A MICROFLUIDIC DEVICE FOR TEMPERATURE-TRIGGERED DNA AMPLIFICATION IN AGAROSE MICROBEADS

Linda Desbois, Adrien Padirac, Yanick Rondelez and Teruo Fujii*
LIMMS/CNRS-IIS, Institute of Industrial Science, The University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8505, Japan.

ABSTRACT
This paper reports the development of a microfluidic device for the generation of solidified microbeads, and on-chip monitoring of compartmentalized biochemical reactions. We generated agarose microdroplets using a push-pull system, which consists in pushing oil and pulling emulsion. Agarose sample is introduced in inlet and aspirated consecutively. Ultralow volumes of agarose have been used (5 to 10µl) and after microdroplets generation, no dead volume remains in inlet. Once microdroplets have been generated, they are cooled in agarose microbeads thanks to two cooling copper wires. Finally, to validate our system, we performed and monitored a DNA isothermal amplification on-chip.

KEYWORDS
Microfluidics, microdroplets, agarose microbeads, on-chip DNA amplification.

INTRODUCTION

Previous reports on generation of monodisperse agarose microdroplets for high efficiency PCR [1] required a syringe heating system [2], off-chip drops collection and cooling, as well as a thermocycler for *ex situ* amplification reaction [1].
The specific features of the present integrated device are the push/pull microdroplets generation system that drastically downscales reagent consumption, the integration of the heating/cooling system necessary to make agarose microbeads and the possibility to observe an isothermal DNA amplification directly on-chip.
The basic structure is a flow-focusing microchannels adapted for the push/pull fluid handling. Only oil mix is pushed in the device (Figs.1 and 2). Because the emulsion is pulled at the outlet with a higher rate, the agarose sample (containing the reaction mix, i.e. DNA template, inhibitor, primers and enzymes) gets aspirated and segmented into droplets. This setup reduces the consumption of sample down to 5mL. Moreover, this process can be performed at 37ºC. After generation, the emulsion is cooled by refrigerated wires (Fig.1) to allow the microbeads to solidify. After 10 min cooling, agarose beads are then heated to 43ºC: they remain in the gel state, and the rise in temperature triggers the start of the DNA amplification.

EXPERIMENT

Material and methods

Microfluidic device fabrication

The microfluidic devices have been done using standard soft photolithography. A 65µm thick photoresist SU-8 mold has been made, and PDMS chip molded on it. PDMS chip is bonded on 135µm thick slide glass with O_2 plasma.

Agarose microbeads generation

In order to generate agarose microdroplets, we designed a device with a focusing flow nozzle (Fig.1). Oil is pushed with 1ml syringe (Terumo, Japan) and syringe pump. Emulsion is pulled with another syringe. 1.5% ultralow gelling agarose (Type IX-A, Sigma, Japan) microdroplets are generated at 37ºC thanks to a thermoplate (Tokai, Japan). Two copper wires are inserted at 500µm from both sides of the incubation chamber and inserted in ice for cooling step.

DNA isothermal amplification

Reactions are done in a buffer containing 10mM KCl, 10mM $(NH_4)_2SO_4$, 50mM NaCl, 2mM $MgSO_4$, 45mM Tris–HCl, 5mM $MgCl_2$, 6mM DTT, 100μg/ml bovine serum albumin (New England Biolabs) and dNTPs (100μM each). *Bst* DNA polymerase, large fragment and NtBstNBI nicking endonuclease are purchased from New England Biolabs and used at 11.2U/mL and 200U/mL, respectively.
DNA oligonucleotides were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA), with high performance liquid chromatography purification. For the amplification reaction, 300nM of a template strand (5'-AACAGACTCGAAACAGACTCGA-3') with a 3'-terminal TAMRA NHS ester modification is put in the presence of 300nM of an inhibitor strand (5'-GTCTGTTTCGAGTAA-3') and 0.01nM of an input strand (5'-TCGAGTCTGTT-3'). 10μl of sample are introduced in the device for agarose microdroplets generation and 10μl are introduced in thermal cycler for control.

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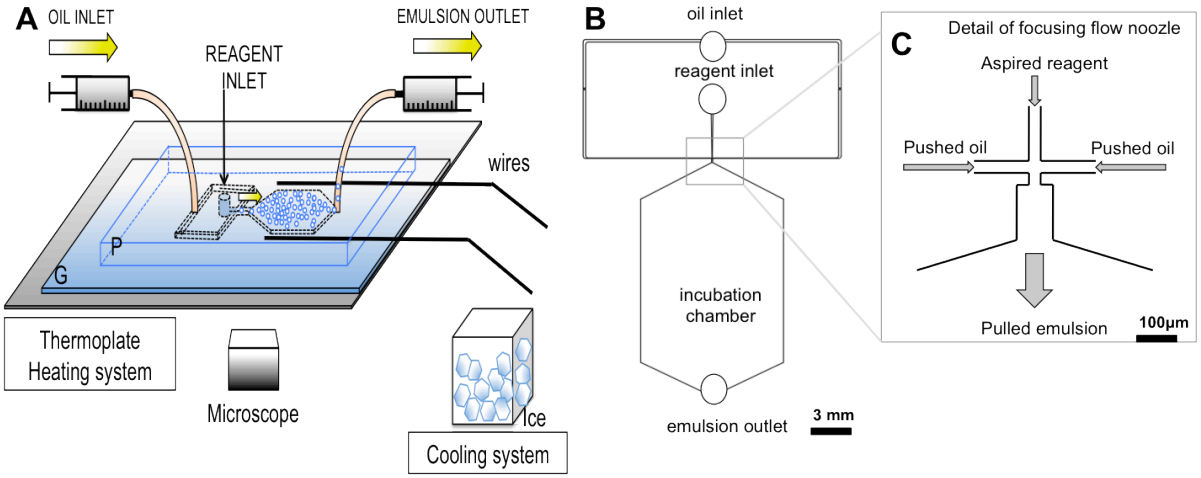

Figure 1: Schematic of the microfluidic device. (A) Device setup: Oil is pushed with syringe pump while emulsion is aspirated with another syringe pump. Reagent (agarose) introduced in inlet is aspirated. For agarose microdroplets generation, the device is warmed at 37ºC with a thermoplate. In order to gelify microdroplets in agarose microbeads, the thermoplate is turned off and the device is cooled thanks to two copper wires introduced in ice. When agarose microbeads are gelified, temperature is increased with thermoplate for DNA amplification. (B) Microchannel network: we designed a focusing flow orthogonal nozzle to generate microdroplets. Microdroplets are generated at 37ºC and incubated in a large incubation chamber. (C) Detail of focusing flow nozzle. Pushed oil segments aspirated agarose flow into monodisperse microemulsion.

Results and discussion

Agarose microdroplets generation and cooling in agarose microbeads

Pushed oil flow is focused on the nozzle and segments aspirated agarose flow in monodisperse microemulsion (diameter of microdroplets= 55µm), as shown in Figure 2. Because agarose sample is deposited and aspirated in inlet, low volume of sample is used (5 to 10µl) and no dead volume remains in inlet. After generation, temperature is decreased in the device with the two copper wires until agarose gelation point (17ºC). Agarose microdroplets are successfully gelified in agarose microbeads (Fig.2,B).

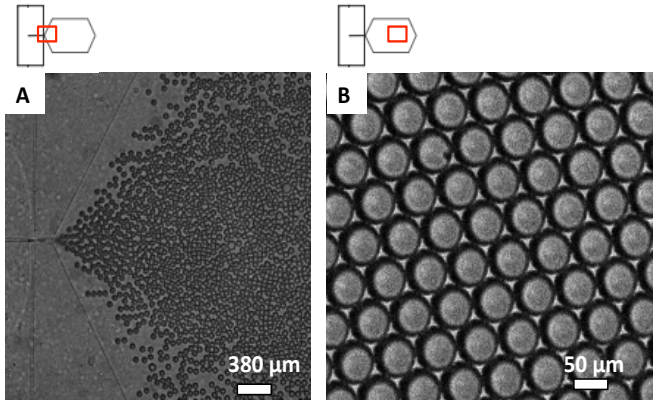

Figure 2: Agarose microdroplets generation. (A): Monodisperse agarose microemulsion generation at 37ºC. (B): Gelified agarose microbeads in incubation chamber after sol-to-gel switch.

Temperature triggered DNA isothermal amplification

This isothermal DNA amplification is based on the use of a DNA polymerase and a nicking enzyme [3] and was made temperature-dependent by the addition of an "inhibitor" DNA molecule (Fig.3). At low temperature (37ºC), the reaction is blocked by the stable inhibitor, whose 3' end is mismatched, preventing the polymerase to extend it. At 43ºC the inhibitor is less stable, allowing the DNA primer to start the reaction.

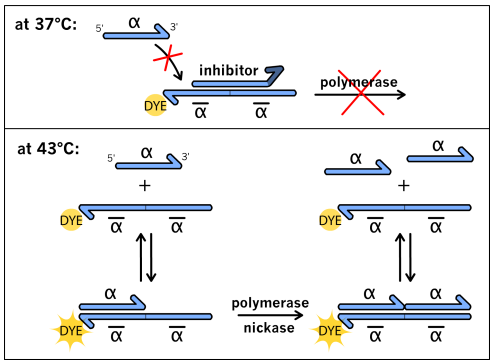

Figure 3: Autocatalytic DNA amplification reaction. At 43°C, DNA amplification occurs: as strand α hybridizes to the template, it is elongated by DNA polymerase. The nascent strand is cut in its middle by a nicking endonuclease, releasing two α strands. The template 3' end is modified with a fluorescent dye, allowing the monitoring of presence of α. At 37°C, a stable inhibitor strand blocks the reaction by displacing strand α, and preventing any polymerase activity.

Using this setup, we have generated stable and monodisperse emulsion (Fig.2,A) and, for the first time, observed DNA amplification in solidified agarose microbeads *in situ* (Fig.2,B). The design requires no valves. Because of solidification, it becomes possible to keep the drops on chip and to incubate them during 115 min. DNA amplification satisfyingly compared to a control experiment performed in a thermocycler (Fig.4).

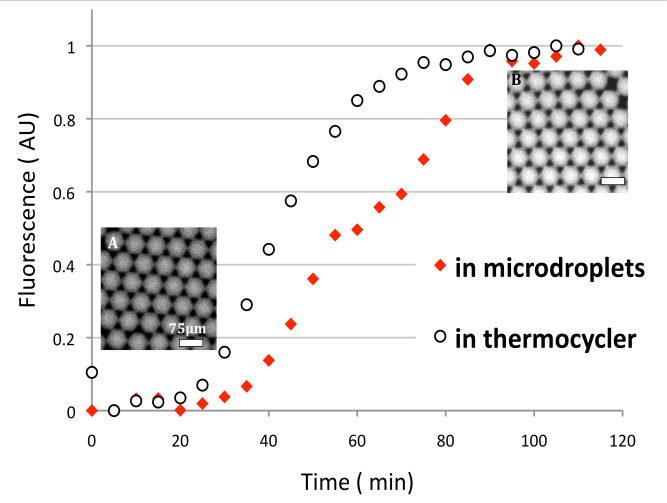

Figure 4: Temperature triggered DNA amplification. The mean fluorescence of 10 microbeads is plotted to establish fluorescence variation curve during incubation on-chip (microdroplet curve). Control experiment (using the same reaction mix) run simultaneously at 43°C in thermal cycler (thermocycler curve). (A): Fluorescence in agarose microbeads at 0 min, (B): at 115 min. Scale bare: 75µm.

Thanks to the on-chip cooling and real-time monitoring capability, the system could enable us to detect specific DNA sequences in real-time without any extra processes. By adopting a combination of different fluorescent probes, the present system can further be applied to the immediate detection of multiple target sequences that is so useful in the field of medical diagnostics, environmental monitoring, food security, etc.

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CONTACT

**tfujii@iis.u-tokyo.ac.jp*