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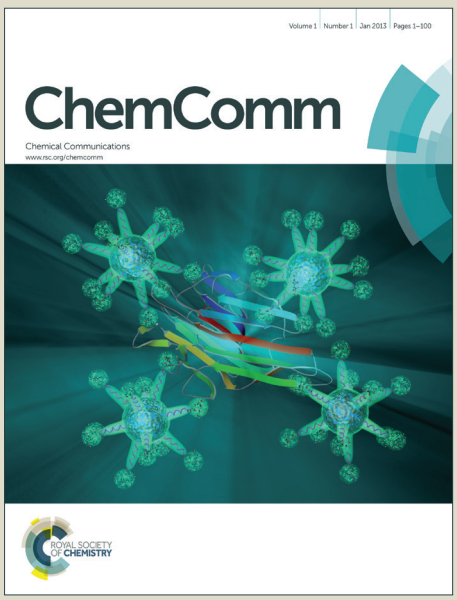

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Magnetic Ionic Liquids as PCR-compatible Solvents for DNA Extraction from Biological Samples

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Kevin D. Clark,[a] Melissa M. Yamsek,[b] Omprakash Nacham[a] and Jared L. Anderson*[a]

A polymerase chain reaction (PCR) buffer was systematically designed to relieve the inhibition caused by hydrophobic magnetic ionic liquids (MILs). We describe a simple, rapid method for MIL-based plasmid DNA extraction from crude bacterial cell lysate in which DNA-enriched MIL is transferred directly to a PCR tube for analysis.

Nucleic acid analysis is rapidly becoming a mainstay for clinical diagnostics,[1] food safety,[2] genomics,[3] and microbiology.[4] Bioanalytical techniques including PCR and sequencing methods are capable of selectively detecting very small quantities of nucleic acids. However, these techniques are limited by their low tolerance to interfering constituents within complex biological or environmental sample matrices.[5,6] Consequently, the isolation of sufficiently purified nucleic acids often requires time-consuming sample preparation steps and represents a significant bottleneck in this field.

In an effort to increase sample throughput and minimize user intervention, automated methods for nucleic acid testing have received substantial attention.[7–9] Magnet-based approaches are particularly attractive platforms that utilize a magnetoactive sorbent to rapidly extract and manipulate nucleic acid samples.[10] Precise control of sample motion is achieved by application of a magnetic field, circumventing the need for tedious centrifugation steps. Functionalized magnetic particles have been explored in pathogen detection,[11] forensics,[12] drug discovery applications,[13] and genomic studies[14] to dramatically reduce overall analysis time. Although magnetic particles are readily applied for high throughput nucleic acid sample preparation, the purity and yields obtained using these substrates can be variable.[12] Furthermore, the high cost of functionalized magnetic particles has prevented their widespread use. Hence, the development of inexpensive materials for rapid nucleic acid analysis is highly desirable.

Magnetic ionic liquid (MIL) solvents offer a promising new magnet-based approach for the selective analysis of nucleic acids. MILs are molten salts that exhibit paramagnetic behaviour in an applied external magnetic field.[15,16] Similar to conventional ionic liquids (ILs), the physicochemical properties of MILs can be controlled by tailoring the structure of the cation/anion.[17–19] While ILs have been successfully applied as sorptive phases for DNA,[20–22] nucleic acid preservation media,[23,24] and PCR additives,[25] hydrophobic MILs were only recently reported as solvents capable of performing highly efficient DNA extractions from aqueous solutions.[26] An important advantage of MIL-based DNA extraction is the ease with which the MIL microdroplet can be manipulated by application of a magnetic field, providing rapid enrichment of DNA. Unfortunately, recovering the nucleic acid from the MIL-based extraction phase has proven to be a time-consuming process that can require considerable user intervention. An ideal nucleic acid sample preparation technique would not only provide a rapid extraction step, but also feature a recovery process involving minimal sample work-up prior to analysis.

Here, we report a method for MIL-based extraction of bacterial plasmid DNA (pDNA) followed by immediate PCR amplification and detection of a target gene. By carefully engineering the components within a PCR mixture, the pDNA-enriched MIL could be added directly to a PCR tube for gene amplification without additional sample purification. The results demonstrate the feasibility of interfacing MIL extraction solvents with biochemical assays to achieve rapid enrichment and analysis of DNA.

Bacterial pDNA containing the 879 bp 5'-methylthioadenosine phosphorylase (MTAP) gene was selected as a model nucleic acid template for the MIL-mediated PCR inhibition studies. The standard PCR mixture components and thermal conditions for amplification of the MTAP gene are described in the ESI. In order to examine the effects of hydrophobic MILs on PCR amplification, the reaction mixture was spiked with 0.5 µL of either the trihexyl(tetradecyl)-phosphonium tetrachloroferrate(III) ($[P_{6,6,6,14}^{+}][FeCl_4^{-}]$) MIL or the trioctylbenzylammonium bromotrichloroferrate(III) ($[(C_8)_3BnN^{+}][FeCl_3Br^{-}]$) MIL. Chemical structures for these two investigated MILs are depicted in Fig. 1a and b. As shown in lanes 3 and 4 of Fig. 1c, the addition of MILs to the PCR mixture completely inhibited the reaction and no amplicon was observed on the agarose gel. In order to address this challenge, a systematic

[a] Department of Chemistry, Iowa State University, Ames, IA 50011, USA.
[b] Department of Chemistry and Biochemistry, University of Toledo, Toledo, OH 43606, USA.
Email: andersoj@iastate.edu; Tel: +1 515-294-8356

Electronic Supplementary Information (ESI) available: PCR conditions, $FeCl_3$ inhibition experiments, DNA sequencing data, cell culture and cell lysis conditions, and schematic of MIL-based DDE. See DOI: 10.1039/x0xx00000x

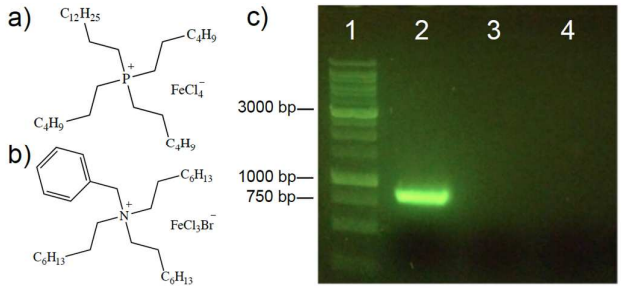

Fig. 1 Chemical structures of the a) $[P_{6,6,6,14}^+][FeCl_4^-]$ and b) $[(C_8)_3BnN^+][FeCl_3Br^-]$ MILs. Agarose gel showing the effect of MILs on the PCR amplification of the MTAP gene from pDNA is shown in pane (c). Lane 1 is a DNA ladder, lane 2 shows the amplicon from a 25.9 ng pDNA standard, lane 3 represents PCR with the addition of $[P_{6,6,6,14}^+][FeCl_4^-]$, and lane 4 shows the result of adding $[(C_8)_3BnN^+][FeCl_3Br^-]$ to the reaction mixture.

approach was undertaken in which the components within the PCR mixture were tailored to mitigate the inhibitory effects of the MILs.

Hydrophobic MILs often consist of a cation possessing long alkyl chains and/or aromatic moieties and a transition metal-based anion. Although iron(III)-based anions impart useful paramagnetic properties to the $[P_{6,6,6,14}^+][FeCl_4^-]$ and the $[(C_8)_3BnN^+][FeCl_3Br^-]$ MILs, haloferrates are known inhibitors of PCR.[27] To simulate the anionic component of the MILs, $FeCl_3 \cdot 6H_2O$ was added at various concentrations to the standard PCR mixture. The minimum inhibitory concentration of $FeCl_3$ under the PCR conditions studied was determined to be 20 µM, which is in good agreement with previously reported values (ESI, Fig. S1).[27,28] In an effort to identify reagents capable of relieving $FeCl_3$ inhibition, bovine serum albumin (BSA), ovalbumin, $MgCl_2$, and iron(III) chelators including EDTA and deferoxamine were investigated as PCR additives. As shown in Fig. 2a, amplification of the MTAP gene from pDNA in solutions containing 20 µM $FeCl_3$ was successful when any of the aforementioned compounds were added to the PCR mixture. This may be due to the sequestration of Fe^{3+} by the PCR additives or, in the case of $MgCl_2$, the outcompeting of Fe^{3+} for binding with reaction components.[27,28] It is important to note that the molar ratio of EDTA and deferoxamine to Fe^{3+} did not exceed 1:1 in order to avoid chelation of essential Mg^{2+} cofactors. Although an amplicon was observed for each reaction in Fig. 2a, the most cost-effective reagents for relieving PCR inhibition caused by $FeCl_3$ were determined to be ovalbumin, $MgCl_2$, and EDTA.

The cationic components of the two MILs examined in this study represent another possible source of interference in PCR assays. To investigate this effect, the corresponding halide salts for each MIL were spiked into the PCR buffer system. As shown in Fig. 2b, adding

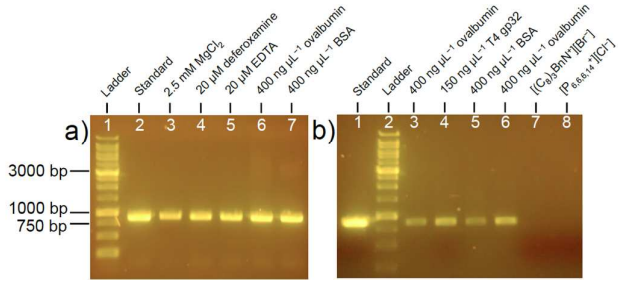

Fig. 2 Amplification of the MTAP gene within PCR buffers spiked with a) 20 µM $FeCl_3$, b) 0.5 µL of $[(C_8)_3BnN^+][Br^-]$ (lane 3) or 0.5 µL of $[P_{6,6,6,14}^+][Cl^-]$ (lanes 4-6). Additives to the standard PCR buffer are noted above each lane. The composition of the standard PCR mixture is described in the ESI.

0.5 µL of either the $[P_{6,6,6,14}^+][Cl^-]$ or $[(C_8)_3BnN^+][Br^-]$ salt completely inhibited PCR amplification of the MTAP gene. Initial attempts to mitigate the cation-induced PCR inhibition by increasing the concentration of $MgCl_2$ from 1.5 mM to 2.5 mM proved unsuccessful. However, lane 3 of Fig. 2b shows that when the reaction mixture was prepared with the $[(C_8)_3BnN^+][Br^-]$ salt and spiked with 400 ng μL^{-1} ovalbumin, a band corresponding to the MTAP amplicon was observed. A reasonable explanation for this result is that ovalbumin may be capable of engaging in hydrophobic interactions with the $[(C_8)_3BnN^+][Br^-]$ IL, thereby preventing the halide salt from interfering with PCR.[27] Ovalbumin, BSA, and the single-stranded DNA binding protein T4 gp32 were also found to reduce PCR inhibition by the $[P_{6,6,6,14}^+][Cl^-]$ IL.

Upon independently establishing conditions suitable for the PCR amplification of the MTAP gene in the presence of $FeCl_3$, $[P_{6,6,6,14}^+][Cl^-]$, and $[(C_8)_3BnN^+][Br^-]$, a combination of PCR additives were selected and applied to reaction mixtures containing MIL. First, a PCR mixture was prepared by spiking 0.5 µL of the $[P_{6,6,6,14}^+][FeCl_4^-]$ MIL into a PCR buffer along with 2.5 mM $MgCl_2$, 1 mM EDTA, and 400 ng μL^{-1} ovalbumin. Unfortunately, PCR amplification was once again inhibited by the $[P_{6,6,6,14}^+][FeCl_4^-]$ MIL and no amplicon was detected on the agarose gel, as shown in lane 3 of Fig. 3. A recent report by Xie and Taubert indicated that aqueous solutions of the hydrophilic 1-butyl-3-methylimidazolium tetrachloroferrate(III) ($[BMIM^+][FeCl_4^-]$) MIL generated acidic pH at elevated temperature due to hydrolysis of the $[FeCl_4^-]$ anion.[29] Low solution pH is known to significantly decrease primer extension rates of DNA polymerase, thereby inhibiting PCR.[30] Although the $[P_{6,6,6,14}^+][FeCl_4^-]$ MIL possesses hydrophobic character, it is possible that the thermal programming during PCR influences the solubility of MIL in the reaction mixture, promoting hydrolysis of the haloferrate anion. To examine this hypothesis, the pH of a PCR mixture containing 2.5 mM $MgCl_2$, 1 mM EDTA, 400 ng μL^{-1} ovalbumin, and 0.5 µL of the $[P_{6,6,6,14}^+][FeCl_4^-]$ MIL was measured before and after thermal cycling. Initially, the PCR mixture was tested using pH paper and found to be between pH 8 and 9. However, after the sample underwent temperature programming the solution exhibited a substantially lower pH (between pH 3 and 4), suggesting hydrolysis of the $[FeCl_4^-]$ anion during PCR. To compensate for the acidic conditions, the reaction mixture was buffered with 80 mM Tris (pH 8). As shown in lane 4 of Fig. 3, amplification of the MTAP gene was successful with a PCR mixture

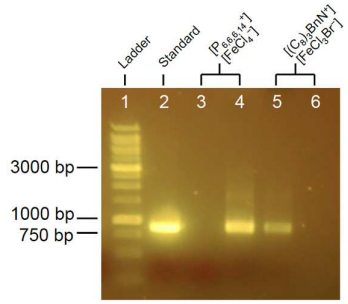

Fig. 3 PCR amplification of the MTAP gene within PCR buffers spiked with MIL. Lane 1 shows a DNA ladder and lane 2 is a control reaction without MIL. Lane 3 represents the PCR amplification of the MTAP gene in a solution spiked with 0.5 µL of the $[P_{6,6,6,14}^+][FeCl_4^-]$ MIL using 2.5 mM $MgCl_2$, 1 mM EDTA, and 400 ng μL^{-1} ovalbumin as additives. Lane 4 shows the amplicon obtained from a PCR solution with identical composition as lane 3, but also includes 80 mM Tris (pH 8) as a buffer component. Lane 5 represents PCR amplification in the presence of 0.5 µL $[(C_8)_3BnN^+][FeCl_3Br^-]$ MIL under the same conditions as lane 4 with 0.4 µM of each primer. Lane 6 shows the result from PCR amplification under the same conditions as lane 5, but with 0.2 µM primers.

consisting of 2.5 mM $MgCl_2$, 1 mM EDTA, 400 ng μL^{-1} ovalbumin, 80 mM Tris (pH 8), and 0.5 µL of $[P_{6,6,6,14}^+][FeCl_4^-]$. When the same reaction conditions were applied for a PCR mixture spiked with 0.5 µL of the $[(C_8)_3BnN^+][FeCl_3Br^-]$ MIL, an amplicon was observed only when the primer concentration was increased from 0.2 µM to 0.4 µM, as shown in lanes 5 and 6 of Fig. 3. Sequence analysis confirmed that the amplicons from PCR samples containing MIL were unaltered when compared to a standard (ESI, Fig. S2-S4).

Upon designing a PCR buffer capable of accommodating MIL, the feasibility of performing MIL-based DNA extraction followed by immediate PCR amplification of a target gene was investigated. *E. coli* cells containing pDNA were subjected to alkaline lysis and the crude cell lysate subsequently extracted using the $[P_{6,6,6,14}^+][FeCl_4^-]$ MIL with a dispersive droplet extraction (DDE) approach.[26] Detailed cell culture and cell lysis conditions as well as a schematic of MIL-based DDE are shown in the ESI. Following a 1 min extraction step, the pDNA-enriched MIL microdroplets were retrieved using a rod magnet and rinsed with deionized water to remove residual salts and cell debris. An aliquot of the pDNA-enriched MIL was then transferred directly into the PCR buffer for amplification of the MTAP gene. As shown in lane 2 of Fig. 4a, an amplicon was readily detected. Similarly, lane 3 of Fig. 4b shows that the $[(C_8)_3BnN^+][FeCl_3Br^-]$ MIL extracted sufficient pDNA within 1 min from bacterial cell lysate for PCR amplification of the MTAP gene without employing any additional tedious purification steps.

In summary, a PCR buffer was systematically designed to enable the amplification of a target gene from pDNA-enriched MIL. The results show that PCR inhibition caused by the cationic and anionic components of two studied MILs could be mitigated using albumin, iron(III) chelators, and by increased buffer capacity of the PCR mixture. Importantly, MILs were capable of extracting PCR amplifiable pDNA from crude bacterial cell lysate without the need for time-consuming sample purification or DNA recovery procedures. This study demonstrates the compatibility of MIL solvents with bioanalytical techniques to dramatically reduce the time required for DNA analysis, making these materials particularly attractive for food safety or other high throughput applications.

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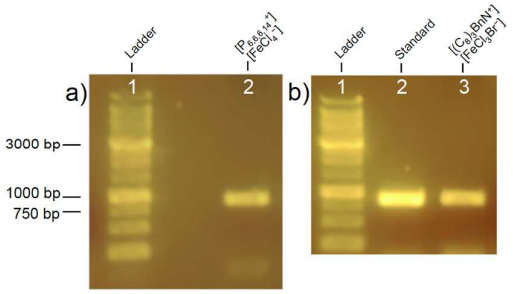

Fig. 4 PCR amplification of the 879 bp MTAP gene following MIL-based extraction of crude bacterial cell lysate using the a) $[P_{6,6,6,14}^+][FeCl_4^-]$ MIL and the b) $[(C_8)_3BnN^+][FeCl_3Br^-]$ MIL. Details regarding the extraction and PCR conditions are shown in the ESI.

Notes and references

1. T. G. Drummond, M. G. Hill and J. K. Barton, *Nat. Biotech.*, 2003, **21**, 1192-1199.
2. H. P. Dwivedi and L.-A. Jaykus, *Crit. Rev. Microbiol.*, 2011, **37**, 40-63.
3. L. A. Garraway and E. S. Lander, *Cell*, 2013, **153**, 17-37.
4. P. C. Y. Woo, S. K. P. Lau, J. L. L. Teng, H. Tse and K. Y. Yuen, *Clin. Microbiol. Infect.*, 2008, **14**, 908-934.
5. I. G. Wilson, *Appl. Environ. Microbiol.*, 1997, **63**, 3741-3751.
6. R. Alaeddini, *Forensic Sci. Int.: Genet.*, 2012, **6**, 297-305.
7. M. U. Kopp, A. J. d. Mello and A. Manz, *Science*, 1998, **280**, 1046-1048.
8. U. Lehmann, C. Vandevyver, V. K. Parashar and M. A. M. Gijs, *Angew. Chem. Int. Ed.*, 2006, **45**, 3062-3067.
9. D. C. Leslie, J. Li, B. C. Strachan, M. R. Begley, D. Finkler, L. A. L. Bazydlo, N. S. Barker, D. M. Haverstick, M. Utz and J. P. Landers, *J. Am. Chem. Soc.*, 2012, **134**, 5689-5696.
10. S. Berensmeier, *Appl. Microbiol. Biotechnol.*, 2006, **73**, 495-504.
11. O. Olsvik, T. Popovic, E. Skjerve, K. S. Cudjoe, E. Hornes, J. Ugelstad and M. Uhlén, *Clin. Microbiol. Rev.*, 1994, **7**, 43-54.
12. C. J. Frégeau and A. De Moors, *Forensic Sci. Int.: Genet.*, 2012, **6**, 511-522.
13. J. G. Bruno and J. L. Kiel, *BioTechniques*, 2002, **32**, 178-180, 182-173.
14. M. L. Metzker, *Nat. Rev. Genet.*, 2010, **11**, 31-46.
15. R. E. Del Sesto, T. M. McCleskey, A. K. Burrell, G. A. Baker, J. D. Thompson, B. L. Scott, J. S. Wilkes and P. Williams, *Chem. Commun.*, 2008, DOI: 10.1039/B711189D, 447-449.
16. M. Li, S. L. De Rooy, D. K. Bwambok, B. El-Zahab, J. F. DiTusa and I. M. Warner, *Chem. Commun.*, 2009, DOI: 10.1039/B917683G, 6922-6924.
17. P. Brown, C. P. Butts, J. Eastoe, E. Padron Hernandez, F. L. d. A. Machado and R. J. de Oliveira, *Chem. Commun.*, 2013, **49**, 2765-2767.
18. E. Santos, J. Albo and A. Irabien, *RSC Advances*, 2014, **4**, 40008-40018.
19. O. Nacham, K. D. Clark, H. Yu and J. L. Anderson, *Chem. Mater.*, 2015, **27**, 923-931.
20. J.-H. Wang, D.-H. Cheng, X.-W. Chen, Z. Du and Z.-L. Fang, *Anal. Chem.*, 2007, **79**, 620-625.
21. O. Nacham, K. D. Clark and J. L. Anderson, *Anal. Methods*, 2015, **7**, 7202-7207.
22. N. Nishimura and H. Ohno, *J. Mater. Chem.*, 2002, **12**, 2299-2304.
23. R. Vijayaraghavan, A. Izgorodin, V. Ganesh, M. Surianarayanan and D. R. MacFarlane, *Angew. Chem. Int. Ed.*, 2010, **49**, 1631-1633.
24. R. R. Mazid, U. Divisekera, W. Yang, V. Ranganathan, D. R. MacFarlane, C. Cortez-Jugo and W. Cheng, *Chem. Commun.*, 2014, **50**, 13457-13460.
25. Y. Shi, Y.-L. Liu, P.-Y. Lai, M.-C. Tseng, M.-J. Tseng, Y. Li and Y.-H. Chu, *Chem. Commun.*, 2012, **48**, 5325-5327.
26. K. D. Clark, O. Nacham, H. Yu, T. Li, M. M. Yamsek, D. R. Ronning and J. L. Anderson, *Anal. Chem.*, 2015, **87**, 1552-1559.
27. C. A. Kreader, *Appl. Environ. Microbiol.*, 1996, **62**, 1102-1106.
28. W. A. Al-Soud and P. Rådström, *J. Clin. Microbiol.*, 2001, **39**, 485-493.
29. Z.-L. Xie and A. Taubert, *ChemPhysChem*, 2011, **12**, 364-368.
30. J. L. Montgomery, N. Rejali and C. T. Wittwer, *Anal. Biochem.*, 2013, **441**, 133-139.