Sensors and Actuators B 258 (2018) 148–155

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

journal homepage: www.elsevier.com/locate/snb

Grinding Lysis (GL): A microfluidic device for sample enrichment and mechanical lysis in one

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ARTICLE INFO

Article history:
Received 25 August 2017
Received in revised form 9 November 2017
Accepted 17 November 2017
Available online 21 November 2017

Keywords:
Grinding lysis (GL)
Sample preparation
Spores
Bacteria
Filtration
Purification
PCR

ABSTRACT

Rapid identification of health threatening bacteria and/or spores present in small concentration in sample fluids is of utmost importance. Efficient sample preparation and molecular detection aims to achieving this goal. Two processes must be conducted successively: the concentration of the targets in a small volume with simultaneous purification, followed by their lysis to provide accessible DNA templates. Conventional PCR is then used in situ to identify the targets.

In this work we present an original approach combining an efficient concentration and purification of the bacteria and spores, a rapid and efficient grinding lysis step, working even for polluted samples, and the integration of the process in a semi-automated device. The method is very efficient and rapid: it can concentrate and detect less than 10 targets in 1 mL of sample, even if the sample is contaminated by some environmental contaminants. The most resistant spores are successfully lysed.

In this study, we successively present the principle and performances of the method, and its integration on a in a semi-automated device. Perspectives to fully integrated system are discussed.

1. Introduction

Molecular detection is the method of choice for rapid pathogens recognition due to its high sensitivity and specificity. This method requires preliminary sample preparation to have access to analyzable nucleic acids compounds [1,2].

This mandatory sample preparation step needs to be efficient to allow DNA or RNA extraction with high yields and good purity from various samples and organisms (bacteria, spores). In order to have a rapid diagnostic, the sample preparation step has to be fast. It should also be portable to be used in the field in order to avoid shipment delays and gain time. Finally single use avoids contamination between samples.

Lysis of bacteria and spores is the next step of the process [3]. Different lysis techniques have been experimented and are reported in the literature: chemical, thermal, enzymatic and mechanical lysis [1,4,5]. Usually the choice of the method depends on the species analyzed (Gram+, Gram- or spore) and a generic method is still lacking.

Chemical and enzymatic lysis involves mixing a cocktail of buffers, salts, surfactants, enzymes and solvents, with the biological sample of interest [6,7]. The harsh chemicals contained in the solvent promote the degeneration of the cell membrane. This allows for easy access to the biological sample's genetic material, intracellular proteins or cellular lipids, but additional DNA purification is required for molecular analysis [8].

Enzymatic lysis is another approach for lysis. It consists in using cell-wall lytic enzymes to disrupt the cell membrane [9]. However, this method is expensive and limited because the enzymatic cocktail has to be tailored to specific pathogens. Some organisms such as spores still remain resistant to chemical and/or enzymatic lysis, and DNA purification is generally required, since the chemical lysis agents inhibit the downstream PCR detection.

Thermal lysis consists in heating cells to high temperature in order to cause cellular cell membrane breakdown [10–12]. This method is not ideal because the thermal lysis does not breach the envelopes of many very resistive spores [11,12]. Besides, high temperature levels are hardly compatible with microsystem materials and sealing. Glass microsystems have been reported to be compatible with thermal lysis [10]. However, plastic is the most usual substrate for low-cost devices, and the glass temperature (Tg) is low and easily reached.

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https://doi.org/10.1016/j.snb.2017.11.082
0925-4005/

A promising approach to cell lysis is mechanical lysis [13,14]. Vandeventer et al. [13] mention that mechanical methods, like bead-beating and sonication, were much more efficient to lyse thick-walled microorganisms such as *Bacillus anthracis* spores and *Mycobacterium tuberculosis* cells [15,16]. Conventionally, these techniques use a crushing action to disrupt the bacteria and spore cell walls. However these approaches require a power supply that limits their use on the field.

Concentration is also an important issue directly linked to the sensitivity of detection. At the present time, concentrations of 10^2 or less bacteria or spores per milliliter are targeted. Even if the lysis step is efficient—which is expected from mechanical lysis devices [4,17] — large volumes cannot be directly processed in PCR reaction. A concentration step is therefore required. Without concentration, the use of sample volumes of typically 10–15 μL taken from the 1 mL reservoir reduces the probability of the presence of a bacteria in the amplification reaction due to low bacteria concentration. Another method for increasing DNA material consists in growing bacteria samples before DNA extraction but it considerably increases the time from sample collection to pathogen identification and does not allow initial bacteria quantification.

Few commercial microfluidic devices exist and all need power supply and are part of relatively complex systems [18,19].

In this work, we present a simple, portable, inexpensive, manual method to concentrate, lyse and purify the DNA of spores and bacteria, contained in a 1 mL sample over a very wide range of concentrations (5–10.000 pathogens/mL).

Sample concentration and purification are achieved by a filter and lysis by grinding the targets against a frosted glass, using a spatula. The method is robust to contaminants present in the sample and we have shown that sensitivity achieved using this approach is higher than state-of-the-art systems such as the Precellys [17].

We first demonstrate the method analytically using a simple device actuated manually. The different steps are detailed. Then, a comparison with the Precellys for the detection of bacteria and spores is performed showing the advantages of the system. Finally, it is shown that the method can be integrated in an autonomous microsystem.

2. Materials and methods

2.1. Spores and bacteria

Bacillus subtilis (Bs) endospores 23857™ from ATCC (ATCC®, UK) and Bacillus globigii (Bg) endospores 49760™ from ATCC (ATCC®, UK) have been used at an initial concentration of 10^7 spores/μL.

Escherichia coli bacteria 9637™ from ATCC (ATCC®, UK), Bacillus globigii (Bg) bacteria 49760™ from ATCC (ATCC®, UK) have been used as target model for bacteria at an initial concentration of 10^8 bacteria/μL.

2.2. Bacteria and spores culture

Petri dishes are used to cultivate bacteria and spores. TSB and TBX agar gels are obtained by mixing powder agar (20 g/L) and respectively TSB media (30 g/L) or TBX media (36.6 g/L) in purified water. The solutions are autoclaved and inserted in the Petri dishes.

Escherichia coli and bacillus globigii are cultivated in 25 mL of culture media during one night at 37 and 30 °C respectively.

Dilutions of spores and bacteria initial solutions to the hundred and thousand have been performed and the resulting samples were introduced in Malassez counting chambers. After 15 min, the spores or bacteria have settled down on the bottom of the chambers. Counting under microscope is then performed.

Spore quality is checked by direct observation under the microscope and by qPCR. Under the microscope, absence of vegetative bacteria in the spore solution is checked. Quantitative polymerase chain reaction (qPCR) is used to determine the ratio of vegetative bacteria in the spore solution. A first sample is generated by heating of the spore solution at 95 °C for 10 min, which leads to the detection of vegetative bacteria (if any). A second sample is generated by mechanical lysis, of the solution using a Precellys, known to allow spore lysis. The ratio of vegetative bacteria in spore sample is then determined.

2.3. Sample

Spore and bacteria are washed once before use. Briefly, a volume of 2 mL of the culture is centrifuged during 3 min at 3500 rpm. The supernatant is removed and the pellet resuspended in a volume of 1 mL of sterile distilled water.

After counting, 1 mL sample solutions at concentrations from 5 to 10^5 pathogens per milliliter are generated in a buffer which can be either pure water, or sample water from the environment such as a lake, or taken in the vicinity of a location polluted by diesel fumes.

2.4. Precellys control sample

Each sample concentrated and lysed by our device is also lysed using a Precellys® 24 instrument. A volume of 50 μL of the sample is introduced in an Eppendorff containing zirconium beads and 1 μL of BSA 1%. The resulting vial is introduced in the Precellys instrument with a program consisting of 2 cycles of 20 s of vibrations at 6400 rpm. Finally 15 μL of the solution is added to 15 μL of a 2X PCR mix and analyzed by qPCR.

2.5. Device

The bottom of the inlet chamber is an abrasive surface with a surface roughness on the order of the dimensions of the targets to be lysed. Typically, a frosted glass slide has the right surface roughness to efficiently lyse bacterial spores.

The filter on top of the inlet chamber is a Millipore isopore DTTP membrane (Merck Millipore, Germany) with monodispersed pores of 0.6 μm diameter. The filter thickness is 20 μm approximately and the porosity is in the range of 5–20%. The filter is slightly flexible and extensible to be compatible with the grinding.

The ceiling of the outlet chamber is constituted by a flexible and extensible membrane which makes it compatible with the grinding. The membrane Microamp® Applied Biosystems 4311971 was purchased from Thermo Fisher Scientific.

The inlet channel is cylindrical with a diameter of 1.6 mm and is connected to the inlet chamber which has a height of 150 μm and a diameter 5 mm. The outlet chamber has a height of 300 μm and a diameter of 5 mm and is connected to the outlet channel, which is geometrically identical to the inlet channel.

In the manual method, the fluids are introduced and recovered using pipettes. Approximately 30 s are required for full injection of the 1 mL sample. Grinding is performed manually using a spatula. A scraping motion of the spatula during approximately 30 s is sufficient to lyse spores/bacteria. In the automated method, fluid driving is taken care of by peristaltic pumps (400 μL/min) and the grinding action is performed using a slowly rotating pin (60 rpm).

2.6. PCR detection

Once the spores/bacteria are lysed, their DNA is eluted to a standard PCR device by a solution containing a PCR mix. The PCR mix contains the primers and Taqman probes corresponding to the