Journal of Bioscience and Bioengineering
VOL. 121 No. 4, 471–476, 2016

ELSEVIER

www.elsevier.com/locate/jbiosc

Handmade microfluidic device for biochemical applications in emulsion

Marsel Murzabaev,[1] Takaaki Kojima,[1,*] Takuro Mizoguchi,[1] Isao Kobayashi,[2] Brandon J. DeKosky,[3] George Georgiou,[3] and Hideo Nakano[1]

Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan,[1] Food Engineering Division, National Food Research Institute, NARO, Tsukuba 305-8642, Japan,[2] and Department of Chemical Engineering, University of Texas at Austin, Austin, TX 78712, United States[3]

Received 11 May 2015; accepted 2 August 2015
Available online 19 September 2015

A simple, inexpensive flow-focusing device has been developed to make uniform droplets for biochemical reactions, such as *in vitro* transcription and cell-free protein synthesis. The device was fabricated from commercially available components without special equipment. Using the emulsion droplets formed by the device, a class I ligase ribozyme, bcl 23, was successfully synthesized from DNA attached to magnetic microbeads by T7 RNA polymerase. It was also ligated with an RNA substrate on the same microbeads, and detected using flow cytometry with a fluorescent probe. In addition, a single-chain derivative of the lambda Cro protein was expressed using an *Escherichia coli* cell-free protein synthesis system in emulsion, which was prepared using the flow-focusing device. In both emulsified reactions, usage of the flow-focusing device was able to greatly reduce the coefficient of variation for the amount of RNA or protein displayed on the microbeads, demonstrating the device is advantageous for quantitative analysis in high-throughput screening.

[**Key words:** Microfluidics; Flow-focusing; *In vitro* transcription/translation; *In vitro* compartmentalization; Monodisperse emulsion]

High-throughput systems for screening large libraries of macromolecules, including DNA, RNA, and proteins have been developed that use water-in-oil emulsion droplets as microreactors (1,2), a technique referred to as *in vitro* compartmentalization (IVC). In these systems, enzymatic reaction and detection is performed in femtoliter volume droplets, and the recovery of the desired products is typically accomplished using fluorescence-activated cell sorting (FACS). However, oil phase cannot be used with FACS; there are three methods to circumvent this limitation: the use of double emulsions, microfluidic devices for fluorescence-activated droplet sorting (FADS), or microbeads as carriers. Water-in-oil-in-water double emulsions (W/O/W) that are compatible with FACS can be obtained through homogenization (3) or membrane-extrusion of W/O emulsions. Homogenization is rapid and straightforward; however, it results in a low signal to noise ratio due to low uniformity of droplets (4). This limitation is, to a large extent, avoided using membrane-extrusion technology. FADS is a particularly attractive technology as it allows direct sorting of droplets based on their fluorescence (5), and it can also be applied for single-cell screening (6). Using a delay line ensures that the reagent incubation time is equal for all droplets, which is difficult to achieve when the droplet collection and sorting steps are completed on separate devices. An alternate approach for high-throughput screening uses microbeads as the solid support component (7). The major advantages of this technique are that molecules, such as DNA, are both confined in a droplet and bound to the microbeads, a linkage that is maintained even after breaking an emulsion. This enables multiple steps of screening using the same microbeads: DNA immobilization, transcription/translation, fluorogenic assays, and FACS (8). Strong binding of fluorescent molecules to the microbeads is required for this technology. This technique has been used to select ribozymes (9), generate functional peptides (10), and screen for transcription factor binding sites (11).

Flow-focusing (FF) technology can be used to produce monodisperse droplets or bubbles from different materials (12). Continuous fluid is used to flank or surround another immiscible focused fluid. Both fluids are then pushed through a narrow orifice, and the focused fluid breaks into droplets. In these devices, droplets can be generated in one of two modes: dripping or jetting (13).

Hypothetically, all IVC methods could benefit from using microfluidics, since these devices encapsulate microbeads or single cells into droplets of uniform size using equal amounts of reagents. Though the advantages of microfluidic platforms and devices are well described, it remains challenging for biologists to enter into the field of single-molecule/single-cell analysis (14). Some attempts have been made to develop microfluidic devices that are easy to both manufacture and operate (15–17). However, these were not applied to generating emulsions from biological samples. Recently, a co-flowing axisymmetric device was developed and its utility was proved by encapsulation of living cells and cell-free protein synthesis in emulsion (18).

Here, we developed straightforward and versatile tools for separating biochemical reactions into millions of uniform microreactors. We designed two variants of FF devices that differ in nozzle geometry. Our devices generate droplets at a higher rate than that of a typical microfluidic device (19). Recently, a comparable device was employed for encapsulation of single cells into emulsion droplets (20). In this work, we have demonstrated the

* Corresponding author. Tel.: +81 52 789 4144; fax: +81 52 789 4145.
E-mail address: kojimat@nuagr1.agr.nagoya-u.ac.jp (T. Kojima).

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2015.08.001

utility of the devices for the *in vitro* co-transcriptional ribozyme-ligation reaction and the cell-free protein synthesis reaction, both of which are fundamental reactions in high-throughput screening for novel biological macromolecules.

MATERIALS AND METHODS

Oligonucleotides All oligonucleotides were purchased from IDT-DNA Technologies (Coralville, IA, USA) or Greiner bio-one (Tokyo, Japan). The primer sequences used in this study are as follows: Fw1-T7-F05, CGATCCCGCG AAATTAATAC TTCTAATACG ACTCACTATA GGAACAC; Fw1-SP6-F05, CGATCCCGCG AAATTAATAC TTCATTTAGG TGACACTATA GGAACACTAT ACTAC, bcI-23 Fw2, GATCCCGCGA AATTAACAC; bcI-23-R05-bio, biotin-GCAACAGGAA TATTAATCAC T; Bio-EcoRI, biotin-GTCGAATTCG CTCCGTCACC; R1 primer, TCCGGATATA GTTCCTCCTT TCAG; bcI-23-R02-Cy5, Cy5-GCACAGGAAT ATTAA. Single strand RNA oligonucleotide S-bcI with the sequence biotin-GACUCCAGUA was purchased from IDT-DNA Technologies.

Jet collection device A Pyrex glass capillary (outer diameter of 3 mm and inner diameter of 1.8 mm, Asahi Glass, Tokyo, Japan) was sealed on one end using a flame. Then, the sealed end was abraded with sandpaper until an orifice of approximately 100 μm in diameter was formed. The capillary was then washed in an ultrasonic bath, dried, and rendered hydrophobic by treating with Sigmacote (Sigma–Aldrich, St. Louis, MO, USA). A 19-gauge stainless steel needle was used to deliver the water phase. All parts were connected using a tee column end fitting TCEF211T (Valco Instruments, Houston, TX, USA), as shown in Fig. 1A. KDS-100 syringe pumps (KD Scientific, Holliston, MA, USA) were used to supply water and oil phases.

Capillary collection device Plastic parts from a Terumo NANOPASS 33 needle (Terumo, Tokyo, Japan) were removed, and the needle was cut with ultrasonic cutter ZO-40B (Honda Electronic, Toyohashi, Japan) on both ends to generate blunt ends. Then, the needle was attached by the thick end to 1/16″ PTFE tubing, 35 mm in length, using Cemedine PPX adhesive (Cemedine, Tokyo, Japan). This was then inserted into a Pyrex glass capillary 35 mm in length and fixed with Cemedine PPX adhesive. A glass capillary with an outer diameter of 1 mm and an inner diameter of 0.6 mm (G-1, Narishige Scientific Instrument Laboratory, Tokyo, Japan) was rendered hydrophobic with Sigmacote. The capillary was inserted into a tee column end fitting, as shown in Fig. 1B. KDS-100 syringe pumps were used to pump in water and oil phases.

Assessment of droplet generation Mineral oil for infrared spectroscopy (Sigma–Aldrich) containing 4% (wt/vol) Sunsoft No. 818SK (Taiyo Kagaku, Yokkaichi, Japan) and 1% (wt/vol) Span 80 was used as the oil phase. Phosphate buffer saline (PBS) with 0.05% bromophenol blue was used as water phase. Two syringe pumps (KD Scientific) were used to drive the flow of oil and water phases. The resultant emulsion was examined under a microscope (Olympus BX21, Olympus, Tokyo, Japan), and at least 100 droplets were assessed using Motic Image Plus (Motic, Hong Kong, China) to calculate the average diameter and coefficient of variation (CV). The process of droplet generation by the jet collection device was taken on video using a high-speed camera with 36000 fps (Fastcam SA 1.1, Photron, Tokyo, Japan).

Cell-free protein synthesis in emulsion *Escherichia coli* S30 extract was prepared as previously described (21). Biotinylated DNA template was prepared by PCR with Bio-EcoRI and R1 primers as shown in Fig. S1. DNA template (1.4 ng) was incubated for 15 min with 5×10^5 streptavidin-coated Dynabeads M-280 (Dynal Biotech, Invitrogen, Carlsbad, CA, USA) followed by two washes with PBS. *E. coli* S30 extract was added to the microbeads and emulsified immediately. Oil phase consisted of 4.5 % (wt/vol) Span 80 and 0.5 % (wt/vol) Triton X-100 in mineral oil (Sigma–Aldrich). The aqueous mixture was gradually added to the oil phase while stirring with a magnetic stirrer (F-606N, Tokyo Glass Kikai, Tokyo, Japan) at dial setting 4.5 (50 Hz) for 30 s on ice. Microfluidic emulsions were prepared as follows. One ml of mineral oil was loaded into a 2.5 ml syringe with Luer lock. Samples (20 μl) were then loaded into the same syringe as a single drop in mineral oil. Oil and water flow rates were set as 136 ml/h and 10 ml/h, respectively. The resulting emulsion was collected at a distance of approximately 5 cm from the tip of nozzle and subsequently incubated at 37°C for 1 h. The droplets were precipitated by centrifuging at 17,400 ×*g* for 1 min at 4°C. The oil phase was removed. The remaining droplets were suspended in 500 μl hexane and centrifuged at 17,400 ×*g* for 1 min at 4°C. The resulting oil phase was removed, and the emulsion was disrupted by adding 500 μl PBS, mixing, and centrifuging at 17,400 ×*g* for 1 min at 4°C. To remove any residual oil phase, the microbead suspension was washed with 500 μl hexane, mixed, and centrifuged at 17,400 ×*g* for 1 min at 4°C, with subsequent removal of the oil phase. This process was repeated twice. Microbeads were then washed twice with 100 μl PBS and incubated with 50 μl (1 ng/μl) of Penta-His Alexa Fluor 647 Conjugate (QIAGEN, Venlo, Netherlands) at 37°C for 1 h. After removing the supernatant, the microbeads were resuspended in 500 μl PBS and subjected to flow cytometry analysis (JSAN, Bay Bioscience, Kobe, Japan). The flow cytometry data were analyzed using FlowJo (Treestar, Ashland, OR, USA).

Co-transcriptional ligation in emulsion Co-transcriptional ligation in emulsion was performed as previously described (8), with some modifications. pGEM/bcI23, which contains class I ligase ribozyme clone 23 (bcI23) (22), was a kind gift from Dr. S. Ohuchi. A T7 promoter was added to bcI23 by PCR amplification using *Extaq* DNA Polymerase (Takara Bio, Otsu, Japan) with Fw1-T7-F05 and bcI23-R01 from pGEM/bcI23 as the template (T7P-bcI23). A SP6 promoter was added to bcI23 using the same process with Fw1-SP6-F05 and bcI23-R01 (SP6P-bcI23). The fragments were cloned into a pGEM-T Easy Vector (Promega, Madison, WI, USA) (pGEM/Fw1-T7P-bcI23 or pGEM/Fw1-SP6P-bcI23, respectively). Each promoter-bcI-23 fragment was amplified from pGEM/Fw1-T7P-bcI23 or pGEM/Fw1-SP6P-bcI23 in a 20 μL PCR mixture using 0.025 U/mL *ExTaq* DNA polymerase (Takara Bio) and 0.25 μM primers (bcI-23-Fw2 and bcI-23-R05-bio, each) using the following temperature protocol: preheat at 94°C for 5 min, 23 cycles consisting of 94°C for 15 s, 50°C for 10 s, and 72°C for 15 s, and an additional extension at 72°C for 7 min. PCR products were purified with the FastGene PCR/Gel Extraction Kit (Nippon Genetics, Tokyo, Japan). The concentrations of purified DNA were measured with NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA) and Quant-iT dsDNA BR Assay (Life Technologies, Carlsbad, CA, USA). Dynabeads M-280 (Life Technologies) (1×10^5) were washed twice with B & W buffer (2 M NaCl, 10 mM Tris–HCl pH 8.0, and 1 mM EDTA). 10 ng of biotinylated DNA template (Fig. S2) was diluted in B & W buffer and added to the microbeads, followed by incubation at 25°C for 30 min with agitation. Microbeads were washed with B & W buffer, and 10 μl of 2 μM biotinylated RNA substrate, S-bcI, in deionized water was added. Following incubation at 25°C for 15 min with agitation, microbeads were washed with deionized water and moved to new tubes. To each reaction, 10 μl of transcription mix containing 40 mM Tris-Acetate buffer with pH 8.0, 5 mM Spermidine, 10 mM $MgCl_2$, 0.5 mM of each NTP, 0.01% BSA, 1 U/μl RNase inhibitor (Toyobo, Osaka, Japan) and 2.5 U T7 RNA polymerase (New England Biolabs) was added. Reactions were emulsified using a vortex or FF device and incubated in a water bath at 37°C for 1 h. Emulsion was disrupted by the addition of 600 μl hexane. After mixing, the microbeads were resuspended in 20 μl blocking solution (1× RT buffer from New England Biolabs, 0.1 mg/ml salmon sperm DNA, 1 U/μl RNase inhibitor), transferred to new tubes, and the supernatant was removed

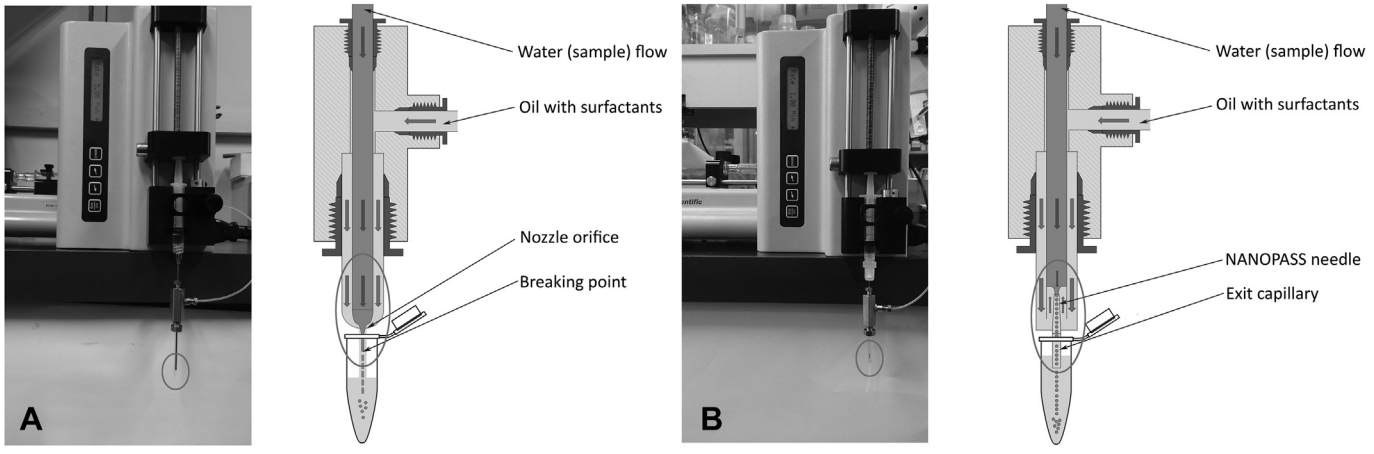

FIG. 1. Schematics of flow-focusing devices: (A) jet collection device; (B) capillary collection device. Circles indicate droplet generation area.