Biosensors and Bioelectronics 36 (2012) 230–235

Contents lists available at SciVerse ScienceDirect

Biosensors and Bioelectronics

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

Macro to microfluidics system for biological environmental monitoring

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ARTICLE INFO

Article history:
Received 18 January 2012
Received in revised form
30 March 2012
Accepted 13 April 2012
Available online 27 April 2012

Keywords:
Biological environmental monitoring
Electrowetting
Sample preparation
Real-time PCR

ABSTRACT

Biological environmental monitoring (BEM) is a growing field of research which challenges both microfluidics and system automation. The aim is to develop a transportable system with analysis throughput which satisfies the requirements: (i) fully autonomous, (ii) complete protocol integration from sample collection to final analysis, (iii) detection of diluted molecules or biological species in a large real life environmental sample volume, (iv) robustness and (v) flexibility and versatility. This paper discusses all these specifications in order to define an original fluidic architecture based on three connected modules, a sampling module, a sample preparation module and a detection module. The sample preparation module highly concentrates on the pathogens present in a few mL samples of complex and unknown solutions and purifies the pathogens' nucleic acids into a few μL of a controlled buffer. To do so, a two-step concentration protocol based on magnetic beads is automated in a reusable macro-to-micro fluidic system. The detection module is a PCR based miniaturized platform using digital microfluidics, where reactions are performed in 64 nL droplets handled by electrowetting on dielectric (EWOD) actuation. The design and manufacture of the two modules are reported as well as their respective performances. To demonstrate the integration of the complete protocol in the same system, first results of pathogen detection are shown.

1. Introduction

Sensitive and specific biological environmental monitoring (BEM) are still far from field applications, even though daily news updates remind us that the need is real. Indeed, in industrialized countries, nosocomial infection occurs in 2%–12% of hospitalized patients (Jarvis et al., 1991) of which a great part is probably due to airborne transmission of pathogens (Cotterill et al., 1996; Gehanno et al., 2009; Schultsz et al., 2003). In hospital rooms for immuno-depressed patients, detection thresholds in the air are very low, with values down to 100 cfu/m^3 for *Streptococcus pneumoniae* and 1 cfu/m^3 (cfu: colony-forming unit) for *Aspergillus*. Other environmental controls could benefit from more frequent monitoring, such as sanitary analysis for swimming conditions in beaches, lakes and rivers (notably fecal coliforms, *Enterococcus spp.*, cyanobacteria producing toxins like microcystin, *Cryptosporidium* oocysts, *Giardia*). European legislation[1] has specified detection thresholds to define the water quality unsafe for swimming, i.e. 185 cfu/L for *Enterococcus* and 250 cfu/L for *Escherichia coli*.

Today's normative methods defined by the safety regulations are slow and costly. Aqueous samples, typically 1–10 L, are manually collected and sent to a central lab where a microbiological test is performed by cultivating and counting bacteria on a Petri dish. It typically takes 2–4 days to have the result with a regulatory approved control. Even if quicker analysis methods, e.g. PCR based detection, are used to reduce the analysis time, a huge part of the delay and cost is due to the manual sampling and sample preparation. As a result BEM are performed at limited frequency, usually once a month, at the most once per week. Therefore, BEM could tremendously benefit from fully automated systems (from sampling to data) performing in a semi-continuous mode several analyses per day and having a complete autonomy of 1 or 2 weeks. In order to be statistically representative, typical samples are 1–100 m^3 for air quality analysis and several liters for water analysis. Thus, elevated concentration factor and purification yield of biological targets coupled to high detection sensitivity and specificity are a requisite.

In order to address these requirements, complex systems have been developed in conjunction with devoted biological protocols. In most cases they combine a sample preparation step and a

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[1] Directive 2006/7/EC of the European Parliament and of the Council of 15 February 2006 concerning the management of bathing water quality and repealing directive 76/160/EEC OJ L 64, p. 46.

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http://dx.doi.org/10.1016/j.bios.2012.04.024

detection step. The latter are commonly based on specific molecular recognition of biological molecules, either proteins (immunodetection) or nucleic acids (molecular biology). Immunoassays are particularly adapted for analysis when speed and simplicity are preferred over sensitivity, as they require very limited sample preparation before analysis (Rider et al., 2003; Schultz et al., 2008). When high sensitivity is needed, real time quantitative Polymerase Chain Reaction (qPCR) is the preferred detection method. Extreme specificity and sensitivity, as low as 1 cfu or nucleic acid molecule, can be obtained using PCR based detection. The Autonomous Pathogen Detection System (APDS) developed by the Lawrence Livermore National Laboratory is the first fully automated tool integrated in the US Biowatch program. It integrates both immunodetection and PCR capability, coupled with an adapted sample preparation module (Regan et al., 2008). The BioHazard Water Analyser is based on extraction of RNA from pathogens, followed by identification using electrochemical detection on a DNA chip. It can detect down to 1–10 pathogens in 10 L of water within 2–3 h. Both systems integrate every analytical step from collection to detection. However, they weigh around 200 kg, which is a drawback for portable and in-the-field applications. As shown by the GeneXpert® (Cepheid), a promising approach is to reduce both weights and footprints by integrating the protocol at a microfluidic scale. Sample preparation and qPCR are performed using a unique cartridge. Despite excellent analytical results, it is limited to one-shot measurement without any capability for semi-continuous monitoring.

To best meet the specific needs of BEM we have been developing an autonomous system integrating generic modules for onsite sampling, sample preparation and PCR based detection. This fully automated and portable system will feature short time-to-result (0.5–1 h), multi-pathogens (1–13) identification and quantification. In this paper we describe a first system that couples sample preparation and PCR detection. A PCR detection module working in small volumes of few tens nL is demonstrated. It represents several important advantages for the BEM applications, such as capability of multiplex analysis, minimized reagents and waste, increased autonomy and portability. Yet, this places a strong constraint on the sample preparation module which has to handle volumes differing by three orders of magnitude, from few mL input sample down to few μL. We present also an original biological protocol based on magnetic beads capture maximizing the concentration factor before PCR. This protocol is integrated into an automated sample preparation module, which is successfully coupled to the PCR detection module. Their common automation demonstrates the feasibility of macro to microfluidic system for BEM. It will be further coupled to a sample collection module depending on the application, i.e. air or water sampling.

2. Material and methods

2.1. Biological models and analytical protocols

We have developed a DNA sample preparation protocol, based on magnetic beads, to address the different needs for integration e.g. very large concentration factor, generic reagent for pathogens capture, no enzyme, no temperature variation and small volumes of reagents. A generic capture was chosen because it allows multi-pathogens detection, e.g. virus, bacteria and spores. However, the counterpart is the potential carryover of PCR inhibitors present in the large sample volume which are also concentrated. To overcome this difficulty, we have worked on an original double capture protocol based on two types of magnetic beads implying different chemical interactions. The probability of having carryover inhibitors binding strongly to both types of beads is thus very low.

The first step of the pathogen capture is done on polycationic beads #1 (SIMAG-ionex ~1 μm beads, Chemicell). Typically 1–10 mL sample volume is mixed with 1–10 μL of beads #1 corresponding to 10^8–10^9 beads. Up to 10^8 microorganisms can be captured on the beads #1 in just a few minutes. Beads #1 are then pelleted by a magnet and the supernatant is removed. 50 μL of lysis buffer (5 M Guanidine thiocyanate, 1% N-Lauryl Sarcosine sodium salt, 20 mM Tris–HCl pH 8) is added on beads #1, which are then dispersed. The lysis step occurs in 2 min. Then the beads #1 are pelleted allowing the supernatant to be removed. The latter is mixed with 200 μL of DNA capture buffer (Qiagen PB buffer—Ref. 19066) and 2.5 μL of silanol beads #2 (SIMAG-basic ~1 μm beads, Chemicell) for the nucleic acids purification. Beads #2 are pelleted and washed twice with the washing buffer (Qiagen AW2 buffer—Ref. 19072) for 1 min. The beads are then resuspended in 1–10 μL of elution buffer (10 mM Tris–HCl, pH 8). Elution step occurs in 3 min. Beads #2 are captured and removed from the solution containing the extract of purified nucleic acids. Otherwise noted, for biological validation the sample volume is 1 mL and the elution volume is 10 μL. This sample preparation protocol is performed at room temperature within 20 min, with no centrifugation steps and only reagents addition and removal. The purified DNA from the sample preparation module is then mixed with the PCR common reagents.

E. coli, *Bacillus subtilis* and *S. pneumoniae* strains have been purchased from ATCC (ATCC 9637 (Amp^R), ATCC-33608, ATCC BAA-255). Human adenovirus2 (Ad2) and baculovirus were generously provided by Dr Pascal Fender (Institut de Biologie Structurale de Grenoble, France). Cultures are made in LB buffer at 37 °C overnight for *E. coli* and *B. subtilis*, and at 30 °C for *S. pneumoniae*. Bacteria concentration was established by standard optical absorbance measurement at 600 nm in PBS buffer and correlated with colony counting on agar plates. Mock samples were prepared by spiking a known quantity of bacteria in 1–10 mL of 10 mM Tris–HCl pH 8, when not specified. The Qiagen QIAamp DNA Mini kit (Ref. 51306) was chosen as the reference sample preparation method for DNA extraction and purification yield comparison.

Reference DNA was purchased from Sigma Aldrich for *E. coli* and from ATCC for the other bacterial strains. Quantification of Ad2 and Baculovirus was obtained by directly running qPCR with the viruses. Primers and probe (Taqman and MGB) molecules were purchased from Applied Biosystems for *S. Pneumoniae* and from MWG for the other strains. Specificity for each biological strain was validated against all other strains. Final PCR mixture contains 0.25 U/μL of AmpliTaq Gold and 1X PCR Gold buffer (Applied Biosystem), 3 mM $MgCl_2$, 200 μM of dNTP, 0.8 mM BSA, 600 nM of primers and probes. A single amplification condition has been used for all the biological models, consisting of an initial step of 10 min at 95 °C, followed by 40 cycles of 30 s. denaturation at 92 °C and 60 s annealing at 60 °C. For all sets of primers and probes, PCR sensitivity of at least ~10 DNA copies was measured with the standard PCR reference method, performed on a MxPro 3005P Stratagene apparatus (Agilent) in 10 μL PCR mixture. Standard qPCR curves were made by 10 fold serial dilutions of Tris buffer DNA (or viruses) solutions starting with 10^5–1 copies/μL concentrations in PCR mixture. PCR efficiency is deduced with the following formula $(10^{(1/\text{slope})}-1)100$, using the slope of the linear relationship between Ct (qPCR cycle threshold) and Log (DNA concentration).

2.2. Sample preparation module

The automated sample preparation module performs all the biological protocol steps in a unique polypropylene chamber of 20 μL (1.4 mm internal) that can handle the required 10 mL input