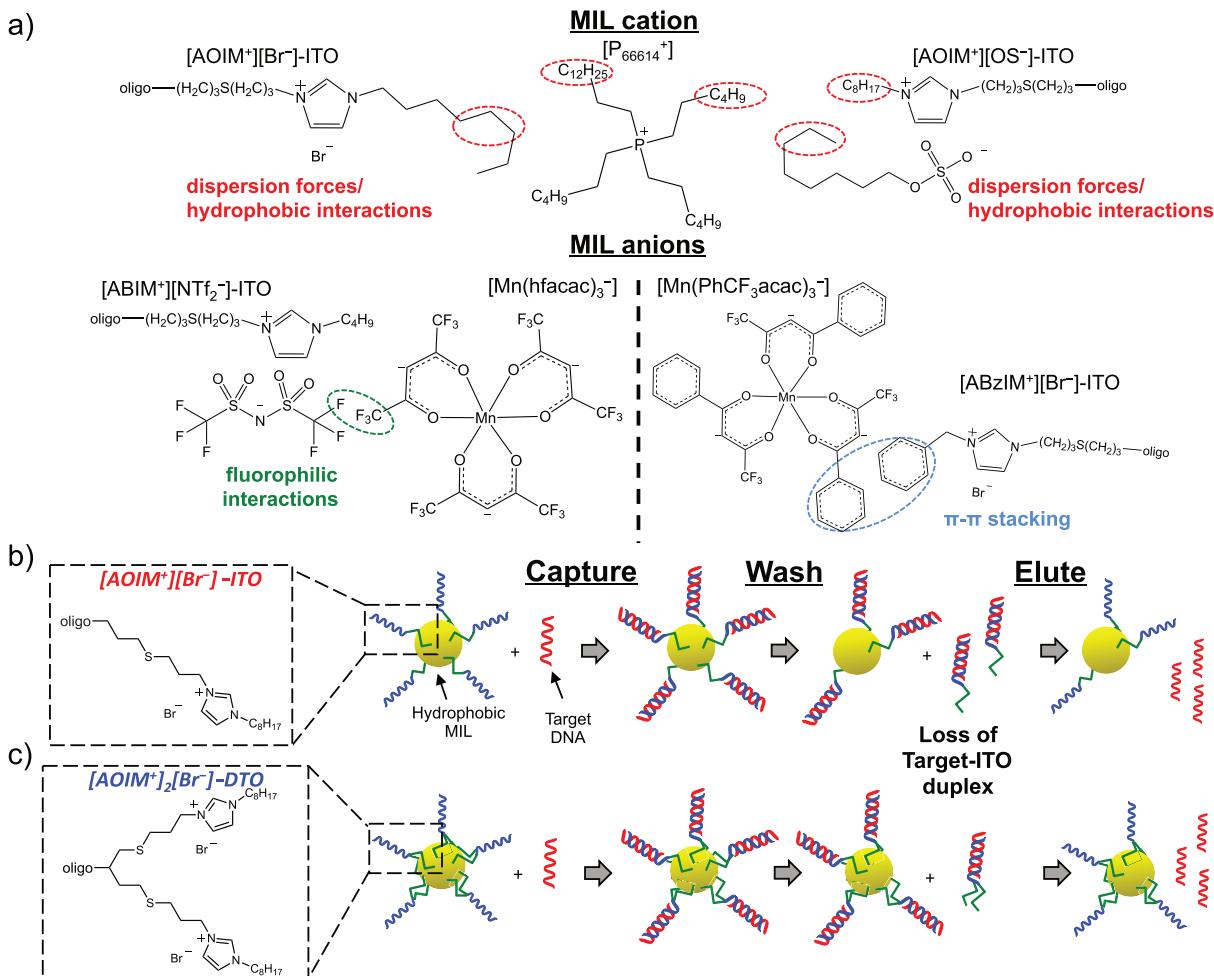


Figure 1. (a) Chemical structures of the MILs used in this study and examples of the intermolecular interactions between the cation/anion components of the ion tag and the MIL support that govern ITO loading efficiency. Schematic showing the sequence-specific DNA extraction procedure using the hydrophobic $[P_{66614}^+][Mn(hfacac)_3^-]$ MIL and the (b) monosubstituted $[AOIM^+][Br^-]$ -ITO or (c) disubstituted $[AOIM^+]_2[Br^-]$ -DTO.

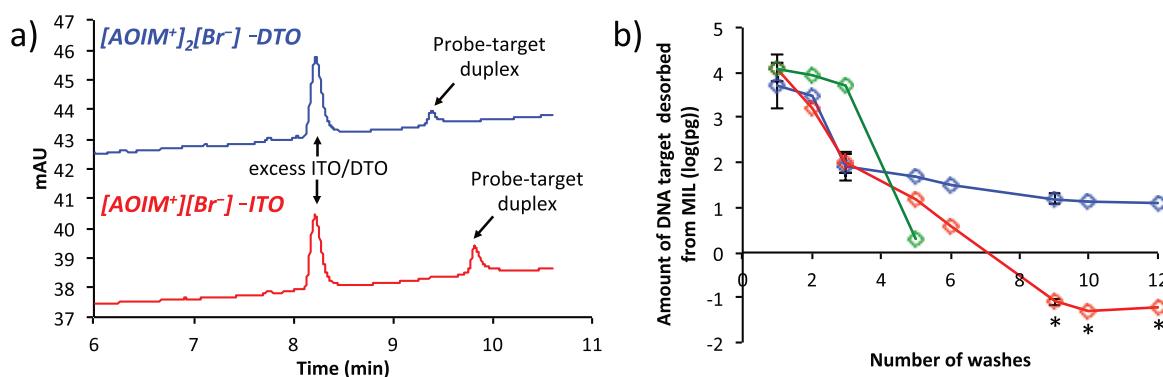


Figure 2. (a) HPLC quantification of the loss of target-ITO duplex from the MIL support after loading a solution containing 60 pmol of duplex onto the MIL and subsequently heating the sample at 37 °C for 10 min in 25 mM NaCl solution. (b) 0.169 pmol (ca. 2.7×10^4 pg) of 261 bp DNA target from aqueous solution using probes substituted with one (red trace) or two (blue trace) $[AOIM^+][Br^-]$ groups or an unmodified probe sequence (green trace). The asterisk indicates that the data were outside the range of the calibration curve and were extrapolated to obtain these values.

revealed nearly quantitative capture ($95 \pm 4\%$) of the $[AOIM^+]_2[Br^-]$ -DTO by the hydrophobic $[P_{66614}^+][Mn(hfacac)_3^-]$ MIL.

Apart from hydrophobic interactions that facilitate loading onto the MIL support, ion tags with benzylimidazolium groups

($[ABzIM^+][Br^-]$ -ITO) were coupled with the $[P_{66614}^+][Mn(PhCF_3acac)_3^-]$ MIL support which possesses phenyl groups within the anion component in order to investigate the effect of $\pi-\pi$ stacking on loading efficiency. The structures of the $[P_{66614}^+][Mn(PhCF_3acac)_3^-]$ MIL and the $[ABzIM^+][Br^-]$ -

Table 2. Quantification Cycle (Cq) Values for Sequence Specific DNA Extraction Using DTO, ITO, or Magnetic Bead-Based Methods Obtained from qPCR Amplification

extraction method	DNA target + 20 mer comp (n = 3)	DNA target + 20 mer 1 nt mismatch (n = 3)	DNA target + 20 mer 2 nt mismatch (n = 3)	DNA target (n = 3)
Dynabeads M-270 ^a	32.98 ± 0.46	32.52 ± 0.26	31.31 ± 0.64	31.23 ± 0.06
[AOIM ⁺] ₂ [Br ⁻] ^b	30.51 ± 0.84	29.30 ± 1.16	31.59 ± 0.10	28.29 ± 0.19
[AOIM ⁺][Br ⁻] ^b	31.29 ± 1.06	30.58 ± 0.34	30.07 ± 0.94	30.36 ± 0.65

^aExtraction conditions: biotinylated probe: 16.9 fmol; Dynabeads M-270: 1.3 μ g; 261 bp DNA target: 0.169 fmol; DTO [AOIM⁺]₂[Br⁻]: 0.845 fmol; ITO [AOIM⁺][Br⁻]: 16.9 fmol. Interfering sequences were all spiked at a 1:1 mol ratio to target. ^b261 bp DNA target was first hybridized in 50 μ L of 25 mM NaCl solution from 90 °C for 5 min to 37 °C for 5 min. One μ L of MIL: [P₆₆₆₁₄⁺][Mn(hfacac)₃⁻] was added in the middle of the solution after hybridization. The extraction was performed for 10 min at room temperature. The 261 bp DNA target was desorbed with 50 μ L of 25 mM NaCl at 37 °C for 10 min.

ITO are shown in **Figures S1b and S2**, respectively. The loading efficiency and selectivity were tested using either a benzyl or C₈-tagged oligo coupled with either the [P₆₆₆₁₄⁺][Mn(PhCF₃acac)₃⁻] MIL or the [P₆₆₆₁₄⁺][Mn(hfacac)₃⁻] MIL to account for nonspecific interactions (i.e., hydrophobic interactions) that may also contribute to loading efficiency of the aromatic tag and/or MIL support. As shown in **Table S1**, no differences in extraction efficiency were observed for the benzyl-tagged oligo and an unlabeled oligo using the [P₆₆₆₁₄⁺][Mn(PhCF₃acac)₃⁻] MIL support. However, duplexes generated from the [ABzIM⁺][Br⁻]-ITO and its complementary sequences were extracted with substantially higher efficiency (40 ± 3%) than untagged duplexes (1 ± 1%), indicating the feasibility of utilizing π -stacking interactions for sequence specific DNA capture.

Binding Affinity and Durability of ITO-MIL Interactions. In order to determine whether high loading efficiency was correlated to better retention of probe molecules within the MIL support solvent, the desorption of either ITOs or DTO from the MIL after successive wash steps commonly employed to remove untargeted DNA in sequence-specific extraction procedures was tested. Similar to the procedures shown in **Figure 1b** and **c**, the [AOIM⁺][Br⁻]-ITO or [AOIM⁺]₂[Br⁻]-DTO was loaded onto the MIL support and subsequently immersed in a solution containing complementary 20 mer DNA. The probes and targets were annealed and then washed with 25 mM NaCl at 40 °C for 10 min, and the aqueous phase was removed for anion-exchange HPLC analysis. **Figure 2a** shows chromatograms obtained for the ITO (red trace) or DTO (blue trace) experiments. As indicated by the area of the later eluting peak, 2.7 ± 0.6-fold more probe-target duplex was desorbed from the MIL support when the [AOIM⁺][Br⁻]-ITO was used to capture the DNA target. These findings are in good agreement with the results obtained for the loading efficiency data (**Table 1**) and show a positive correlation between loading efficiency and stability of the ITO-MIL interactions.

Many relevant nucleic acid targets are much longer sequences than the initially studied 20 mer and may potentially impact the partitioning behavior of the probe-target duplex from the MIL support. The affinity between the ITOs and DTOs for the MIL was also tested following the capture of a model 261 bp DNA sequence. For these experiments, a procedure similar to **Figure 1b** and **c** was employed, using qPCR amplification to detect very small quantities of nucleic acid desorbed from the MIL. After extraction of a solution containing 0.169 pmol of target using either ITO, DTO, or an unmodified oligonucleotide as hybridization probes, the MIL phase was subjected to successive washes with 25 mM NaCl at 37 °C in order to maintain base pairing within the probe-target

duplex. Wash fractions were collected for qPCR analysis until no detectable target DNA remained, or the amount of desorbed target DNA from wash to wash remained constant. As shown in **Figure 2b**, the amount of DNA target desorbed from the MIL decreased after the first three wash steps when using either the ITO or DTO. The initial rapid decrease in DNA target desorption was also observed when using unmodified oligonucleotide probes, indicating that the phenomenon is due to the release of DNA nonspecifically extracted by the MIL support. It is important to note that a greater amount of untagged probe-target duplex was initially released from the MIL phase than for ITO- or DTO-target duplexes, likely due to weaker interactions between the untagged probe and MIL support. Moreover, no DNA was detected for any subsequent washes for the sample extracted using the unmodified oligonucleotide probe. After 9 wash steps, the amount of DNA target released by the [AOIM⁺][Br⁻]-ITO was lower than the limit of quantification. However, the [AOIM⁺]₂[Br⁻]-DTO continued to release a small amount of DNA target into the subsequent wash solutions and retained a detectable amount of DNA target for up to 12 successive washes. These results indicate that the DTO is retained within the MIL phase longer and may be subjected to more rigorous washing or hybridization conditions than the monosubstituted ITO.

Distinguishing Double- and Single-Nucleotide Variants Using ITOs with MIL-Based Capture and Comparison to a Commercial Magnetic Bead-Based Method.

Single-nucleotide polymorphisms are the most common type of alteration to genomic DNA and are widely recognized to provide meaningful diagnostic information for numerous diseases. These modifications are invariably colocalized with high levels of background nucleic acids that differ by a single base pair. The selectivity of the ITO and DTO probes for complementary sequences and mismatched sequences was initially tested using melt curve analysis. As shown in **Table 1** and **Figures S9 and S10**, the T_m value of the [AOIM⁺]₂[Br⁻]-DTO probe and complement was comparable to the monosubstituted [AOIM⁺][Br⁻]-ITO, indicating little influence of the second alkyl chain on hybridization behavior. Similarly, both ITO and DTO showed only minor melt curve features for the 1 and 2 nt mismatches, compared to the more prominent and higher melting temperature peaks observed for the unmodified oligonucleotide probe (**Figure S11**). For [AOIM⁺][OS⁻], a slightly higher melting temperature with the complementary sequence was observed compared to the unmodified oligo probe, while ITOs with [NTf₂⁻] and [PFBS⁻] anions resulted in much lower melting temperatures (**Table 1**). Although ITOs with [NTf₂⁻] and [PFBS⁻] anions showed minimal peaks for 1 and 2 nt mismatches, the melt

curves were very broad (ca. 20 °C), indicating noncanonical base pairing or nonspecific interactions.²⁷ For this reason, we focused on the DTO and $[\text{AOIM}^+][\text{Br}^-]$ -ITOs for extraction applications.

Cell-free nucleic acid biomarkers in plasma cover a wide range of concentrations from no detectable amount to 1000 ng μL^{-1} with the average concentration near 200 ng μL^{-1} .^{28,29} Due to the presence of highly similar interfering DNA, low abundance target DNA sequences are particularly challenging to detect. As shown in Table S2, the MIL-based extraction conditions were optimized for low target concentrations, resulting in a 5:1 ratio of probe:target sequence (0.845 fmol of DTO) and 1 μL of MIL. Next, the extraction selectivities of the $[\text{AOIM}^+]_2[\text{Br}^-]$ -DTO and $[\text{AOIM}^+][\text{Br}^-]$ -ITO were studied by spiking a 3.4 pM (0.027 ng μL^{-1}) solution of 261 bp target DNA with interfering sequences (20 mers) with either 0, 1, or 2 nt mismatches to the 20 mer probes. The samples were then subjected to extraction using the procedures shown in Figures 1b and c. Extractions were also performed using a commercial method based on magnetic beads according to the manufacturers instructions. As shown in Table 2, the ITO and DTO methods exhibited superior extraction performance compared to the magnetic bead-based approach when single nucleotide variants were added to solution. These findings are likely due to higher nonspecific DNA extraction observed for magnetic beads when compared to the $[\text{P}_{66614}^+][\text{Mn}(\text{hfacac})_3^-]$ MIL support.¹⁹

Sequence-Specific DNA Capture from Bacterial Cell Lysate and Human Plasma Using DTO Probes and MIL Support. In order to investigate the influence of a complex sample matrix on the performance of the DTO-based DNA capture method, approximately 1×10^6 *E. coli* cells were lysed by ultrasonication, and the crude lysate was spiked with target DNA. Two approaches were studied for sequence-specific DNA extraction: 1) a hybridize-first method where the probe and target are hybridized prior to capture by the MIL support and 2) a load-first method where the DTO probe is first loaded onto the MIL support and subsequently used for extraction of target DNA. For both methods, the optimal ratio of DTO:target (5:1) was used. Figure 3 shows that when the hybridize-first method was employed for the extraction of 0.3 ng of target DNA from the crude cell lysate, a Cq value of approximately 33 was obtained using the DTO probe. However, a much lower Cq value (23.5 ± 0.2) was observed

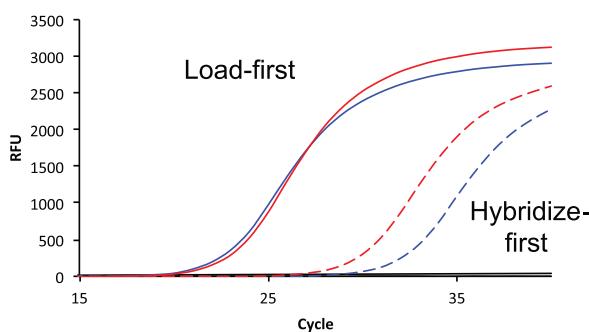


Figure 3. qPCR amplification of 261 bp DNA target isolated from *E. coli* cell lysate using the ITO-MIL approach either in hybridize-first (dashed lines) or load-first (solid lines) mode. Samples spiked with 0.3 ng of target were subjected to each mode using either the monosubstituted $[\text{AOIM}^+][\text{Br}^-]$ -ITO (red traces) or the disubstituted $[\text{AOIM}^+]_2[\text{Br}^-]$ -DTO (blue traces).

for the load-first method, indicating an approximate 1000-fold increase in the amount of DNA recovered compared to the hybridize-first method. The observation that greater quantities of DNA target were recovered using the load-first method may be due to nonspecific interactions between the alkyl chains of the DTO and lipophilic components of the cell lysate that compete with the hydrophobic MIL. Figure 3 also shows a similar trend for the monosubstituted $[\text{AOIM}^+][\text{Br}^-]$ -ITO, albeit less pronounced due to the ITO possessing one fewer C₈ alkyl group.

The detection of cell-free nucleic acids in human plasma has diagnostic and prognostic potential for a variety of cancers.²⁸ Although it is an appealing alternative to conventional tissue biopsies, plasma samples contain proteins and globulins that inhibit the direct PCR amplification of DNA targets.³⁰ The extraction performance of the DTO-MIL method was investigated in human plasma samples spiked with 6 fmol of a 120 bp fragment from a known oncogene, KRAS, as the DNA target. The MIL and DTO-based method was also compared to an approach using commercially available streptavidin-coated magnetic beads and biotinylated oligonucleotides (20-mer) complementary to the KRAS fragment. Prior to extraction, samples were diluted 10-fold in order to minimize the nonspecific extraction due to matrix interferences. As shown in Figure 4, the diluted plasma sample

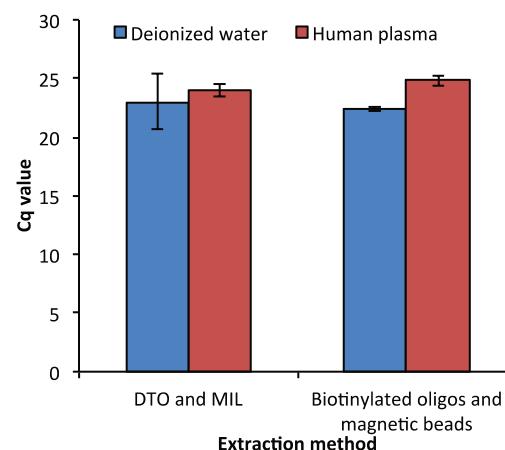


Figure 4. Comparison of sequence-specific DNA capture methods from aqueous solution and human plasma samples. A 120 bp fragment of human KRAS gene was extracted using either commercially available magnetic beads and biotinylated oligo probes or the DTO-MIL method and subjected to qPCR amplification.

resulted in diminished extraction efficiency for the DTO-MIL method compared to an aqueous DNA solution, likely due to competing interactions between matrix components and the MIL. A similar trend was observed for the magnetic bead-based approach, which exhibited little to no difference in Cq values (24.82 ± 0.45) when compared to the DTO-MIL method (23.97 ± 0.60) for the extraction of KRAS fragment from plasma.

CONCLUSIONS

The analysis of specific DNA sequences invariably requires a selective sample preparation procedure that eliminates or dramatically reduces the quantity of untargeted biomolecules from the sample. The recognition of target DNA using ITOs coupled with MIL-based capture represents a powerful

approach for the enrichment of specific DNA sequences. In order to capitalize on the selectivity and high DNA target yields afforded by the ITO-MIL method, we investigated a series of ITO probes with various hydrophobic and fluorophilic functional groups in the cation and anion moieties of the ion tag. The best performing probe possessed two alkyl groups (C_8) that facilitated dense loading of the DTO onto the MIL support. When applied for the capture of specific DNA sequences from aqueous solution, the high affinity of the DTO for the MIL support allowed for more stringent wash conditions than a monosubstituted ITO ($[AOIM^+][Br^-]$ -ITO). Furthermore, the DTO-MIL system was capable of extracting target DNA from solutions containing single nucleotide variants with higher DNA yields than a commercial magnetic bead-based approach. The DTO-based method also selectively extracted DNA from crude bacterial cell lysate, demonstrating the tolerance of the sample preparation method toward interferences in complex samples.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.analchem.9b00350](https://doi.org/10.1021/acs.analchem.9b00350).

Synthesis of magnetic ionic liquids and imidazolium-based ion tags; LC-TOFMS characterization of ITOs/DTO; melt curve conditions; magnetic bead-based DNA extraction; figures of chemical structures, mass spectra, melt curves, and ion chromatogram; and table of single- and double-stranded DNA loading efficiencies (PDF)

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Notes

The authors declare no competing financial interest.

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