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The use of ionic liquids for cracking viruses for isolation of nucleic acids

S. Fister [a], S. Fuchs [a], P. Mester [a], I. Kilpeläinen [c], M. Wagner [b], P. Rossmanith [a,b,*]

[a] Christian Doppler-Laboratory for Monitoring of Microbial Contaminants, Institute for Milk Hygiene, Milk Technology and Food Science, Department of Farm Animal and Public Health in Veterinary Medicine, University of Veterinary Medicine, Veterinärplatz 1, 1210 Vienna, Austria
[b] Institute for Milk Hygiene, Milk Technology and Food Science, Department of Farm Animal and Public Health in Veterinary Medicine, University of Veterinary Medicine, Veterinärplatz 1, 1210 Vienna, Austria
[c] Laboratory of Organic Chemistry, Department of Chemistry, University of Helsinki, P.O. Box 55, FI-00014 Helsinki, Finland

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ABSTRACT

The most important molecules in the life sciences are nucleic acids. This is especially important in the context of virus research where nucleic acids are primarily analysed. In general, there are two methods for extracting nucleic acids: solution-based and column-based. In many cases quantitative isolation of nucleic acids is necessary. In the present paper, ionic liquids have been tested for the first time for disintegration of virus particles and separation of the nucleic acids in a liquid phase system. $[(OH)^2C_2C_1C_1NH][C_2CO_2]$ and several $[NTf_2]$-based ionic liquids were tested as well as $[C_1C_1im][C_1PO_2OH]$, $[C_8C_8C_8C_1N]$ and $[C_8C_1im]$-based ionic liquids. With $[(OH)^2C_2C_1C_1NH][NTf_2]$, $[C_6C_6C_6C_{14}P][FAP]$ and $[C_6C_1im][FAP]$ a significant higher recovery was obtained in comparison to the control. In particular, $[C_1C_1im][C_1PO_2OH]$ achieved promising results with respect to recovery rates and purity. The isolation protocol is fast, easy and column free. An additional advantage is the deactivation of nucleases such as DNase I and RNase H.

1. Introduction

In the life sciences, particularly in molecular biology, one of the most important classes of molecules are nucleic acids such as deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). While DNA encodes the genetic instructions used in the development and functioning of all known living organisms and some viruses, ribonucleic acid (RNA) has various biological functions such as coding, decoding, regulation, and expression of genes, and is also a DNA equivalent in some viruses. Indeed, due to enzymatic activities of RNA and the possibility of self replication, RNA is considered the likely starting molecule for evolution on earth [1]. Due to their prominent role in all known living organisms, various methods have been developed to analyse nucleic acids to address biological questions in both diagnostics and research and they are starting points for many downstream processes.

DNA was isolated from leukocytes for the first time by the Swiss physician Friedrich Miescher in 1869 [2]. Initially, extraction was complicated and time-consuming. Today there are many extraction kits available and isolation is fast and practical. In general, there are two possibilities for extracting nucleic acids: solution-based methods, that include mostly organic solvents followed by alcohol precipitation, and column-based methods [3]. Both methods require two general steps: The first step is lysis of the cell, bacterium or virus particle; the second step is separation, in which the desired nucleic acid (genomic DNA, plasmid DNA, cDNA, or genomic RNA, mRNA, mi RNA, rRNA, etc.) is extracted out of the cell debris and sample residues.

Most of the state of the art commercially available kits are column-based. After disruption of the cells or viruses, nucleic acids are bound on silica matrices, nitrocellulose or polyamide columns or membranes by hydrogen-bonding. After several washing steps the nucleic acid is eluted [3]. Column-based methods are well established and the recovered nucleic acid is usually of a high level of purity. On the other hand, these kits are not the best choice if DNA/RNA should be isolated from complex matrices, because the columns are easily clogged resulting in a biased recovery. Moreover, column-based methods provide 75–80% recovery at best and have been reported to depict a high intra-experimental deviation in actual recovery rate (User manual NucleoSpin® Extract II (Macherey–Nagel) April 2004/ Rev.01).

* Corresponding author at: Christian Doppler-Laboratory for Monitoring of Microbial Contaminants, Institute for Milk Hygiene, Milk Technology and Food Science, Department of Farm Animal and Public Health in Veterinary Medicine, University of Veterinary Medicine, Veterinärplatz 1, 1210 Vienna, Austria. Tel.: +43 25077 3527; fax: +43 25077 3590.

E-mail addresses: Susanne.fister@vetmeduni.ac.at (S. Fister), Sabine.Fuchs80@gmx.at (S. Fuchs), Patrick-julian.mester@vetmeduni.ac.at (P. Mester), Ilkka.kilpelainen@helsinki.fi (I. Kilpeläinen), Martin.Wagner@vetmeduni.ac.at (M. Wagner), peter.rossmanith@vetmeduni.ac.at (P. Rossmanith).

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For viruses, the most important conventional solution-based nucleic acid extraction method is guanidinium thiocyanate phenol chloroform extraction, which was originally developed by Ulrich et al. (1977) [3] and then further improved [4]. Liquid–liquid extraction is a simple and fast technique that is highly flexible and can achieve high yields [5], especially from complex matrices where it is the preferred method compared to column-based extraction. Moreover, the use of guanidinium thiocyanate provides some protection against nucleases, which degrade free nucleic acids and can cause to false results. This is especially true in the case of RNases, which are ubiquitously present in environmental or clinical samples and are not completely inactivated by guanidinium thiocyanate. Another disadvantage of guanidinium thiocyanate phenol chloroform based isolation methods is their toxicity and volatility, which requires special working areas with fume hoods.

In many cases, the quantitative isolation of nucleic acids is necessary. This is especially true in diagnostics where legal regulations define the number of pathogens permitted to be present in the sample. In this context, the lysis step of the cells or viruses is the most crucial one and the time that is required for this step also plays an important role.

To the best of our knowledge, ionic liquids have not previously been used for the isolation of nucleic acids from viruses. Nevertheless, their unique properties appear to make them a promising tool. Ionic liquids have been used to break up yeast cells and to extract proteins [6] as well as double stranded DNA [7]. The first successful application of ionic liquids for both lysis of cells and extraction of DNA from bacteria was presented by Fuchs et al. [8]. This group developed a lysis method for quantitative and fast DNA isolation of bacteria based on ionic liquids and high temperature. For quantitative cell lysis and DNA release, the bacteria had to be incubated at 120 °C for 1 min in $[C_4C_1pyrr][NTf_2]$. Afterwards the DNA could simply be extracted into water and the DNA-containing aqueous phase could be directly used for analysis without inhibiting any subsequent methods such as Real time PCR (qPCR).

In the paper presented here, ionic liquids have been tested for the first time for fast and quantitative disintegration of virus particles for subsequent isolation of either RNA or DNA. Based on the results of Fuchs et al. [8] the impact of different incubation temperatures was determined. Most of the ionic liquids tested in this study were water-immiscible, as from such ionic liquids the nucleic acids should be extractable into water without an additional precipitation step.

2. Material and methods

2.1. Ionic liquids

In this study, a total of 16 different ionic liquids were tested and the respective abbreviations and structures are presented in Fig. 1. Six water-immiscible ionic liquids ($[C_4C_1pyrr][NTf_2]$, $[C_6C_6C_6C_{14}P][NTf_2]$, $[(OH)^2C_2(OH)^2C_2C_4NH][NTf_2]$, $[(OH)^2C_2C_1C_1NH][NTf_2]$, $[C_6C_1im][FAP]$ and $[C_6C_6C_6C_{14}P][FAP]$) as well as the water-miscible ionic liquid $[(OH)^2C_2C_1C_1NH][C_2CO_2]$ were provided from Merck KGaA (Darmstadt, Germany). Water-miscible $[C_1C_1im][C_1PO_2OH]$ was provided from Solvionic (Toulouse, France). In addition, seven ionic liquids based on the $[C_8C_8C_8C_1N]^+$ cation combined with $[IO_3]^-$, $[H_2NSO_3]^-$, [orotate]$^-$, $[FeCl_4]^-$, [calconate]$^-$, $[(COOH)^4(OH)^{2,3}C_3CO_2]^-$ and $[Cl^4C_6H_6OC_1CO_2]^-$, as well as $[C_8C_1im][IO_4]$, were provided by Proionic GmbH (Grambach, Austria) with a nominal purity higher than 95%, or synthesised using the CBILS® [1] route [9]. In short, exactly one molar equivalent of the corresponding acid (iodic acid, 4-chlorophenoxyacetic acid, sulphamic acid (all Sigma–Aldrich Chemie GmbH, Steinheim, Germany) and periodic acid, L(+)-Tartaric acid, orotic acid and calconcarboxylic acid (all Merck KGaA, Darmstadt, Germany)) was added to the $[C_8C_8C_8C_1N][C_1CO_2]$ (provided by Proionic). Carbon dioxide was released and the ionic liquid was formed. The ionic liquids were isolated from respective solvents *in vacuo* to yield typically 98–99% of the theoretical amount using a SPC-SpeedVac® (Thermo Savant, Thermo Scientific Inc.).

2.1.1. Selection of special water-immiscible ionic liquids

In this study, our aim was to find water-immiscible ionic liquids which form two-phase systems with DNA suspended in water. For handling reasons, it is preferable to have two-phase systems in which the DNA stays in the upper phase. For this reason, several water-immiscible ionic liquids were mixed with DNA that was stained with SYBR® Safe DNA Gel Stain (Life Technologies, Carlsbad, USA). The sample was then shortly centrifuged to accelerate phase separation and photographed under UV-light. Ionic liquids which formed two-phase systems with fluorescence in the upper phase were tested for cracking of viruses and are listed in Section 2.1 and shown in Fig. 1.

2.2. Viruses

Feline calicivirus (FCV) was used as a model for RNA viruses; it being a well-established surrogate for human noroviruses [10–14], one of the most important food-borne pathogens. A virus stock and corresponding CRFK (Crandel feline kidney) cells were obtained from Dr. Reimar Johne (BfR, Berlin, Germany). The viruses were replicated in CRFK cells in DMEM (Gibco®, Life Technologies™, UK) plus 10% foetal bovine serum gold, 1% L-glutamine (both PAA Laboratories GmbH, Pasching, Austria), 2% Anti–Anti (antibiotic–antimycotic; Gibco®, Life Technologies™, Grand Island, NY, USA) and MEM Non-Essential Amino Acids (PAA Laboratories GmbH, Pasching, Austria). Harvested viruses were aliquoted and frozen at −20 °C. Working stocks were thawed and stored at 4 °C.

The phage P100 was used as a model for DNA viruses. This phage was purchased as Listex™ P100 solution (Batch 12G26, Lot: 308; Micreos, Wageningen, NL). The phage solution was used for plaque assays [15]. A single plaque was isolated and used for replication in a log-phase culture of the phage-sensitive *L. monocytogenes* EGDe (ATCC BAA-679). Thereafter the infected culture was centrifuged at 8,000 rpm for 2 min. The supernatant was treated with chloroform, aliquoted and stored at 4 °C. The phage was confirmed as phage P100 by qPCR (for details, see Section 2.5.2).

2.3. Cracking of viruses and extraction of nucleic acid isolation

2.3.1. Procedure

In a ratio of 1:10 (v/v), 5–10 µl of virus suspension (FCV: 10^4–10^5 RT qPCR units; P100: approx. 10^7 qPCR units) was added to either 45 or 90 µl of ionic liquid, mixed and incubated for either 1 min at 120 °C, 10 min at 70 °C or 10 min at room temperature. Subsequently 200 µl of ddH_2O was added, mixed by pipetting and used for precipitation of nucleic acid. If phase formation was observed (water-immiscible ionic liquids), only the upper phase was used for precipitation. All ionic liquids were first tested for cracking of DNA viruses and promising candidates also tested for lysis of RNA viruses.

All experiments included a negative control (water instead of virus suspension) and were repeated at least three times, except ionic liquids that were clearly unsuitable.

[1] CBILS® is a registered trademark of Proionic GmbH.