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Magnetic ionic liquids: interactions with bacterial cells, behavior in aqueous suspension, and broader applications

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Abstract

Previously, we demonstrated capture and concentration of Salmonella enterica subspecies enterica ser. Typhimurium using magnetic ionic liquids (MILs), followed by rapid isothermal detection of captured cells via recombinase polymerase amplification (RPA). Here, we report work intended to explore the broader potential of MILs as novel pre-analytical capture reagents in food safety and related applications. Specifically, we evaluated the capacity of the ([P66614+][Ni(hfacac)3−]) ("Ni(II)") MIL to bind a wider range of human pathogens using a panel of Salmonella and Escherichia coli O157:H7 isolates, including a "deep rough" strain of S. Minnesota. We extended this exploration further to include other members of the family Enterobacteriaceae of food safety and clinical or agricultural significance. Both the Ni(II) MIL and the ([P66614+][Dy(hfacac)4−]) ("Dy(III)") MIL were evaluated for their effects on cell viability and structure-function relationships behind observed antimicrobial activities of the Dy(III) MIL were determined. Next, we used flow imaging microscopy (FIM) of Ni(II) MIL dispersions made in model liquid media to examine the impact of increasing ionic complexity on MIL droplet properties as a first step towards understanding the impact of suspension medium properties on MIL dispersion behavior. Finally, we used FIM to examine interactions between the Ni(II) MIL and Serratia marcescens, providing insights into how the MIL may act to capture and concentrate Gram-negative bacteria in aqueous samples, including food suspensions. Together, our results provide further characterization of bacteria-MIL interactions and support the broader utility of the Ni(II) MIL as a cell-friendly capture reagent for sample preparation prior to cultural or molecular analyses.

Keywords

Magnetic ionic liquids, Cell capture, Cell concentration, Gram-negative bacteria, Enterobacteriaceae, Flow imaging microscopy

Disciplines

Food Chemistry | Food Science | Human and Clinical Nutrition | Medical Biochemistry | Molecular, Cellular, and Tissue Engineering | Molecular, Genetic, and Biochemical Nutrition | Other Life Sciences

Comments

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Magnetic Ionic Liquids: Interactions with Bacterial Cells, Behavior in Aqueous Suspension and Broader Applications

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Abstract

Previously, we demonstrated capture and concentration of *Salmonella enterica* subspecies *enterica* ser. Typhimurium using magnetic ionic liquids (MILs), followed by rapid isothermal detection of captured cells *via* recombinase polymerase amplification (RPA). Here, we report work intended to explore the broader potential of MILs as novel pre-analytical capture reagents in food safety and related applications. Specifically, we evaluated the capacity of the ($[P_{66614}^+][Ni(hfacac)_3^-]$) ("Ni(II)") MIL to bind a wider range of human pathogens using a panel of *Salmonella* and *Escherichia coli* O157:H7 isolates, including a "deep rough" strain of *S.* Minnesota. We extended this exploration further to include other members of the family *Enterobacteriaceae* of food safety, clinical or agricultural significance. Both the Ni(II) MIL and the ($[P_{66614}^+][Dy(hfacac)_4^-]$) ("Dy(III)") MIL were evaluated for their effects on cell viability and structure-function relationships behind observed antimicrobial activities of the Dy(III) MIL were determined. Next, we used flow imaging microscopy (FIM) of Ni(II) MIL dispersions made in model liquid media to examine the impact of increasing ionic complexity on MIL droplet properties as a first step towards understanding the impact of suspension medium properties on MIL dispersion behavior. Finally, we used FIM to examine interactions between the Ni(II) MIL and *Serratia marcescens*, providing insights into how the MIL may act to capture and concentrate Gram-negative bacteria in aqueous samples, including food suspensions. Together, our results provide further characterization of bacteria-MIL interactions and support the broader utility of the Ni(II) MIL as a cell-friendly capture reagent for sample preparation prior to cultural or molecular analyses.

Keywords: Magnetic ionic liquids; cell capture; cell concentration; Gram-negative bacteria; *Enterobacteriaceae*; Flow Imaging Microscopy

Introduction

The family *Enterobacteriaceae* is a related grouping of Gram-negative, facultatively anaerobic rod-shaped bacteria. The family contains several genera of importance to agriculture, food safety and human health, including *Cronobacter*, *Enterobacter*, *Erwinia*, *Escherichia*, *Klebsiella*, *Pantoea*, *Pectobacterium*, *Salmonella*, *Serratia*, *Shigella* and *Yersinia* (1). From a food safety perspective alone, four of these groups or species - nontyphoidal *Salmonella* spp., *Escherichia coli* O157:H7, *Shigella* spp. and *Yersinia enterocolitica* are estimated to be responsible for a combined 1,319,624 foodborne illnesses each year in the US, resulting in 23,463 hospitalizations and 437 deaths (2) (Electronic Supplementary Material Table S1). Apart from the human suffering caused, foodborne disease also has very real economic impacts stemming from lost wages, lost productivity and the costs of hospitalization, product recalls and litigation. The estimated economic burden of foodborne disease caused each year by *Salmonella* spp. in the US alone is $3.4 billion; for *E. coli* O157:H7 it is $271 million (3). These figures highlight the need for detection of enterobacterial pathogens such as *Salmonella* and *E. coli* O157:H7 in foods, and by extension, detection of agricultural pests and clinically important members belonging to this family in crop and soil matrices or in clinical samples across the farm-to-fork-to-physician continuum (4, 5).

Because problematic bacteria may be present at low levels in foods or other samples, environmental, food and clinical microbiologists rely heavily on the use of growth-based enrichment steps prior to downstream analysis. Selective media for enrichment and identification of *Enterobacteriaceae* exist, primarily for their detection in foods as indicators of proper hygienic practices or in nonsterile pharmaceutical products for ensuring compliance with quality or

regulatory standards (6). Unfortunately, growth-based enrichment of bacteria is extremely time-consuming and therefore represents a major hurdle to moving quickly from sample to answer (4).

Approaches for growth-independent, physical enrichment of bacteria include "brute force" methods such as centrifugation and filtration, but these often result in co-extraction of matrix-associated debris, which can interfere with downstream detection assays. To circumvent this, magnetic separation technologies can be used for rapid preconcentration and isolation of bacteria. For example, functionalized magnetic particles can be added to complex samples and allowed to bind to bacteria *via* electrostatic interactions, glycan binding, antigen-antibody pairing or other capture modalities of varying specificities. Subsequent application of a magnetic field allows manipulation of particle-bound bacteria, enabling their concentration and separation from matrix-associated contaminants (4, 7, 8). Although this approach is selective, drawbacks include the high cost of particle functionalization and issues such as particle aggregation or limited physical access to microniches occupied by target bacteria (8).

Magnetic ionic liquids (MILs) are magnetoactive solvents comprised of organic/inorganic cation and anion pairs. A paramagnetic component is integrated into either the cation or anion moiety, conferring susceptibility to magnetic fields (9, 10). MILs may also have the additional advantages of being nonvolatile and nonflammable, with tunable physicochemical properties. The hydrophobic and fluid nature of MILs allows for their distribution throughout aqueous samples as liquid microdispersions, which facilitates interactions with and capture of biochemical or cellular analytes. Due to these novel properties, MILs are emerging as a powerful and versatile reagent platform for extraction of a wide variety of bioanalytes, including hormones, nucleic acids and viable bacterial cells (11-13). In bacterial applications, MILs have enabled preconcentration of viable, nonpathogenic *E. coli* from fluid milk, followed by downstream detection *via*

microbiological culture or quantitative polymerase chain reaction (qPCR) (9). More recently, MILs have been paired with recombinase polymerase amplification (RPA) for the rapid preconcentration and detection of *Salmonella* Typhimurium from 2% milk, almond milk and liquid egg product (8). However, despite the success to date of MILs in microbiological applications, little is known about MIL-bacterial interactions and the physicochemical principles responsible for capture and concentration of viable cells. In order to further the development of MILs as whole-cell sample preparation agents, a greater understanding is needed of how they behave in aqueous suspensions both alone and in the presence of bacteria, whether or not they possess intrinsic antibacterial activities that might limit their use in applications where maintaining cell viability is critical, and how broadly they may be applied for capture of bacteria of concern across the farm-to-fork-to-physician continuum.

In this study, we evaluated the capacity of the Ni(II) MIL to capture a broad range of Gram-negative bacteria, including *Salmonella* serovars, *E. coli* O157:H7 and other representatives of the family *Enterobacteriaceae* of importance to global agriculture, food safety and human health. We also plated MIL-exposed cells in parallel onto selective and non-selective agars to explore whether or not MIL-based capture causes cellular injury in either *Salmonella* or *E. coli* O157:H7. We determined extent of and the chemical and structural bases of observed antibacterial activity of the Dy(III) MIL, and we directly compared the antimicrobial activities of the Ni(II) MIL and ($[EMIM^+][SCN^-]$), a non-magnetic IL used previously by others for extraction of *Salmonella* and other pathogens from foods (14). Finally, we used Flow Imaging Microscopy (FIM) to capture data on the physical properties of aqueous Ni(II) MIL suspensions under different ionic conditions and to characterize the behavior of the Ni(II) MIL in the presence of the pigmented enterobacterial strain *S. marcescens*. This study underscores the promise of the Ni(II) MIL as an emerging sample

preparation reagent by providing a more complete picture of its utility for capture of various enterobacterial pathogens, demonstrating its generally non-injurious nature and offering new insight into potential mechanisms behind its ability to physically enrich bacteria from complex samples.

Materials and Methods

Reagents and Magnetic Ionic Liquid Preparation: Chemical structures of the two MIL solvents examined in this study are shown in Fig. 1a. Synthesis and characterization of the MILs was performed as previously described (15). A brief description of MIL synthesis is also included in the accompanying Electronic Supplementary Material. MIL solvents were purified by liquid-liquid extraction with acetonitrile/hexane and dried *in vacuo*. Prior to all experiments, MILs were stored in a desiccator for at least 24 h.

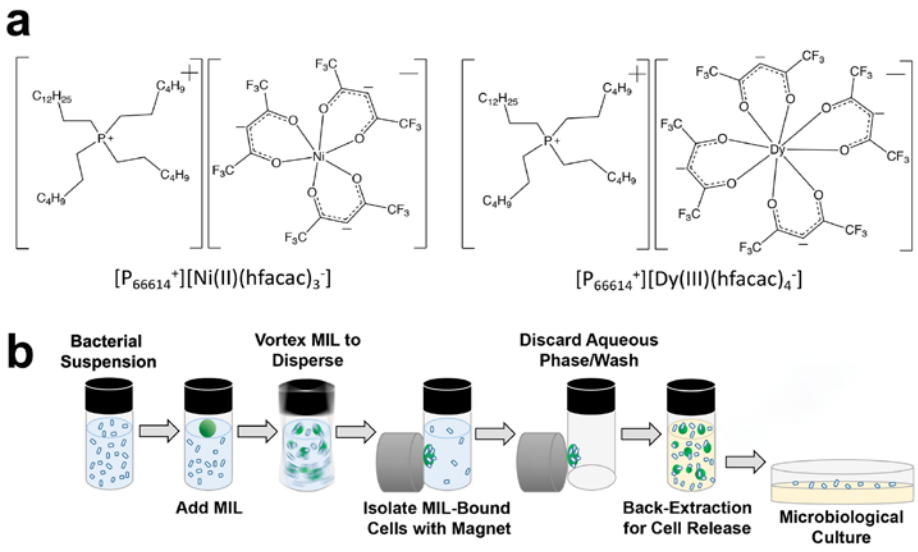


Figure 1 (a) Structures of the MILs used in this study. (b) Schematic for the capture, concentration and recovery of enterobacteria from aqueous samples, followed by downstream analysis by microbial culture using non-selective and/or selective media. Figure 1b adapted from Hice et al., 2019.

Bacteria and Culture Conditions: All of the bacterial strains used in this study belong to the family *Enterobacteriaceae*, and are listed in Table 1. All growth media were from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). Cultures of *S. marcescens* were grown as previously described (8). *Salmonella* and *E. coli* were grown 24 h in 10 mL volumes of Tryptic Soy Broth (TSB) at 37 °C. *K. aerogenes* and *C. sakazakii* were grown in 5 mL volumes of TSB at 30 °C and *Y. enterocolitica* strains were grown at 37 °C, with shaking (190 rpm) on a Shel Lab Shaking incubator (Sheldon Manufacturing, Inc., Cornelius, OR, USA). Plant pathogens were grown in 10 mL volumes of Columbia Broth (CB) at 28 °C with shaking at 190 rpm. Depending on the experiment, bacteria were enumerated using Tryptic Soy Agar (TSA), Columbia Agar (CA), Bismuth Sulfite Agar (BSA) or Violet Red Bile Glucose Agar (VRBGA) plates as described below under "Plating and Enumeration".

Table 1 Enterobacterial strains used in this study

Organism	**Source**
Cronobacter sakazakii 01088P (derived from ATCC 29544)	Microbiologics[a]
Erwinia amylovora Ea935	ISU PP[b]
Escherichia coli O157:H7 N886-71	OHA[c]
Escherichia coli O157:H7 N366-2-2	OHA
Escherichia coli O157:H7 N549-3-1	OHA
Escherichia coli O157:H7 N317-3-1	OHA
Escherichia coli O157:H7 N192-5-1	OHA
Escherichia coli O157:H7 N192-6-1	OHA
Escherichia coli O157:H7 N336-4-1	OHA
Escherichia coli O157:H7 N405-5-8	OHA
Klebsiella aerogenes ATCC 29940	ISU PP
Pantoea eucalypti 299R (formerly *Pantoea agglomerans* 299R)	ISU PP
Pantoea stewartii Rif9A	ISU PP
Pectobacterium carotovorum pv. *carotovorum*	ISU PP
Salmonella bongori SA4410	SGSC[d]
Salmonella enterica subsp. *arizonae* SA4407	SGSC
Salmonella enterica subsp. *diarizonae* SA4408	SGSC
Salmonella enterica subsp. *enterica* ser. Minnesota SLH 157	SLH[e]
Salmonella enterica subsp. *enterica* ser. Minnesota mR613	SGSC
Salmonella enterica subsp. *enterica* ser. Typhimurium ATCC 14028	ATCC[f]
Salmonella enterica subsp. *houtenae* SA4409	SGSC

Salmonella enterica subsp. *indica* SA4411	SGSC
Salmonella enterica subsp. *salamae* SA4406	SGSC
Serratia marcescens	CBS[g]
Yersinia enterocolitica subsp. *enterocolitica* ATCC 9160	ATCC[f]
Yersinia enterocolitica subsp. *enterocolitica* ATCC 23715	ATCC

[a]Microbiologics (St. Cloud, MN); ISU PP, [b]Iowa State University Plant Pathology; [c]OHA, Oregon Health Authority, Public Health Division (Portland, OR, USA); [d]SGSC, *Salmonella* Genetic Stock Centre (Calgary, Alberta, Canada); [e]SLH, Wisconsin State Laboratory of Hygiene (Madison, WI, USA); [f]ATCC, American Type Culture Collection (Manassas, VA, USA); [g]CBS, Carolina Biological Supply (Burlington, NC)

MIL-Based Whole-Cell Extraction: A universal schematic for MIL-based cell extraction is depicted in Fig. 1b. A 1 mL volume of diluted cell suspension was added to a flat-bottomed 4-mL screw cap glass vial. Fifteen microliters of either the Ni(II) or Dy(III) MIL was added and dispersed into microdroplets by vortex agitation for 30 s (9). The aqueous phase was decanted following dispersive extraction, and the MIL was subjected to a brief wash step using 1 mL of nuclease-free water (Integrated DNA Technologies, Coralville, IA, USA) to ensure adequate removal of loosely or incidentally bound cells (9). After washing, release of viable cells from the MIL extraction phase was carried out using a "back-extraction" step accomplished through addition of 1 mL of an ionically-rich nutritive medium comprised of 20 g L^{-1} tryptone and 10 g L^{-1} NaCl, followed by a 120 s vortex step (8). After back-extraction, aliquots of the cell-enriched back-extraction medium were enumerated using the track dilution method described by Jett et al. (16) using 100 x 100 mm square, gridded TSA, BSA, CA or VRBGA plates.

Plating and Enumeration: Following back-extraction, aliquots of the cell-enriched back-extraction medium were serially diluted in 0.1% peptone water and a 10 µL aliquot of each dilution was applied to a separate lane on square plates containing an appropriate agar medium. The plates were then tilted at an ~80° angle for 15 min to allow the droplets to travel toward the opposite end of the plate (16). The plates were incubated for 24 h at 37 °C (TSA, VRBGA), for 48 h at 37 °C

(BSA) or for 24 h at 37 °C (CA). Colonies were counted and colony forming units (CFU) were determined. The enrichment factor (E_F) for MIL-based extraction was calculated using Equation 1, where C_{MIL} represents the concentration of bacteria in suspension following extraction using the MIL and C_S is the initial concentration of bacteria in the sample.

$$E_F = \frac{C_{MIL}}{C_S} \quad \text{(Equation 1)}$$

Exposure to Ni(II) MIL as a Function of Time and Evaluation of [EMIM$^+$][SCN$^-$] IL Toxicity: To examine whether exposure time to the Ni(II) MIL affected cell viability, MIL-based whole-cell extraction was performed using the Ni(II) MIL, and cell-enriched back-extraction media were enumerated at 0, 5, 10 and 15 min on both TSA and BSA. To evaluate toxicity of the [EMIM$^+$][SCN$^-$] IL, one milliliter of diluted *S.* Typhimurium ATCC 14028 cell suspension was added to a 4-mL screw cap glass vial. A 5% (vol/vol) or 50% (vol/vol) aqueous solution of 1-ethyl-3-methylimidazolium thiocyanate ("[EMIM$^+$][SCN$^-$]") (IoLiTec, Tuscaloosa, AL, USA) was added and mixed by vortexing for 30 s (14). Aliquots were enumerated at 0, 5, 10 and 15 min using TSA and BSA.

Comparison of Air-Displacement and Positive-Displacement Pipettes for MIL Handling: A 1 mL volume of diluted *S.* Typhimurium ATCC 14028 cell suspension was added to a 4-mL screw cap glass vial. Fifteen microliters of the Ni(II) MIL was added using either a Pipetman Classic P20 air-displacement pipette (Gilson, Middleton, WI, USA), or a Microman E M25E positive-displacement pipette (Gilson) and dispersed into microdroplets by vortex agitation for 30 s. MIL-based extraction and enumeration was carried out as previously described.

Exposure to Dy(III) MIL, $DyCl_3$ and ([NH_4^+][Dy(hfacac)$_4^-$]): One milliliter of diluted *S.* Typhimurium ATCC 14028 cell suspension was added to a 4-mL screw cap glass vial. Fifteen

microliters of either the Dy(III) MIL or 2-10 µL of 100 mM $DyCl_3$ solution or 10 mg of the ammonium tetra(hexafluoroaceto)dysprosium salt (15) ($[NH_4^+][Dy(hfacac)_4^-]$) ("Dy(III) ammonium salt") was added and dispersed by vortex agitation for 30 s. Aliquots of the Dy(III) MIL- or Dy(III) ammonium salt-exposed cell suspension were enumerated using square TSA or BSA plates (BD).

Impact of Ionic Environment on Ni(II) MIL Dispersion Properties and Bacteria-MIL Interactions by Flow Imaging Microscopy: For analysis of MIL dispersion properties as a function of ionic environment, microdroplet suspensions of the Ni(II) MIL were generated as above for whole-cell extraction of bacteria and analyzed with a FlowCam 8000 instrument (Fluid Imaging Technologies, Inc., Scarborough, ME). Briefly, three peptone water (PW) formulations representing multiples of the manufacturer's basal formulation for this medium were added to 4-mL screw cap glass vials. Fifteen microliters of the Ni(II) MIL were added to each PW formulation and the mixture dispersed with vortexing for 30 s for microdroplet formation. Samples were analyzed immediately using the FlowCam instrument using the 10x objective (100 µm field of view). Samples (20 µL) were taken from the top portion of each tube with a ~25 s collection time. PW formulations evaluated were 1x (0.5% NaCl/1% peptone), 5x (2.5% NaCl/5% peptone) and 10x (5% NaCl/10% peptone). Data were analyzed with VisualSpreadsheet® software (v. 5.0, Fluid Imaging Technologies, Inc.). A basic size filter of 5 µm (minimum droplet size) to 10,000 µm (maximum droplet size) was applied, and droplet distributions were plotted as volume (%) vs. equivalent spherical diameter (ESD). Key measurements tabulated for each ionic condition include mean droplet diameter (ESD), maximum droplet size, D_{50} (median droplet size; 50% are smaller and 50% are larger than this value), D_{90} (90% of droplets are smaller than this value) and number of droplets mL^{-1}. The same settings were used for analysis of bacteria-MIL interactions, with high

concentrations (~10^7 – 10^8 CFU mL^{-1}) of *S. marcescens* suspended in 1x PW prior to addition of the MIL and sampling.

Statistical analysis: The following statistical analysis was performed in SAS 9.4. Four master suspensions of bacteria were prepared for four *Salmonella enterica* serovars: subsp. Typhimurium, subsp. *arizonae*, subsp. *diarizonae* and subsp. *houtenae*. Two master suspensions were made for the remaining three *Salmonella* serovars or species: *S. enterica* subsp. *indica*, subsp. *salamae* and *S. bongori*. For *E. coli* O157:H7, two master suspensions of bacteria were prepared for each of the eight strains studied. For all bacteria tested, two replicate extractions from the same master suspension were performed, using the 10^{-3} dilution. Following MIL-based capture, back-extraction solutions were plated to non-selective agar (TSA) and selective agar (BSA) for *Salmonella* and SMAC for *E. coli*. To identify whether significant differences existed for the capture of *Salmonella* and *E. coli* O157:H7, we applied a linear mixed model for the response variable (enrichment factor) with log transformation in order to reduce skewness. We treat both the strain and the medium as fixed effects and the suspension of bacteria as the random block.

In order to compare potential injury caused by the MIL across genera (*Salmonella* serovars and STEC *Escherichia*), a medium suitable for growth of both organisms (VRBGA) was used in parallel with TSA and resulting counts were compared in experimental settings repeated across five different days. In each experiment, three *Salmonella* serovars were tested: *S.* Typhimurium ATCC 14028, *S.* Minnesota SLH 157, and deep rough mutant *S.* Minnesota mR613. Two *E. coli* strains were also tested: *E. coli* O157:H7 N192-6-1 and *E. coli* O157:H7 N192-5-1. We applied the linear mixed model for the log-transformed enrichment factors with fixed strain and agar effects and random blocks of the experiment days as samples from a population of days. Additional

statistical analyses regarding precision measurements and CFU counts vs. time for the MIL capture experiments depicted in Fig. 2 are also provided in the accompanying Electronic Supplementary Material, including Electronic Supplementary Material Table S2.

Results

Exposure to Ni(II) MIL as a Function of Time and Evaluation of [EMIM$^+$][SCN$^-$] IL Toxicity: Because culture-based methods depend on sample preparation steps that preserve bacterial viability, potential deleterious cytotoxic effects imparted by MIL extractants must be considered. To begin our further study of MIL-bacterial interactions, we selected *S.* Typhimurium ATCC 14028 as a model Gram-negative pathogen and the Ni(II) MIL as extractant, as most of our work to date has used this combination (8). Briefly, a 1 mL aliquot of TSB was inoculated with $1x10^5$ CFU mL^{-1} of bacteria and spiked with 15 µL of the Ni(II) MIL, and the general schematic for MIL-based extraction and recovery followed as shown in Fig. 1b. After extraction, 10 µL aliquots of the back-extraction solution were enumerated at 0, 5, 10 and 15 min using TSA and BSA. Average CFU counts were compared to a standard that was not exposed to the Ni(II) MIL. As shown in Fig. 2, average CFU counts for *S.* Typhimurium exposed to the Ni(II)-based MIL appeared to be similar on both TSA and BSA for exposure periods ranging from 0 to 15 min. To better understand the data in Fig. 2, statistical analyses of Ni(II) MIL capture precision and CFU count variation as a function of MIL exposure time were performed and are provided in the Electronic Supplementary Material. Variation in MIL capture may stem from inherent randomness of MIL dispersion and coalescence behaviors.

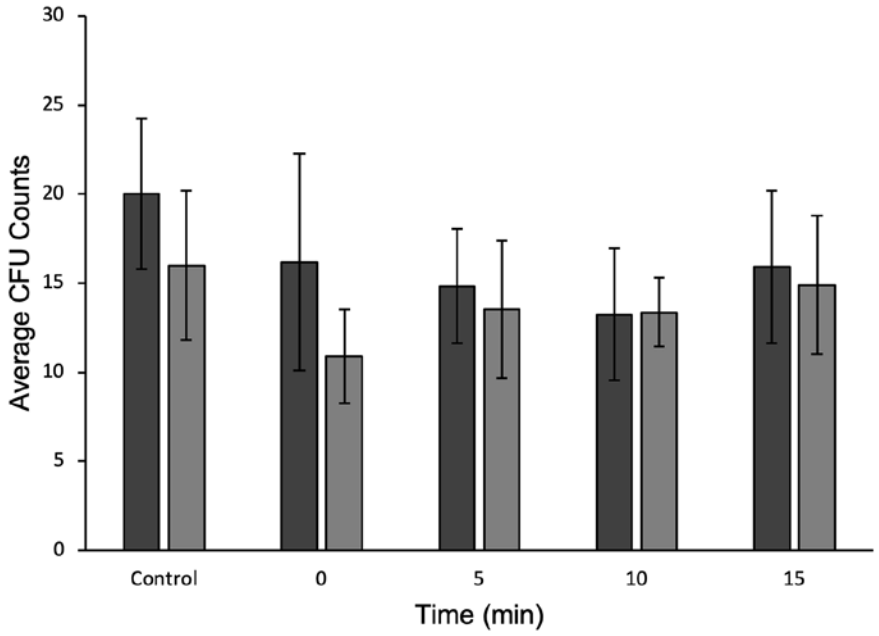


Figure 2 Recovery of *Salmonella* Typhimurium extracted with Ni(II) MIL as a function of time (min). Average colony forming unit (CFU) counts recovered from the aqueous Ni(II) MIL back-extraction phase over time (0 - 15 min). A suspension of *S.* Typhimurium ATCC 14028 was prepared and captured with the Ni(II) MIL as described in the text, back-extracted using modified LB broth containing 20 g L^{-1} tryptone and 10 g L^{-1} NaCl, then plated on TSA (dark bars) and BSA (light bars). Also shown are results for a standard not treated with the MIL (Control). Statistical analyses of these data (precision, variation over time) are provided in Electronic Supplementary Materials.

Enrichment factors were calculated as a function of time and are reported in Table 2. For TSA, the resulting average E_F value was 7.2 ± 0.6 (n=4); for BSA, the average E_F value was 8.2 ± 1.0 (n=4).

Table 2 Enrichment factors for *S.* Typhimurium as a function of exposure time to the Ni(II) MIL

Time (min)	Enrichment Factor (TSA)	Enrichment Factor (BSA)
0	8	7
5	7	8
10	6	8
15	8	9

The effects of 5% or 50% (vol/vol) aqueous solutions of the [$EMIM^+$][SCN^-] IL on the viability of the cells were also evaluated (14). Average CFU counts were compared to a standard not exposed to [$EMIM^+$][SCN^-]. In our hands, when *S.* Typhimurium was exposed to 50% (vol/vol) solutions of [$EMIM^+$][SCN^-], no recovery was observed on either TSA or BSA after 5 min of exposure.

Evaluation of Capture and Recovery of Wild Type and Mutant *Salmonella* Minnesota Strains Using the Ni(II) MIL: To further evaluate the importance of the OM to serve as a surface to which the MIL can bind and as a protective layer from potentially deleterious activities of the Ni(II) MIL, capture and recovery of two physiological variants of *S.* Minnesota was performed. The strains compared were *S.* Minnesota SLH 157 (wild type, functional OM) and *S.* Minnesota mR613 (OM mutant). *S.* Minnesota mR613 is considered a "deep rough" mutant, possessing a truncated OM core. Compared to the wild type, cells with a truncated OM are dramatically more susceptible to damage from antimicrobial agents or chemically harsh environments (17). To study the effect of the Ni(II) MIL exposure on the recovery of wild type and mutant strains of *S.* Minnesota, a 1 mL aliquot of TSB was inoculated with $1x10^5$ CFU mL^{-1} of bacteria and spiked with 15 µL of the Ni(II) MIL. MIL-based extraction was performed and 10 µL aliquots of the back-extraction solution were enumerated using TSA and BSA plates. Average CFU counts were compared to a standard that was not exposed to the Ni(II) MIL. Extraction and recovery of *S.* Typhimurium was also assessed. Enrichment factors were calculated and are reported in Table 3.

Table 3 Enrichment factors for *S*. Typhimurium, *S*. Minnesota SLH 157 (wild type) and *S*. Minnesota mR613 (mutant)

Strain	Enrichment Factor (TSA)	Enrichment Factor (BSA)
S. Typhimurium	10	14
S. Minnesota SLH 157	17	11
S. Minnesota mR613	4	*

* For both the standard and the MIL-treated cells, no growth was observed on BSA using the *S*. Minnesota mR613 "deep rough" mutant strain.

While the extraction efficiency using the Ni(II) MIL was greatly reduced for *S*. Minnesota mR613, capture and recovery of viable cells was observed on TSA. As expected, no growth of mR613 was seen on BSA due to the inherent susceptibility of this OM mutant to selective agents. Likewise, growth of the *S*. Minnesota mR613 standard was observed on TSA but not on BSA.

Capture and Recovery of Seven Representative DNA Subgroups of *Salmonella* and Eight Strains of *E. coli* O157:H7: Previous work has demonstrated successful capture of *E. coli* K12, *Serratia marcescens* and *Salmonella* Typhimurium using MILs (8, 9). In order to explore the broader utility of our approach, evaluation of additional cell types is needed. We began this extended evaluation of the Ni(II) MIL with seven representative DNA subgroups of *Salmonella* and eight strains of *E. coli* O157:H7. Briefly, a 1 mL aliquot of TSB was inoculated with $1x10^6$ CFU mL^{-1} of bacteria, and spiked with 15 µL of the Ni(II) MIL. MIL-based extraction was performed and 10 µL aliquots of the back-extraction solution were enumerated using TSA and BSA plates (*Salmonella*) or TSA plates (*E. coli* O157:H7). Initially, we examined MacConkey Agar with Sorbitol (SMAC) as a common selective medium for parallel evaluation of injury in *Salmonella* and *E. coli* O157:H7, but *Salmonella* did not grow well on this medium (data not shown). We later determined that VRBGA was a suitable common medium for this purpose and used this in our statistical analysis. Average CFU counts were compared to standards that were not

exposed to the Ni(II) MIL. Enrichment factors were calculated for *Salmonella* on both non-selective (TSA) and selective agars (BSA) and for *E. coli* O157:H7 on non-selective agar (TSA), as reported in Table 4.

Table 4 Enrichment factors for seven representative DNA subgroups of *Salmonella* and eight strains of *E. coli* O157:H7

Strain	Enrichment Factor (TSA)	Enrichment Factor (BSA)
S. enterica subsp. *salamae*	12	9
S. enterica subsp. *diarizonae*	7	11
S. enterica subsp. *houtenae*	8	8
S. Typhimurium	8	6
S. bongori	4	4
S. enterica subsp. *arizonae*	3	2
S. enterica subsp. *indica*	3	4
E. coli O157:H7 N192-6-1	9	-
E. coli O157:H7 N549-3-1	8	-
E. coli O157:H7 N192-5-1	7	-
E. coli O157:H7 N886-71	4	-
E. coli O157:H7 N366-2-2	4	-
E. coli O157:H7 N317-3-1	4	-
E. coli O157:H7 N336-4-1	3	-
E. coli O157:H7 N405-5-8	2	-

Comparison of the Initial Wash and Full MIL Extraction Procedure on the Recovery of *S.* Typhimurium: Although all of the strains assessed were capable of being enriched by the MIL, some species were physically enriched to greater extents than others with extraction. In order to further examine the cause for this finding, the number of captured cells lost to the wash solution was investigated. The wash step is performed after the MIL enrichment step in order to remove any incidentally-adsorbed bacteria prior to back-extraction. It is hypothesized that the cells with lower enrichment factors have lower affinities for the MIL and are therefore lost in greater number to the wash solution than those having higher enrichment factors. To test this, five different

Salmonella strains exhibiting varying degrees of enrichment in initial experiments were examined. The percent-loss during the wash step ranged from 47-79%. These data, along with inferred relative affinities for the MIL are shown in Table 5. E_F data from Table 4 for three overlapping *Salmonella* strains are superimposed to highlight trends in percent-loss, relative affinity and E_F. The data for *S*. Minnesota mR613 (79% cell loss to wash) support the conclusion that the lower E_F values observed for this strain result from lower affinity to the MIL, rather than from antimicrobial effects.

Table 5 Percent-loss of cells to wash, relative affinity for MIL and E_F of select *Salmonella* spp.

Strain	Percent-Loss[a]	Relative Affinity for MIL[b]	E_F TSA/BSA[c]
S. enterica subsp. *diarizonae*	47±7	+++++	7/11
S. Typhimurium	53±3	++++	8/6
S. Minnesota SLH 157	59±3	+++	-
S. Minnesota mR613	69±5	++	-
S. enterica subsp. *arizonae*	79±1	+	3/4

[a] Percent-loss was calculated by dividing the counts obtained from the wash solution by the sum of the counts of the wash and back-extraction solution, multiplied by 100. [b] Relative affinity (RA) for MIL assumes higher losses during wash step are due to lower cellular affinity for the MIL. [c] E_F data from Table 4 for both TSA and BSA are provided here to show parity in RA- E_F trends for select *Salmonella* spp.

Capacity of the Ni(II) MIL for Capture of other Members of the Family *Enterobacteriaceae* of Food Safety, Clinical or Agricultural Significance: Our results show that all of the additional *Enterobacteriaceae* examined here could be concentrated from aqueous suspension using the Ni(II) MIL. These bacteria are ranked (Table 6) in descending order according to E_F, with *P. eucalypti*, *K. aerogenes* and *P. carotovorum* pv. *carotovorum* yielding much higher E_F than seen with other bacteria tested, either here or in our previous work (8, 9).

Table 6 Enrichment factors for other enterobacterial strains

Strain	Enrichment Factor
P. eucalypti 299R[a]	169
K. aerogenes ATCC 29940[b]	71
P. carotovorum pv. *carotovorum*[a]	24
C. sakazakii 01088P[b]	12
E. amylovora Ea935[a]	10
Y. *enterocolitica* ATCC 2371[b]	5
Y. *enterocolitica* ATCC 9160[b]	5

[a] E_F determined using Columbia Agar. [b] E_F determined using Tryptic Soy Agar.

Investigating Mechanisms for Observed Antimicrobial Activities of the Dy(III) MIL: Incorporation of a rare-earth metal into the MIL structure is of significant interest as these metals possess greater magnetic moments. In principle, this should allow for improved magnetic manipulation compared to transition metal-based MILs. However, when the Dy(III) MIL was previously examined for the capture of bacteria, recovery of viable cells was not observed (9). To further explore these results and to determine if they result from intrinsic antimicrobial activity of this MIL, bacterial suspensions of *S.* Typhimurium were exposed to various structural components of the MIL.

Within 30 s of vortexing a cell suspension to which 15 µL of the Dy(III) MIL was added, we observed extensive flocculation and no growth after plating, suggesting that this MIL may have intrinsic antimicrobial activity, resulting in rapid cell lysis.

To gain further insight into this phenomenon, the effect of free elemental dysprosium was evaluated by subjecting the cells to 0.1 mM and 1.0 mM solutions of $DyCl_3$. The cells were exposed to the metal salt solutions for 30 s and subsequently plated on selective and non-selective media. Results shown in Fig. 3 demonstrate that growth can be observed on both types of plates.

However, substantially lower counts were observed on BSA than TSA, particularly at the higher $DyCl_3$ concentration.

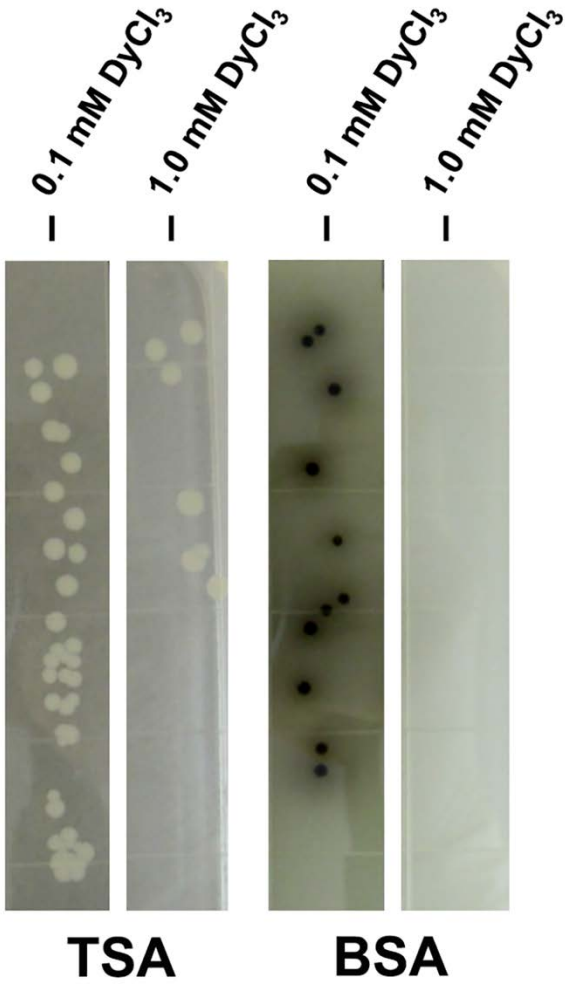
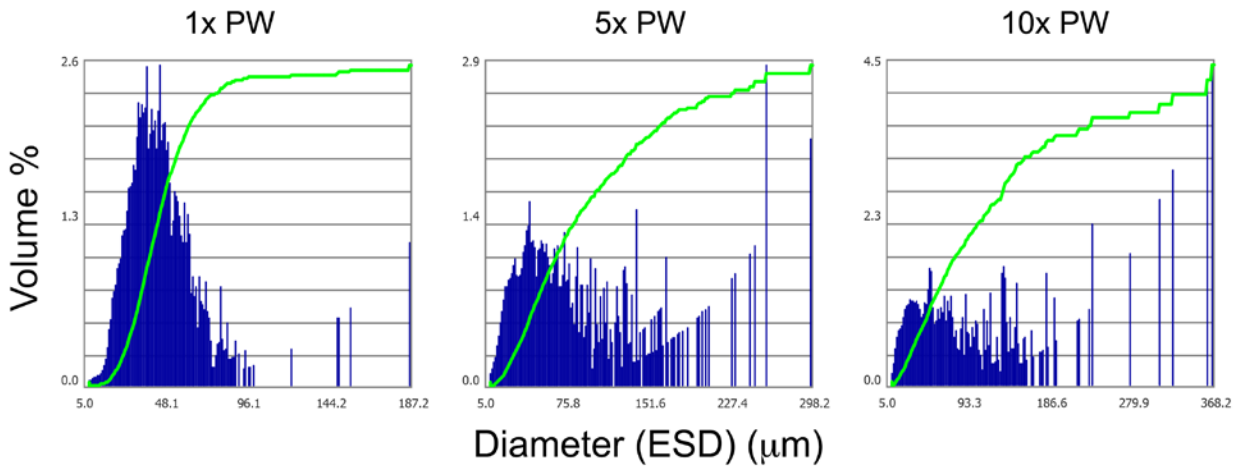


Figure 3 Recovery of *Salmonella* Typhimurium following 30 s exposure to 0.1 mM and 1.0 mM $DyCl_3$. Viable CFU counts recovered on non-selective TSA (left) and selective BSA (right) from the $DyCl_3$-exposed cells following a 30 s treatment.

Comparison of Positive- and Air-Displacement Pipettes for MIL Delivery: We found that handling and delivery of the Ni(II) MIL was challenging when using traditional air-displacement (AD) pipettes, due to both MIL viscosity and incomplete delivery of aspirated MIL. We therefore

sought to compare AD pipetting with piston-driven positive-displacement (PD) pipetting and the impact, if any, on bacterial extraction results. Briefly, 15 µL of MIL was delivered from each pipette and weighed using an analytical balance (n=3). The PD pipette was able to deliver a substantially greater mass of MIL (17.1±0.6 mg) compared to the AD pipette (11.3±0.4 mg), with similar reproducibility (Electronic Supplementary Material Fig. S1). To test whether or not this difference impacted extraction performance, extractions were performed, using each pipette to dispense 15 µL of MIL. The calculated E_F did not differ according to the pipetting method used, despite the disparity in the amount pipetted (data not shown).

Characterization of MIL Dispersions Under Differing Ionic Conditions Using Flow Imaging Microscopy: As summed in Figure 4 below, FIM measurements of Ni(II) MIL dispersions in aqueous samples of increasing ionic complexity (1x, 5x and 10x PW) showed that as solute concentration increased, droplet size decreased. As would be expected, these trends towards smaller particle sizes were directly correlated to increasing numbers of total particles per unit volume (particles mL^{-1}). An additional trend towards larger outlier droplets (overall droplet heterogeneity) was seen as ionic complexity increased.



Treatment	Mean Diameter (ESD)	Max Droplet Size	D50	D90	Droplets mL^{-1}
1x PW	22.44 µm	187.19 µm	20.29 µm	41.45 µm	392,896
5x PW	16.69 µm	298.18 µm	12.08 µm	32.76 µm	1,338,275
10x PW	14.41 µm	368.20 µm	10.46 µm	26.53 µm	1,370,130

Figure 4 Visualization of MIL-bacteria interactions *via* Flow Imaging Microscopy (FIM). Key metrics from FIM analyses of Ni(II) MIL suspension characteristics under three different concentrations of peptone water (PW) are shown. VisualSpreadsheet® software (v. 5.0, Fluid Imaging Technologies) was used for to analyze sample data. A trend toward smaller droplet size and greater extremes of maximum droplet size was seen as the ionic complexity of the medium increased.

***Serratia marcescens*-Induced Aggregation of Ni(II) MIL Droplets - Implications for MIL-Mediated Bacterial Concentration:** In general, cell-free suspensions of Ni(II) MILs under various conditions of ionic complexity yielded distributions of individual or minimally-aggregated droplets (Electronic Supplementary Material Fig. S2). In some cases, apparent surface granularity of MIL droplets suggested a MIL coalescence mechanism similar to oil-in-water emulsions through aggregation and merging of smaller particles. In the presence of *S. marcescens* cells, however, we observed a radically different presentation of MIL droplets (Fig. 5), with the

formation of multi-droplet aggregates and chains under the "food-like" conditions of 1x PW (0.5% NaCl, 1% peptone), which may suggest a physical mechanism for MIL-based concentration of bacteria.

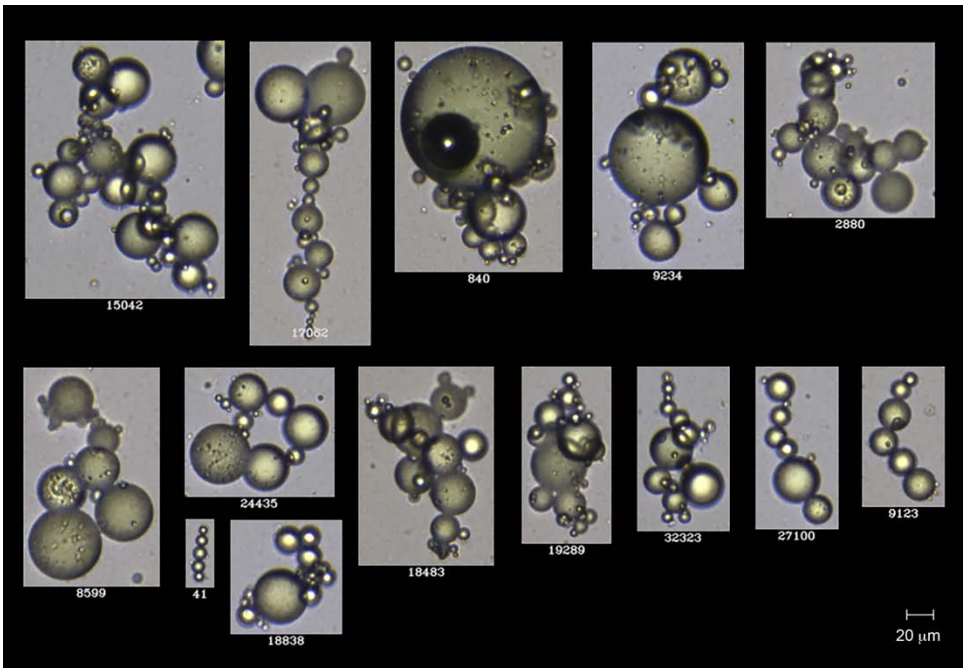


Figure 5 *Serratia marcescens*-Induced Aggregation of Ni(II) MIL Droplets. The presence of *S. marcescens* resulted in a dramatic shift in the presentation of Ni(II) MIL droplets in 1x peptone water (PW). In the presence of *S. marcescens*, MIL droplets formed large and complex aggregates, which may suggest a physical mechanism for MIL-based concentration of bacteria.

Discussion

Exposure to Ni(II) MIL as a Function of Time and Evaluation of [EMIM$^+$][SCN$^-$] IL Toxicity: Cell injury can be detected by plating MIL-treated cells in parallel on both non-selective and selective media and evaluating growth under each condition (18). Gram-negative cells possess an outer membrane (OM), which protects them against the diffusion of otherwise toxic molecules into the cell. As a result, physiologically intact Gram-negative cells are able to tolerate exposure to toxic agents such as bile salts, crystal violet or brilliant green used in selective agars designed

to limit the growth of Gram-positive cells. Injury to Gram-negative cells caused by exposure to deleterious physical or chemical conditions is typically characterized by damage to the OM, which causes these cells to become "leaky" and show impaired growth on selective agars. Injury can therefore be detected by plating treated cells in parallel on both non-selective and selective agars and comparing the results (18). When we exposed suspensions of *S.* Typhimurium to the Ni(II) MIL for times ranging from 0 min (essentially our standard 30 s extraction protocol) to 15 min, the resulting average CFU counts on TSA and BSA appeared similar, regardless of exposure time. We explored these data further using the statistical analyses reported in the Electronic Supplementary Material. For TSA, no statistically significant differences were shown in the CFU counts over time. For cases that were plated to BSA, we did find significant evidence that the CFU counts at time 5 min, 10 min, and 15 min are statistically greater compared to time 0 counts. We hypothesize that if the Ni(II) MIL was chemically injurious to *S.* Typhimurium, counts on BSA would be lower than those observed on TSA, and further, that BSA counts would continue to fall as a function of exposure time. Although we found that CFU counts on BSA were statistically different from counts at time zero, the observation that counts at 5, 10 and 15 min were greater than those at time zero support our conclusion that the Ni(II) MIL did not cause detectable injury to *S.* Typhimurium with this media pairing, even after exposure periods thirty times longer than our standard 30 s extraction time.

The $[EMIM^+][SCN^-]$ IL has been previously investigated for solubilization of protein-rich food matrices as a means for sample preparation (14). The $[EMIM^+]$ cation potentially acts as a detergent, while the $[SCN^-]$ anion is chaotropic. The ability to essentially obliterate difficult food matrices with this IL, then collect released bacterial cells for analysis represents a novel advance in sample preparation. However, these authors have reported that $[EMIM^+][SCN^-]$ is injurious to

Salmonella Typhimurium in this application, with only 34 – 45% of inoculated *S.* Typhimurium recovered after IL-mediated matrix lysis when plated to a selective agar (14). In our hands, when *S.* Typhimurium was exposed to [$EMIM^+$][SCN^-] as originally described for IL-based extraction of this pathogen from foods (14), no recovery was observed on either TSA or BSA, confirming the injurious nature of this IL in stark contrast to and in direct comparison with our "cell-friendly" Ni(II) MIL.

It is possible that a major contributor to the innocuous behavior of our Ni(II) MIL with Gram-negative bacteria stems from the inherent capacity of the OM to exclude hydrophobic compounds – which the MIL clearly is. We therefore sought to further investigate the role of the OM 1) as a key cell structure of potential importance in mediating MIL-based binding and capture of cells and 2) in protecting Gram-negative cells against potential MIL toxicity, as discussed further below.

Evaluation of Capture and Recovery of Wild Type and Mutant *Salmonella* Minnesota Strains Using the Ni(II) MIL: Our ability to capture this mutant strain demonstrates two key points: 1) the Ni(II) MIL has the capacity to capture and concentrate a strain of *S.* Minnesota that displays a drastically different external surface than wild type cells, and 2) the post-capture growth behavior of this physiologically sensitive strain suggests that the Ni(II) MIL capture process is not overtly antimicrobial. Apparent absence of a toxic impact for the Ni(II) MIL on the "deep rough" mutant *S.* Minnesota strain may result from a lack of intrinsic chemical toxicity, from low diffusivity of the hydrophobic MIL across whatever remaining barrier is offered by this strain's truncated OM, the tendency of the insoluble, hydrophobic MIL to quickly sequester itself into large, non-diffusible aggregate structures in aqueous media, or any combination of these potential phenomena. These data suggest that Gram-negative cell surface molecular diversity and character

are likely important factors mediating successful cell binding and capture by MILs. We expect that other enterobacterial OM mutants, and possibly mucoid strains (see further discussion below) may also vary in their capacity for capture with the Ni(II) MIL.

Capture and Recovery of Seven Representative DNA Subgroups of *Salmonella* and Eight Strains of *E. coli* O157:H7: Our results for capture of the various strains of *Salmonella* and *E. coli* O157:H7 (Table 4) show that all strains of both pathogens could be captured to some degree, when plated onto TSA. *Salmonella* strains representing the seven DNA subgroups belonging to this genus also showed very similar results when plated to BSA (Table 4), suggesting a lack of MIL-imparted injury. To delve beyond superficial visual interpretation of the data, we evaluated a subset of the strains thus far examined and applied statistical analyses to determine 1) whether capture of bacteria varied significantly as a function of strain and 2) if selective agars used revealed the presence of MIL-conferred injury. Our analysis confirmed significant serotype or strain effects for capture of *Salmonella* (p-value < 0.0001) and *E. coli* O157:H7 (p-value = 0.0721). Regarding MIL-conferred injury, no significant differences (p-value = 0.4491) were seen between TSA and VRBGA for recovery of *E. coli* O157:H7, indicating that for this agar pairing, no injury could be detected. Interestingly, while no statistically significant differences (p-value = 0.7248) were seen for *Salmonella* serovars on TSA or BSA (i.e. no detectable injury), recovery of *Salmonella* on VRBGA was significantly lower than on TSA (p-value = 0.0004). These results suggest that the choice of selective agar is important for both revealing the presence of injury and for informing practical application of MIL-based capture for cultural detection, especially if selective agars are to be used. Comparing the two selective agars, VRBGA contains two selective agents – crystal violet and bile salts, while the sole selective agent in BSA is brilliant green dye. Bile salts are generally agreed to be membrane-active amphiphilic “detergents”, and Gram-negative bacteria

with an intact OM can exclude crystal violet from the cell, avoiding its deleterious effects. It is possible that the barrier function of the OM is altered in some way through its interaction with the MIL during capture, that VRBGA's bile salts and crystal violet act cooperatively on the impaired OM and that *Salmonella* is more susceptible to these effects than *E. coli* O157:H7. It is important to note that BSA is a robustly selective agar. The fact that MIL-exposed *Salmonella* serovars were not impaired for growth on BSA indicates that MIL-based capture can be paired with selective plating onto this medium without interference from the capture process.

Comparison of the Initial Wash and Full MIL Extraction Procedure on the Recovery of *S.* Typhimurium: This experiment provides evidence that differences in E_F values for the various bacteria tested could be due to intrinsic differences in affinity for the MIL extraction phase. If this is true, bacteria with lower affinities for the MIL may be weakly bound, and are therefore easily removed by the wash step compared to the strains with higher observed enrichment factors.

Capacity of the Ni(II) MIL for Capture of other Members of the Family *Enterobacteriaceae* of Food Safety, Clinical or Agricultural Significance: Our work with additional enterobacterial strains of concern clearly highlights the broader utility of the Ni(II) MIL for capture and concentration of these economically important bacteria. Of particular interest was the extremely high E_F result seen for *P. eucalypti* 299R (formerly *P. agglomerans* 299R), which yielded an E_F of 169 - approximately 20x greater than that for many of the other enterobacteria examined in this study. The reason for this result is not yet known, although this strain was visually more pigmented than other enterobacteria tested, suggesting a possible connection between carotenoid content and higher binding. Another potential reason for this result may be the formation of "symplasmata" by this strain. Symplasmata are multicellular aggregates (hence this strain's previous epithet "*agglomerans*", meaning "forming into a ball") that confer competitive advantages to this

bacterium. Symplasmata are comprised of many (potentially hundreds of) clonal cells bound within a thick polysaccharide envelope and are known to form in laboratory media, as well as on plant surfaces (19). It is reasonable to suggest that our remarkable E_F results for *P. eucalypti* 299R could be due to the presence of symplasmata in our culture of this organism – a possibility that we plan to investigate in the future. If this is the case, it would demonstrate the exciting potential of our MIL-based approach to capture and concentrate unique multicellular structures of importance to plant health in additional to individual bacterial cells.

The broad applicability of MIL-based capture and concentration to enterobacteria occurring across the production-to-consumption continuum underlines the potential value of this approach to rapid detection methods aimed at mitigating human disease and preventing crop loss. A brief overview of the significance of the bacteria included in this study to agriculture, food safety and human health is provided below. Foodborne and clinically-important enterobacteria include *Cronobacter sakazakii*, which is problematic in powdered infant formula, causing neonatal infections with mortality as high as 40% (1). Pathogenic *Escherichia coli* can be divided into several important groups based on pathology, with *E. coli* O157:H7 and five other Shiga toxin-forming *E. coli* (STEC) forming the "Big Six" – bacteria regarded by regulatory agencies as "zero tolerance" food adulterants. In 2018 there were two multistate outbreaks of *E. coli* O157:H7 in Romaine lettuce; at the end of 2019, another such outbreak and recall of Romaine lettuce from the Salinas Valley occurred, affecting 167 people in 27 states (20). *Salmonella* spp. represent one of the most pervasive bacterial threats to the food system, in terms of the estimated number of infections (Electronic Supplementary Material Table S1) and breadth of foods affected. The genus *Yersinia* includes *Y. pestis* – the cause of the plague (the "Black Death") and *Y. enterocolitica*, which is transmitted through undercooked pork infections (Electronic Supplementary Material

Table S1). On the clinical side, carbapenem-resistant *Enterobacteriaceae*, which includes some *Klebsiella* strains, has been prioritized as an "urgent threat" by the Centers for Disease Control and Prevention (CDC), meaning that urgent and aggressive action is required to counter this threat to public health (21). Enterobacterial plant pathogens include *Erwinia amylovora*, the cause of fire blight, which can decimate entire apple or pear orchards. *Pantoea* spp. cause infections in both humans and plants and, like *Cronobacter*, have been isolated from powdered infant formula (1). *P. eucalypti* is an epiphyte on many plants and causes disease in others, including pea, sweet corn and wheat, while *Pantoea stewartii* causes wilt in corn and seed rot in cotton, among others (22). *Pectobacterium carotovorum* pv. *carotovorum* is a ubiquitously distributed pathogen causing bacterial soft rot in various plants and blackleg disease in potato (1).

The family *Enterobacteriaceae* is a large group of genetically- and physiologically-related Gram-negative bacteria existing in a wide variety of niches of importance to and overlapping with human activities. Although there is considerable diversity within the family, these bacteria share several structural and biochemical features that are of importance in defining their surface characteristics, and therefore, their potential to interact with MILs. These are discussed further below.

As Gram-negative bacteria, the *Enterobacteriaceae* all possess a lipopolysaccharide outer membrane (OM) comprised of a lipid element, a conserved oligosaccharide core and a highly variable polysaccharide sequence, termed the "O-antigen" (23-25). The OM serves as barrier to the diffusion of toxic compounds such as antibiotics, and the O-antigen plays roles in avoiding phagocytosis and protecting cells against complement-mediated cell lysis (24). There are two notable types of O-antigen variants in *Salmonella* and other enterobacteria – "rough" and "mucoid". Rough mutants have a truncated LPS and do not possess an O-antigen; mucoid variants

have an O-antigen, but it is obscured by a capsule that obscures it from immunologic detection. The surface antigens displayed by the *Salmonella* strains representative of the seven DNA subgroups that comprise this genus are shown in Electronic Supplementary Material Table S3. This table highlights the considerable surface molecular diversity of the *Salmonella* strains used in this study. Despite this molecular diversity and its expected impact on diversity of cell surface charge, all of these salmonellae could be captured by the Ni(II) MIL, with 4 of the 7 strains displaying E_F on par with what we have previously observed for *S.* Typhimurium and nonpathogenic *E. coli* (8). The lower E_F values for *S. bongori*, *S. arizonae* and *S. indica* may be explored in future studies, using whole-cell ς-potential measurements to assess differences in cell surface charge.

Another feature common to the family, as suggested by the name, is the Enterobacterial Common Antigen (ECA), a polysaccharide repeat structure located in the cell envelope that is linked to maintenance of OM integrity and represents a useful target for detection of enterobacterial strains (23, 24). Additional cell surface structures that contribute to the molecular and charge diversity of enterobacteria include porins - transmembrane transport proteins, which also act as receptors for bacteriophage, fimbriae (also referred to as adhesins or pili) - stiff, hair-like appendages uniformly distributed across the cell surface and that mediate bacterial binding to host cells, and flagella. Flagella (H-antigen) are whip-like structures that confer cell motility and whose number and surface arrangement may vary according to cell type (1).

Investigating Mechanisms for Observed Antimicrobial Activities of the Dy(III) MIL: The partially inhibitory effects of $DyCl_3$ indicates that the coordinated metal itself may be partially responsible for the observed deleterious effects of the MIL. While the metal did show cytotoxicity, it cannot be completely responsible for the Dy(III) MIL's effect on the cells, as no flocculation

was seen and growth was still observed. Since both the Ni(II) and the Dy(III) MILs contain identical cations ($[P_{6,6,6,14}^{+}]$), the role of the anion structure was evaluated. The anion of the Dy(III) MIL contains one additional hexafluoroacetylacetonate ligand than the Ni(II) MIL (Fig. 1), making the coordination geometry of the two complexes different. To test the effects of the anion structure, cells were subjected to 10 mg of the Dy(III) ammonium salt. After 30 s vortex, similar flocculation was observed as when the cells were exposed to the native Dy(III) MIL. After plating and 24 h incubation, no growth was seen on either TSA or BSA. These results, combined with those from $DyCl_3$ exposure experiments, provide strong evidence that the anion structure is largely responsible for the antimicrobial effects of this MIL. Ongoing work is focused on the design and synthesis of a non-toxic Dy(III)-based MIL whose strong paramagnetism can be exploited.

Comparison of Positive- and Air-Displacement Pipettes for MIL Delivery: Although the PD pipette delivered substantially more of the target 15 μL volume of Ni(II) MIL, enrichment factors from bacterial extractions were not affected by this ~6 μL difference added to bacterial suspensions (data not shown). These results suggest that, for the number of bacteria present in standardized suspensions, the amount of MIL used is above the carrying capacity of the MIL (the amount at which it is saturated and cannot bind additional bacteria). Apart from the performance equivalence of the two methods, PD pipetting was faster, easier and did not result in loss of MIL due to adherence to the pipette tip, which could be economically advantageous, especially for high-throughput applications.

Characterization of MIL Dispersions Under Differing Ionic Conditions Using Flow Imaging Microscopy: As noted above in Results, as the ionic complexity of PW solutions increased, droplet size decreased, with trends toward smaller droplet size resulting in a commensurate trend towards higher droplet count (droplets mL^{-1}). The effect was not completely uniform, however, as

overall sample heterogeneity, as characterized by increasing outlier particle size, increased along with increasing solute concentration. It is not clear how applicable the principles of traditional oil-in-water emulsion chemistry are to our consideration of MIL behavior in aqueous suspension, as the MILs, while hydrophobic, are not technically "oils". Further, because they are comprised of ion pairs, they are chemically inhomogeneous. As such, the term "emulsion", which also implies an inherently stable structure, can only be loosely applied to their behavior when they are mechanically dispersed in aqueous media.

Of importance to their application as sample preparation reagents, mechanically dispersed MILs form a short-lived "cloud" of particles capable of interacting with different charged species within aqueous matrices such as foods, including solutes and suspended particles, followed by eventual density-based coalescence at the bottom of the sample vial. Because they are paramagnetic, their separation from the aqueous phase, along with any bound species, can be hastened in the presence of a magnet. Our results with differing concentrations of PW suggest that interactions with and partitioning of charged solutes, such as NaCl and the peptidic and amino acid components of this medium into the MIL phase affect droplet size, and subsequently, the number of droplets mL^{-1}.

These interactions of the MIL with sample components may have important impacts on the efficacy of the MIL as a bacterial capture reagent. On one hand, a trend towards smaller droplets and greater overall numbers of droplets per unit volume is expected to favor more efficient collision with colloidal particles such as bacteria. However, we also hypothesize that higher sample ionic complexity may compete with bacterial binding, if such binding is governed solely by electrostatic binding effects. The concentrations of PW used here include levels of NaCl beyond which we would expect the Ni(II) MIL to be effective as a bacterial capture reagent in foods. For

example, we previously used a medium containing only 1% NaCl for desorption of captured bacteria from the MIL phase during our "back extraction" procedure (8). To contextualize these salt levels in terms of model food systems, we used nutritional content panel information from store-bought chicken broth, a perceivably salty food, to calculate its NaCl content as ~0.2% - well below the 1% we used for MIL-desorption. Still, the use of PW-based model matrix formulations yielding NaCl concentrations ranging from 0.5% (food-like) to 5% provided new insights into MIL dispersion/medium composition trends that may inform applications of MILs in various sample types, including non-food samples.

It is not clear what the trend towards larger outlier droplets in the presence of higher ionic concentration indicates, as this could result from poorer initial MIL dispersion or from faster coalescence. In separate work, we have used nonionic polyoxyethylene detergents such as Brij 700 to modify dispersion characteristics of MILs. This approach may be useful for ensuring greater droplet homogeneity, although the impacts of these detergents on extraction characteristics of MILs is unknown. Presumably, because they are nonionic in nature and our working hypothesis is that charge-based interactions are important in governing the partitioning of bacterial surface structures into MILs, they would not interfere with extraction behavior. However, we are also exploring other potential modes through which MILs might interact with bacteria, such as hydrophobic interactions. If these also play a role, the hydrophobic aliphatic chains of such detergents could partition into the MIL and affect extraction behavior. In the absence of such information, purely physical approaches for promoting droplet homogeneity, which could help reduce inter-experimental variability, such as the use of conical or baffled vials for sample dispersion may also be valuable, as they would not result in or depend on chemical modification

of the MIL. It is also worth noting that the intrinsic chemical properties of the aqueous food matrix itself may pose limitations to application of MILs - an additional topic for further exploration.

***Serratia marcescens*-Induced Aggregation of Ni(II) MIL Droplets - Implications for MIL-Mediated Bacterial Concentration:** The dramatically different presentation of MIL particles in the presence of *S. marcescens* was an unexpected result. Although the FlowCam magnification used (10x) was not sufficient for visualizing individual cells, we interpret the formation of large aggregates and chains in the presence of *S. marcescens* as the result of cell-mediated "bridging" of individual droplets. We expect that, although *S. marcescens* produces a hydrophobic pigment (prodigiosin) and is known to avidly partition into hydrocarbons such as *n*-hexadecane (26), as a bacterium, its native environment is largely aqueous and partitioning inside of MIL droplets would not be energetically favorable. We hypothesize that cells of *S. marcescens* are able to bind to and partition into MIL droplet surfaces, leaving one side of the organism in contact with the MIL and the other side exposed to the environment. Subsequent collision with and binding to suspended MIL particles creates a MIL-bacterium-MIL "sandwich" (Electronic Supplementary Material Fig. S3), which, when repeated, results in the formation of the multi-droplet aggregates and chains we observed. These observations may provide a general model for MIL-bacteria interactions, but further work is needed to explore this phenomenon. First, a very high load of *S. marcescens* (~10^7 – 10^8 CFU mL^{-1}) was used here. Additionally, we do not know if other modes of binding, such as hydrophobic interactions, also play a role in MIL-base capture of bacteria. Future FIM-based analysis of MIL-bacteria dynamics using other *Enterobacteriaceae* at various cell concentrations may shed additional light on the microscale interactions governing MIL-based capture of Gram-negative bacteria.

Conclusions

In this work, we explored the further utility of the Ni(II) MIL as a novel, cell-friendly reagent for preanalytical sample preparation. We evaluated the extraction performance and/or physiological effects of this MIL on nine *Salmonella* serotypes, eight *E. coli* O157:H7 strains and nine additional members of the family *Enterobacteriaceae* of importance to agriculture, food safety or human health. We used differential plating on non-selective and selective agars, various MIL exposure times and an O-antigen-deficient *S*. Minnesota mutant to demonstrate that the Ni(II) MIL possesses no overt antimicrobial activities against bacteria tested. These results highlight the potential suitability of the Ni(II) MIL for integration into detection schemes involving the use of selective media screens. Additionally, we determined the key factors behind the observed antimicrobial effects of the Dy(III) MIL, knowledge of which may help design new, less toxic transition metal-based MILs having improved magnetic properties. Finally, we used Flow Imaging Microscopy (FIM) to observe the dispersion behavior of the Ni(II) MIL under various ionic conditions and to demonstrate that MIL coalescence and aggregation behaviors are dramatically different in the presence of *S. marcescens*. These later observations may suggest a general mechanism governing the capture and concentration of Gram-negative bacteria from aqueous samples. This study provides the foundation for further investigation into the capture and recovery of other notable foodborne pathogens, including viruses, and establishes MILs as a viable platform for rapid preconcentration and extraction of Gram-negative bacteria for potential use in environmental, food and clinical applications.

Author Contributions

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Conflict of Interest
The authors disclose no conflicts of interest related to this work.

References

1. Octavia S, Lan R. The Family *Enterobacteriaceae*. In: Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F, editors. The Prokaryotes: Gammaproteobacteria. Fourth Edition ed. Berlin: Spring-Verlag; 2014. p. 225-86.
2. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, et al. Foodborne illness acquired in the United States--major pathogens. Emerg Infect Dis. 2011;17(1):7-15.
3. Hoffman S, Maculloch B, Batz M. Economic burden of major foodborne illnesses acquired in the United States. *Current Politics and Economics of the*

United States, Canada and Mexico. 2015;17(4).
4. Brehm-Stecher B, Young C, Jaykus LA, Tortorello ML. Sample preparation: the forgotten beginning. J Food Prot. 2009;72(8):1774-89.
5. Bisha B, Brehm-Stecher BF. Simple adhesive-tape-based sampling of tomato surfaces combined with rapid fluorescence in situ hybridization for *Salmonella* detection. Appl Environ Microbiol. 2009;75(5):1450-5.
6. Weber C, Stephan R, Druggan P, Joosten H, Iversen C. Improving the enrichment procedure for *Enterobacteriaceae* detection. Food Microbiol. 2009;26(6):565-72.
7. Soo HS, Brehm-Stecher, B.F, Jaykus, L.A. Advances in Separation and Concentration of Microorganisms from Food Samples. In: Sofos J, editor. Cambridge, UK: Woodhead Publishing; 2013. p. 173-92.
8. Hice SA, Clark KD, Anderson JL, Brehm-Stecher BF. Capture, Concentration, and Detection of *Salmonella* in Foods Using Magnetic Ionic Liquids and Recombinase Polymerase Amplification. Anal Chem. 2019;91(1):1113-20.
9. Clark KD, Purslow JA, Pierson SA, Nacham O, Anderson JL. Rapid preconcentration of viable bacteria using magnetic ionic liquids for PCR amplification and culture-based diagnostics. Anal Bioanal Chem. 2017;409(21):4983-91.
10. Clark KD, Nacham O, Purslow JA, Pierson SA, Anderson JL. Magnetic ionic liquids in analytical chemistry: A review. Analytica Chimica Acta. 2016;934:9-21.
11. Clark KD, Varona M, Anderson JL. Ion-Tagged Oligonucleotides Coupled with a Magnetic Liquid Support for the Sequence-Specific Capture of DNA. Angew Chem Int Ed Engl. 2017;56(26):7630-3.

12. Ding X, Clark KD, Varona M, Emaus MN, Anderson JL. Magnetic ionic liquid-enhanced isothermal nucleic acid amplification and its application to rapid visual DNA analysis. Anal Chim Acta. 2019;1045:132-40.
13. Merib J, Spudeit DA, Corazza G, Carasek E, Anderson JL. Magnetic ionic liquids as versatile extraction phases for the rapid determination of estrogens in human urine by dispersive liquid-liquid microextraction coupled with high-performance liquid chromatography-diode array detection. Anal Bioanal Chem. 2018;410(19):4689-99.
14. Mester P, Wagner M, Rossmanith P. Use of ionic liquid-based extraction for recovery of *Salmonella* Typhimurium and *Listeria monocytogenes* from food matrices. J Food Prot. 2010;73(4):680-7.
15. Pierson SA, Nacham O, Clark KD, Nan H, Mudryk Y, Anderson JL. Synthesis and characterization of low viscosity hexafluoroacetylacetonate-based hydrophobic magnetic ionic liquids. New Journal of Chemistry. 2017;41(13):5498-505.
16. Jett BD, Hatter KL, Huycke MM, Gilmore MS. Simplified agar plate method for quantifying viable bacteria. Biotechniques. 1997;23(4):648-50.
17. Nikaido H. Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev. 2003;67(4):593-656.
18. Liao CH, Fett WF. Resuscitation of acid-injured *Salmonella* in enrichment broth, in apple juice and on the surfaces of fresh-cut cucumber and apple. Lett Appl Microbiol. 2005;41(6):487-92.
19. Tecon R, Leveau JH. Symplasmata are a clonal, conditional, and reversible type of bacterial multicellularity. Sci Rep. 2016;6:31914.
20. CDC. Outbreak of *E. coli* infections linked to Romaine lettuce 2019. Available from: https://www.cdc.gov/ecoli/2019/o157h7-11-19/index.html.
21. CDC. Antibiotic Resistance Threats in the United States, 2019. In: U.S. Department of Health and Human Services C, editor. Atlanta, GA2019.
22. Walterson AM, Stavrinides J. Pantoea: insights into a highly versatile and diverse genus within the *Enterobacteriaceae*. FEMS Microbiol Rev. 2015;39(6):968-84.
23. Mitchell AM, Srikumar T, Silhavy TJ. Cyclic Enterobacterial Common Antigen Maintains the Outer Membrane Permeability Barrier of Escherichia coli in a Manner Controlled by YhdP. MBio. 2018;9(4).
24. Kalynych S, Morona R, Cygler M. Progress in understanding the assembly process of bacterial O-antigen. FEMS Microbiol Rev. 2014;38(5):1048-65.
25. Grimont P, A.D., Weill F-X. Antigenic Formulae of the *Salmonella* Serovars. WHO Collaborating Center for Reference and Research on *Salmonella*, Institut Pasteur, Paris, France. 9th ed2007.
26. Rosenberg M. Microbial adhesion to hydrocarbons: twenty-five years of doing MATH. FEMS Microbiol Lett. 2006;262(2):129-34.

Analytical and Bioanalytical Chemistry

Electronic Supplementary Material

Magnetic Ionic Liquids: Interactions with Bacterial Cells, Behavior in Aqueous Suspension and Broader Applications

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Table S1 Estimated numbers of annual foodborne disease cases, hospitalizations and deaths occurring in the US attributable to four key enterobacterial genera. Data excerpted from Scallan et al., 2011

Organism	Foodborne cases	Hospitalizations	Deaths
Nontyphoidal *Salmonella*	1,027,561	19,336	378
Shigella spp.	131,254	1,456	10
STEC 0157	63,153	2,138	20
Y. enterocolitica	97,656	533	29
Total	**1,319,624**	**23,463**	**437**

Synthesis of MILs

The $[P_{6,6,6,14}^{+}][Ni(II)\ (hfacac)_3^{-}]$ $[P_{6,6,6,14}^{+}][Dy(III)\ (hfacac)_4^{-}]$ MILs were synthesized following a previously reported procedure.[1] Briefly, 10 mmol of hexafluoroacetyl acetone was added dropwise into a round bottom flask sealed with a rubber septum containing 10 mmol of ammonium hydroxide in 30 mL of ethanol. After the white vapor formed settled, 3.3 mmol of nickel (II) chloride or 2.5 mmol of dysprosium(III) chloride hexahydrate were added and stirred at room temperature for 5 h. Following evaporation under vacuum, the crude product was dissolved in diethyl ether, filtered, and washed several times with deionized water until no apparent precipitate was observed when a $AgNO_3$ test was performed. Subsequently, diethyl ether was evaporated and the purified salt dried overnight in a vacuum oven at 50 °C. The purified salt was dissolved in methanol and an equimolar amount of trihexyl(tetradecyl)phosphonium chloride was added and stirred overnight. The crude MIL product was dissolved in hexane and filtered. The product was subsequently washed with aliquots of water until no precipitate was observed during a $AgNO_3$ test. Hexane was then evaporated and the purified MIL was dried in a vacuum oven overnight at 50 °C.

1. Pierson, S.A.; Nacham, O.; Clark, K.D.; Nan, H.; Mudryk, Y.; Anderson, J.L. “Synthesis and Characterization of Low Viscosity Hexafluoroacetylacetonate-based Hydrophobic Magnetic Ionic Liquids,” *New Journal of Chemistry*, **2017**, *41*, 5498-5505. DOI: 10.1039/C7NJ00206H

Precision analysis for data reported in Fig. 2 and analysis of the recovery of *Salmonella* Typhimurium ATCC 14028 extracted with Ni(II) MIL as a function of the exposure time (0, 5, 10 and 15 min) when plated to both TSA and BSA.

Supplementary Table 2 below reports the Average CFU counts and the associated precision measurements, which include both the standard deviations (SD (TSA) and SD (BSA)) and the relative standard deviations (RSD (TSA) and RSD (BSA), compared to the mean levels). The sample sizes of cases that were plated to TSA are 11, 11, 9 and 10 for 0, 5, 10 and 15 min timepoints, respectively. The sample sizes of cases that were plated to BSA are 11, 11, 8 and 9 for timepoint 0, 5, 10 and 15 min, respectively. For each time phase, The CFU counts related to BSA have lower averaged values, as well as smaller standard deviations and relative standard deviation values compared to those of TSA. The one standard deviation away from the averaged value is displayed as the interval in Figure 2 (vertical lines).

In order to investigate whether the recovery of *S.* Typhimurium extracted with Ni(II) MIL varies over time, we performed a statistical analysis to compare the CFU counts recovered from the aqueous Ni(II) MIL back-extraction phase over time (5, 10 and 15 min) to the initial CFU counts at time 0. The nonparametric Wilcoxon test, which does not require the normality assumption, was used for our data with positive integer values. For cases that were plated to TSA, no statistically significant differences were shown in the CFU counts over time 5 min (11 samples), 10 min (9 samples) and 15 min (10 samples) compared to time 0 (11 samples). The respective p-values were 0.7842, 0.3570, 0.9035. For cases that were plated to BSA, there is significant evidence that the CFU counts at time 5 min (11 samples), 10 min (8 samples) and 15 min (9 samples) are statistically greater compared to time 0 (11 samples), yielding p-values of 0.0953, 0.0470 and 0.0254.

Table S2 Average CFU counts and precision measurements

Time (min)	Avg. (TSA)	Avg. (BSA)	SD (TSA)	SD(BSA)	RSD(TSA)	RSD(BSA)
Control	20.600	16.200	4.219	4.207	0.205	0.260
0	16.182	10.909	6.096	2.625	0.377	0.241
5	14.818	13.545	3.219	3.857	0.217	0.285
10	13.222	13.375	3.701	1.923	0.280	0.144
15	15.900	14.889	4.280	3.887	0.269	0.261

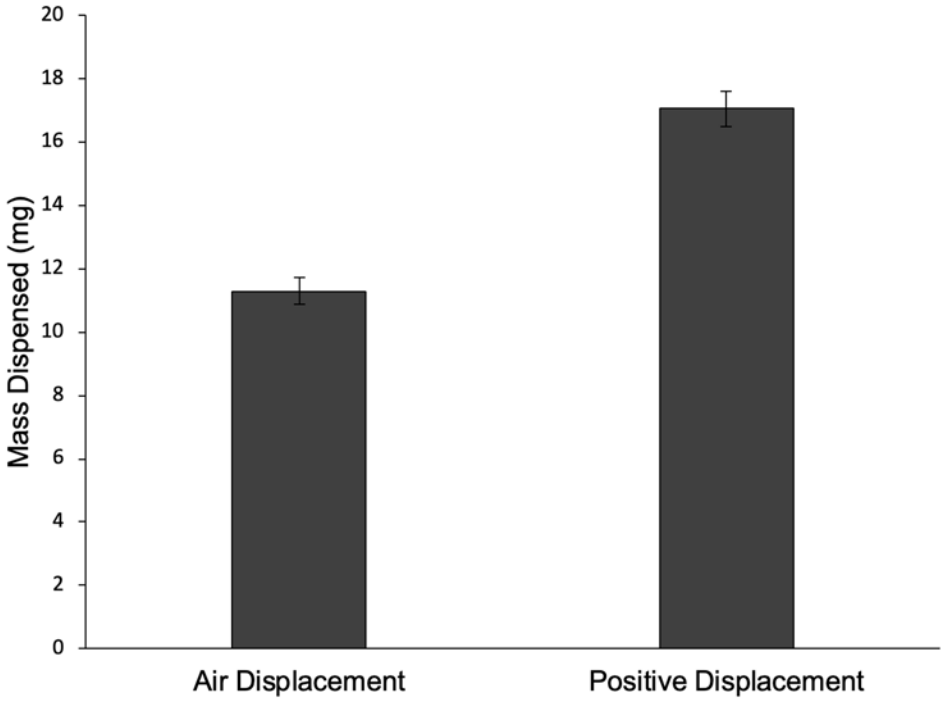

Figure S1 Evaluation of Ni(II) MIL delivery using air displacement (AD) and positive displacement (PD) pipettes. The average mass in mg of Ni(II) MIL dispensed using the AD and PD pipettes is reported. The PD pipette was able to deliver a substantially greater mass of Ni(II) MIL (17.1 ± 0.6 mg) compared to the AD pipette (11.3 ± 0.4 mg).

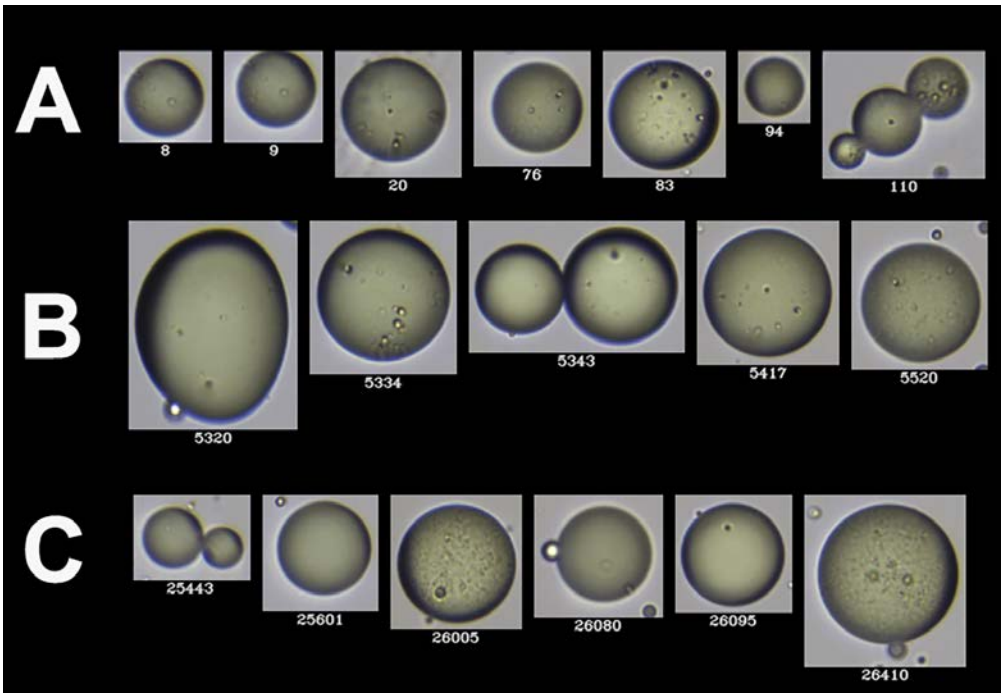

Figure S2 General appearance of Ni(II) MIL suspended in aqueous media of increasing ionic complexity, without added bacteria. A) 1x peptone water (PW); B) 5x PW; C) 10x PW. Apart from the size distribution data noted for each medium, these suspensions consisted generally of well-separated droplets having minimal interactions.

Table S3 Somatic and flagellar antigenic formulae of *Salmonella* spp. used in this study

Strain	DNA Subgroup	Somatic (O) Antigen	Flagellar (H) Antigen	
			Phase 1	Phase 2
S. Typhimurium ATCC 14028	I	4,5,12	i	1,2
S. salamae SA4406	II	1,9,12	1,w	e,n,x
S. arizonae SA4407	IIIa	51	z(4),z(23)	(-)
S. diarizonae SA4408	IIIb	6,7	1,v	z(53)
S. houtenae SA4409	IV	45	g,z(51)	(-)
S. indica SA4411	VI	1,6,14,25	a	e,n,x
S. bongori SA4410	V	66	z(41)	(-)

Key cell surface antigens – lipopolysaccharide-based somatic (O) and flagellar (H) antigens are listed according to the Kauffmann-White scheme (Grimont and Weill, 2007). These data highlight the antigenic diversity (surface biochemical complexity) of these representatives of the seven DNA subgroups comprising the genus *Salmonella*, which all, to some degree, were captured by the Ni(II) MIL. "Diphasic" *Salmonella* are capable of expressing both Phase 1 or Phase 2 flagellar antigens in coordinate, "either/or" fashion. "Monophasic" *Salmonella* – those that only express Phase 1 flagellar antigens – are indicated by "(-)".

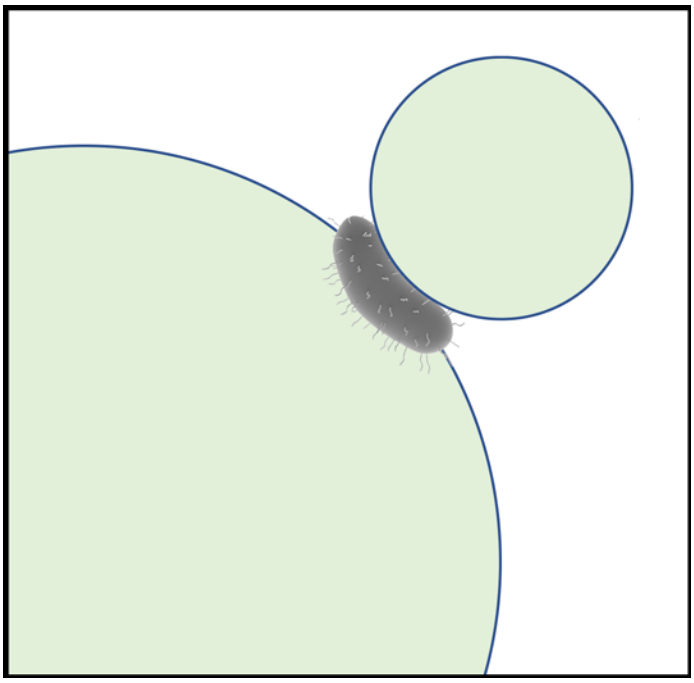

Figure S3 Hypothesized mechanism for cell-mediated MIL aggregation. Based on the radically different aggregation behavior of the Ni(II) MIL when *S. marcescens* is present, we hypothesize that the structures (chains and other aggregates) observed result from interfacial "sandwiching" effects, where bound bacterial cells serve to link and bridge adjacent MIL droplets, leading to cell-mediated aggregation and gravimetric deposition of MIL-cell complexes at the bottom of the sample tube after vortexing.