Continuous-flow ATP amplification system on a chip

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Abstract

In this study, we constructed a novel microfluidic device for continuous-flow ATP amplification, using the SU-8:PDMS method. Sepharose beads immobilized with adenylate kinase and pyruvate kinase was packed into a microfluidic chamber to form lamination layer. Dry film type SU-8 was suitable to form a very thick mold for beads column reactor and its dam structure. A good correlation between amplified luminescence and initial ATP concentration was observed in this system. The gradient of amplification when performing 6 cycles of continuous-flow ATP amplification was 1.72^{N}.

Keywords: Bioluminescence assay; ATP amplification; Continuous-flow system; PDMS chip

1. Introduction

Firefly luciferase-based bioluminescence assay for ATP detection is a well-established technique [1]; it has been employed in many industrial areas, such as clinical analysis and hygiene monitoring, because it is useful for rapidly detecting and quantifying a broad range of ATP concentrations [2]. In order to fully utilize the advantages and potential of bioluminescence technology, several problems must be overcome, the most important being the relatively low sensitivity of the assay. At low concentrations of ATP, bioluminescence is too weak to be detected by a luminometer, thus hindering the development of new technologies. Therefore, it is important to increase sensitivity.

We have enhanced the sensitivity of ATP detection by developing an ATP amplification reaction employing (i) adenylate kinase (ADK) to convert AMP + ATP into 2 molecules of ADP, (ii) pyruvate kinase (PK) or polyphosphate kinase to convert ADP back to ATP (ATP amplification) and (iii) commercially available firefly luciferase (Fig. 1). This technique was effective for detecting low levels of ATP and bacterial contamination [3]. Furthermore, we have also developed an ATP amplification reactor, using a continuous-flow system (Fig. 2) that enabled us to control the number of ATP amplification cycles [4]. This system was an effective way of performing reproducible ATP amplification and increasing the sensitivity of ATP quantification by bioluminescence assay.

However, the system was not optimized to a continuous-flow ATP amplification reaction. Enzymes were immobilized on commercially available columns, and reaction mixtures were injected on high-performance liquid chromatography (HPLC) columns. Although the shortest commercially available column was employed, its volume was still too large for each reaction, thus resulting in excessive back pressure and high enzyme consumption problems. Another drawback of a large flow system is lack of portability and versatility. A specialized device was proposed to solve these problems.

In the last two decades, microfluidic devices have been developed in various areas by applying semiconductor microfabrication technologies, and many biochemical reactions have been performed using these devices [5-7]. For example, Kopp *et al*. developed a continuous-flow system on a chip for multistep enzyme reaction such as polymerase chain reaction (PCR) [8], and they successfully performed PCR at high speed, using a microfluidic

device. Though the PCR device employed unbound enzymes, immobilized enzyme method was also developed in both of open tubular type [9-11] and packed column type [12-14]. Reduction of the reactor size is beneficial in reducing expensive reagents consumption. The microfluidic fields also aim to develop a handy analyzer for on-site measurements. Adoption of the pretreatment amplification reactor to chip format may be useful to future integration into such a portable device. In recent years, microfluidic devices fabricated with SU-8 photoresist and polydimethylsiloxane (PDMS) have been commonly used because of rapid and easy prototyping [15-19]. Though PDMS chips have low pressure durability, they have been applied to packed column reactor [20].

In this study, microfluidic devices for continuous-flow ATP amplification were fabricated by the SU-8:PDMS method. Dry film SU-8 was used to provide a very thick mold structure to reduce the pressure drop. ADK and PK-immobilized Sepharose beads were sequentially packed and layered on a PDMS chip. This microreactor system was useful and effective as a large flow system for ATP amplification control.

2. Experimental

2.1 Reagents

PK, ADK, ATP and phosphoenolpyruvate (PEP) were obtained from Sigma-Aldrich (Missouri, USA). AMP was obtained from Tokyo Chemical Industry (Tokyo, Japan). To remove ATP and ADP contaminations in AMP and PEP, AMP was purified using a

diethylaminoethanol column (Tosoh, Tokyo, Japan) with buffer A [10 mM NaCl and 10 mM citrate buffer (pH 2.85)] and buffer B [1 M NaCl and 10 mM citrate buffer (pH 4.40)]. AMP was eluted after 30 min with a linear gradient of 0%–100% buffer B. Next, 50 nM PEP was manually injected into a 1-ml HiTrap NHS-activated HP column immobilized with 10 U apyrase, according to the manufacturer's instructions. After 2 h of incubation at room temperature in the column, injection of 1 ml buffer containing 5 mM $MgCl_2$ and 50 mM HEPES-NaOH (pH 7.4) extruded PEP from the column. The bioluminescence assay mixture contained 25 μg luciferase (Bioenex, Higashi-Hiroshima, Japan), 2 mM luciferin, 1 mM $(CH_3COO)_2$ Mg, 5% (w/v) trehalose and 50 mM HEPES-NaOH (pH 7.8).

2.2 Enzyme immobilization on Sepharose beads

One millilitre N-Hydroxysuccinimide (NHS)-activated Sepharose beads (average diameter = 34 μm) was extracted from a 1-ml HiTrap NHS-activated HP column (GE Healthcare Ltd., Amersham, UK), suspended in 10 ml isopropanol and stored. Immobilization of PK and ADK was performed as follows. PK and ADK were each dissolved in 0.5 M NaCl and 0.2 M $NaHCO_3$ (pH 8.3). Furthermore, 100 μl of the Sepharose bead suspension was washed with 1.4 ml of ice-cold 1 mM HCl. Each of the Sepharose beads was obtained by centrifugation (6,000 rpm, 25°C), and 200 μl PK solution (144 μg/ml) and 100 μl ADK solution (60 μg/ml) were added to the beads. After 3 h of incubation at 25°C, the supernatants were removed by centrifugation (6,000 rpm, 25°C) and the beads were washed 3 times with 1 ml buffer containing 5 mM $MgCl_2$ and 50 mM HEPES-NaOH (pH 7.4). The amount of immobilized

enzyme was calculated by subtracting the amount of enzyme in the supernatant after immobilization from that present before immobilization. After washing, the PK- and ADK-immobilized Sepharose beads were suspended in 500 and 200 µl washing buffer, respectively.

2.3 PDMS chip fabrication

The reaction chamber chip (pattern code: AA07), which comprised of a flow channel and a dam structure for packing the Sepharose beads, was designed (Fig. 2). The first mask for whole flow path including chambers (simple rectangle 90.0×1.0 mm) and the second mask only for chamber pattern(simple rectangle 85.0×1.0 mm) were designed. The two patterns were aligned with their inlet side. The photomasks were obtained from Mitani Micronics Co., Ltd. (Tokyo, Japan). A 25-µm thick dry-film SU-8 photoresist (Kayaku MicroChem Co., Tokyo, Japan) was laminated on a 4 inch silicon wafer on a hot plate heated at 50°C, and a whole flow path pattern was irradiated with the first photomask for 20 s, using a mask aligner (EMA-400; Union Optical Co., Ltd., Tokyo, Japan). Six sheets of 45-µm dry film were then laminated at 50°C. The irradiated pattern area of the first SU-8 layer got the reddish colour during the lamination process and was hardly succeeded to align with the second pattern mask. After the second irradiation step, the silicon wafer was baked at 60°C for 2 min and then at 90°C for 10 min with a hot plate used for the post-exposure bake process. The silicon wafer was developed for 10 min and thoroughly cleaned with 1-methoxy-2-propyl acetate (SU-8

developer; Kayaku MicroChem, Japan). All photolithography processes were performed in a clean, photolithographically dark room.

Furthermore, 35 g of silicone elastomer from the PDMS kit (Sylgard 184, Dow Corning Co.) was added with 5 g of its curing agent. The mixture was mixed and defoamed by using a centrifugal mixer (AR-100; THINKY, Tokyo, Japan). The mixture was then poured over the silicon wafer with the SU-8 structure as a mold master. After curing at 80°C for 1 h, the PDMS replica was peeled off from the mold. Two-millimetre holes were punched out to create an inlet and outlet on the flow channels. The PDMS replica was bound to a specially ordered glass slide (28×100 mm) with O_2 plasma (4.5 Pa, 5 mA, 45 s), using a plasma etching chamber (SEDE-HM; Meiwafosis Co., Ltd., Osaka, Japan).

2.4 Construction of a flow system and evaluation of the PDMS chip

A flow system was constructed by connecting a syringe pump (KD Scientific Inc., Massachusetts, USA), a digital pressure gauge (GC67; NAGANO KEIKI Co., LTD., Tokyo, Japan), a manual sample injector (rehodyne 7010; sample loop volume, 500 μl; IDEX Corporation, Illinois, USA) and the PDMS chip. Sepharose beads without immobilized enzymes were injected into the reaction chamber for back pressure evaluation. After packing, the back pressure of the chip was measured by running a buffer containing 5 mM $MgCl_2$ and 50 mM HEPES-NaOH (pH 7.4) at various flow rates.

2.5 Continuous-flow enzymatic reaction and bioluminescence assay

PK and ADK-immobilized Sepharose beads were prepared by the above method. Various amounts of the PK and ADK-immobilized beads were injected into the reaction chamber, and the lengths of filled areas were measured. Each chip was equilibrated with 1 ml of running buffer containing 5 mM $MgCl_2$ and 50 mM HEPES-NaOH (pH 7.4) at 0.03 ml/min. After equilibration, 0.5 ml of a mixture containing 100 nM ADP, 1 mM PEP, 5 mM $MgCl_2$ and 50 mM HEPES-NaOH (pH 7.4) for PK and 50 nM ATP, 0.1 mM AMP, 5 mM $MgCl_2$ and 50 mM HEPES-NaOH (pH 7.4) for ADK was injected at room temperature. For each reaction, 1.2 ml of the reaction mixture was collected in 30-µl fractions from the outlet.

The amount of ATP and ADP in each fraction was measured as follows. For ATP, 5 µl of each fraction was mixed with 45 µl of a bioluminescence assay mixture containing 25 µg luciferase, 2 mM luciferin, 1 mM $(CH_3COO)_2$ Mg, 5% (w/v) trehalose and 50 mM HEPES-NaOH (pH 7.8), and the bioluminescence was measured using a luminometer (C-1000; Kikkoman, Chiba, Japan). Control ATP samples were also measured for calibration of each day, and luminescence intensity was converted to ATP concentration. For ADP + ATP, 100 µl of each reaction mixture was mixed with 1 µl of a solution containing 10 U/ml PK, 1 mM PEP, 5 mM $MgCl_2$ and 50 mM HEPES-NaOH (pH 7.4), followed by incubation at 37°C for 30 min. When the PK reaction reached equilibrium, 5 µl of the reaction mixture was added to 45 µl of the bioluminescence assay mixture, and bioluminescence was measured using a luminometer. The amount of ADP in each fraction was calculated by subtracting the amount of ATP from that of ATP + ADP.

2.6 Continuous-flow ATP amplification reaction

PK and ADK-immobilized Sepharose beads were prepared as described above. Filled areas of ADK and PK beads (ADK and PK layers) were layered as follows. A 2-mm layer in the flow direction of the PK beads was injected into the reaction chamber, followed by a 1-mm layer of beads without enzyme to avoid mixing of ADK and PK beads during the packing process. A 6-mm layer of ADK beads was then injected into the chamber, followed by a 1-mm layer of no-enzyme beads. The number of ADK-PK layers was increased by repeating the procedure described above. In the case of 6 ADK-PK layers, 59 mm of a 85 mm chamber was filled with the beads. Reaction mixtures containing 50 nM ATP, 1 mM PEP, 0.1 mM AMP, 5mM $MgCl_2$ and 50 mM HEPES-NaOH (pH 7.4) were injected into the reaction chamber at 0.03 ml/min at room temperature. Following amplification, 1.2 ml of each reaction mixture from the outlet was collected in 30-µl fractions.5 µl from each fraction was mixed with 45 µl of the bioluminescence assay mixture, and bioluminescence was measured using a luminometer.

3. Results and Discussion

3.1 Characteristics of the chips

Other designs were examined before the chip design was adopted. Residual time is a critical factor in the system design, because dead volumes at the connection tubes between injector and chip and between injector and collection port are much larger than the volume of

the reaction column. A wide cross-sectional area perpendicular to flow direction in the reaction column is beneficial to reduce the pressure drop. Mold fabrication requires a microfabrication technique to employ a dam structure to stop enzyme carrier beads at the end of the column. The SU-8:PDMS method is a well-established technique for achieving a flow channel with microstructures. However, it is practically difficult to make the structure thicker than 100 μm. In this research, we employed a newly developed film type SU-8 to achieve a thick structure. A 6-fold lamination was completed within 30 minutes. Preliminary column design experiments revealed that a column of 300 μm thickness was better than one of 100 μm. Column designs (AA01: simple rectangle of 30.0mm column and 10.0mm dam in length, both 1.0mm in width, AA02: two tandem columns with additional beads inlet holes, AA05: twice folded line of 1.0mm in width with 1 mm gap, and and AA09: rectangle of 30.0 mm length and 10.0 mm width with expanding pipe shape at both ends) were fabricated and examined. Figure 3 shows some typical packing results. Separate packing was practically unable for us due to back stream at the branch structure in AA02. Leakage was often observed at folded structure (AA05). Lamination layer packing was unable due to turbulence in the very wide column (AA09). Only AA01 was effective for a few layer experiments, but insufficient in length for more than 3 sets of reactions. Multilayer introduction to AA07 was possible, though the boundaries were slightly unflattened, probably due to the wall effect. Then we finally chose the design AA07.

The pressure durability of the chip was examined by increasing the flow rate. Sepharose beads leaked from the dam structure of the chip at a pressure of >0.3 MPa, and they did form

a contact surface between the PDMS and glass slide at >0.8 MPa. Therefore, the back pressure must not exceed 0.3 MPa on the chip. The maximum flow rate within this capacity range was 0.06 ml/min. Slow flow rate is beneficial for pressure drop problem, but worse for rapid analysis. It took 30 min to flow sample from an injector to a fraction collector in our system, which is longer limit to complete whole process within an hour. Thus, a flow rate of 0.03 ml/min was employed in subsequent experiments.

3.2 Length optimization of the layer of immobilized beads

The amount of immobilized ADK and PK was 0.8 μg and 1.6 μg in the ADK and PK bead suspensions, respectively. The ADK and PK reactions in each layer must reach equilibrium to achieve full and reproducible ATP amplification. This was accomplished in our previous continuous-flow system by manipulating the flow rate to control the reaction time. However, in this study, the flow rate was set at 0.03 ml/min. Therefore, the amount of immobilized ADK and PK beads was manipulated to reach equilibrium in each enzymatic reaction.

A flow chart of the 0.5-ml ATP sample showed a peak ATP concentration with a plateau area at the top, indicating no dilution. Thus, 0.5 ml was enough volume for a reaction on this chip (Fig. 4).

In the ADK and PK layers, the reactants reached equilibrium with lengths of >5.5 and >2 mm, respectively, at a flow rate of 0.03 ml/min (Fig. 5). Thus, 6 mm of the ADK layer and 2 mm of the PK layer were employed in subsequent experiments. Thus the consumption of enzymes for column formation was drastically decreased to 1.2% (PK) and 3.6% (ADK) of

that of the previous report. One week storage of the 10% suspension of the beads at -80, -20, and 4°C showed no decrease in the activities though freeze dry method was not applicable due to gel beads deformation.

3.3 Continuous-flow ATP amplification on the PDMS chip

The concentration of ATP in the reaction mixture was amplified by increasing the ADK-PK layers. A good exponential correlation was observed between the amplified ATP and the number of ADK-PK layers (R^2=0.9866). The gradient of amplification is theoretically 2^N, was 1.87^N in our previous report with large reactor columns, but was 1.72^N in this experiment (N = number of ADK-PK layers; Fig. 6). These results suggest that the proposed reaction to control reaction cycles by the number of ADK-PK layers was realized in the constructed system. However, the bases of exponential function were less than the theoretical value, 2. The results in Figure 6 and some other experiments show the tendency that the highly repeated amplification gave low bioluminescence, though a single layer microreactor converted about 100% of such high concentration sample ATPs.

A system using commercially available columns and an HPLC system has no portability and versatility. In this study, a specialized device was constructed. While the previously reported system required approximately 52 mm immobilized zone for 1 cycle of ATP amplification, this device needed only 9 mm immobilized zone per cycle and allowed 6 cycles of the ATP amplification reaction to be performed on a glass slide-sized chip. Given the improvement in portability, this chip made it possible to perform a continuous-flow ATP amplification reaction on site though further integration with flow and detection system is

required. Furthermore, this system could be employed with other microfluidic devices. Thus, our design system had also improved the versatility of this system.

A continuous-flow system using commercially available columns required the immobilization of large amounts of enzyme (144 μg PK and 30 μg ADK for 1 cycle of ATP amplification) for a single reaction step, thus resulting in high cost of amplification. Miniaturization helped reduce the amounts of immobilized enzyme to only 1.6 μg of PK and 0.8 μg of ADK. The consumption of enzyme was lower than for the system using commercially available columns.

3.4 Back pressure on the chip

The maximum number of ATP amplification cycles was increased, and 6 cycles were performed in the chip system. The previous system relying on commercially available columns had excess enzyme immobilization capacity, which resulted in a higher pressure drop, thus limiting the maximum number of ATP amplification cycles. In this system, the Sepharose bead capacity was fully utilized to reduce the amounts of beads necessary to reach equilibrium in each enzymatic reaction. The pressure drop was reduced to a minimum.

However, the maximum number of ATP amplification cycles was 6 because the back pressure could not exceed the durability of the chip. Even with this specialized continuous-flow system, the pressure drop is still a critical factor that limits the number of ATP amplification cycles. A system with an amplification cycle that does not rely on the number of ADK and PK-immobilized areas could help to decrease the back pressure. For

example, the pressure drop can be further reduced using a circular flow system [21] containing a single set of ADK and PK areas. Further development is needed to reduce the back pressure on the system.

4. Conclusion

We devised and tested a continuous-flow ATP amplification system using a PDMS chip in which the number of ATP amplification cycles could be controlled by the number of ADK-PK layers. A good exponential correlation between amplified luminescence and the number of ADK-PK layers was observed. This system was miniaturized, thus making it possible to perform a continuous-flow ATP amplification reaction on site. Furthermore, this system could be employed with other microfluidic devices, thus also improving its versatility. We tried to immobilize luciferase for future detector integration, but less than 1% original activity was remained after immobilization, which gave too weak luminescence to be detected by highly sensitive CCD camera. To reduce the system complexity, some improvement should be done such as avoiding back pressure increase with amplification cycle increase, integration with a bioluminescence detection part, and luciferase immobilization.

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References

[1] M. DeLuca, W. D. McElroy, Kinetics of the firefly luciferase catalyzed reactions, Biochemistry 26 (1974) 921-925.

[2] D. Champiat, N. Matas, B. Monfort, H. Fraass, Applications of biochemiluminescence to HACCP, Luminescence 16 (2001) 193-198.

[3] T. Satoh, J. Kato, N. Takiguchi, H. Ohtake, A. Kuroda, ATP amplification for ultrasensitive bioluminescence assay: detection of a single bacterial cell, Biosci. Biotechnol. Biochem. 68 (2004) 1216-1220.

[4] T. Satoh, Y. Shinoda, M. Alexandrov, A. Kuroda, Y. Murakami, Continuous-flow ATP amplification system for increasing the sensitivity of quantitative bioluminescence assay, Anal. Biochem. 379 (2008) 1067-1078.

[5] A. Manz, N Graber, H. M. Widmer, Miniaturized Total Chemical Analysis Systems: a Novel Concept for Chemical Sensing, Sens. Actuators, B1 (1990) 244-248.

[6] P. S. Dittrich, K. Tachikawa, A. Manz, Micro Total Analysis Systems. Latest Advancements and Trends, Anal. Chem. 78 (2006) 3887-3907.

[7] K. Ohno, K. Tachikawa, A Manz, Microfluidics: applications for analytical purposes in chemistry and biochemistry, Electrophoresis, 29 (2008), 4443-4453.

[8] M. U. Kopp, A. J. Mello, A. Manz, Chemical amplification: continuous-flow PCR on a chip, **Science** 280 (1998) 1046-1048.

[9] Y. Murakami, T. Takeuchi, K. Yokoyama, E. Tamiya, I. Karube, M. Suda, Integration of Enzyme-Immobilized Column with Electrochemical Flow Cell Using Micromachining Techniques for a Glucose Detection System, Anal. Chem. 65 (1993) 2731-2735.

[10] Y. Murakami, M. Suda, K. Yokoyama, T. Takeuchi, E. Tamiya, I. Karube, Micromachined Enzyme Reactor for FIA System, Microchem. J. 49 (1994) 319-325.

[11] D. L. Huber, R. P. Manginell, M. A. Samara, B. I. Kim, B. C. Bunker, Programmed Adsorption and Release of Proteins in a Microfluidic Device, Science, 301 (2003) 352-354.

[12] I. Karube, K. Yokoyama, Trends in biosensor research and development, Sens. Actuators B13-14 (1993) 12-15.

[13] R. D. Oleschuk, L. L. Shultz-Lockyear, Y. Ning, D. J. Harrison, Trapping of bead-based reagents within microfluidic systems: on-chip solid-phase extraction and electrochromatography, Anal. Chem. 72 (2000) 585-590.

[14] H. Nakamura, Y. Murakami, K. Yokoyama, E. Tamiya, I. Karube, M. Suda, S. Uchiyama, A Compactly Integrated Flow Cell with a Chemiluminescent FIA System for Determining Lactate Concentration in Serum, Anal. Chem. 73 (2001) 373-378.

[15] H. Lorenz, M. Despont, N. Fahrni, N. LaBianca, P. Renaud, P. Vettiger, SU-8: a low-cost negative resist for MEMS, J. Micromech. Microeng. 7 (1997) 121-124.

[16] D. C. Duffy, J. C. McDonald, O. J. A. Schueller, G. M. Whitesides, Rapid Prototyping of Microfluidic Systems in Poly(dimethylsiloxane), Anal. Chem. 70 (1998) 4974-4984.

[17] T. Yamamoto, T. Fujii, T. Nojima, PDMS-glass hybrid microreactor array with embedded temperature control device. Application to cell-free protein synthesis, Lab. Chip. 2 (2002) 197-202.

[18] R. Pantoja, J. M. Nagarah, D. M. Starace, N. A. Melosh, R. Blunck, F. Bezanilla, J. R. Heath, Silicon chip-based patch-clamp electrodes integrated with PDMS microfluidics, Biosens. Bioelectron. 20 (2004) 509-517.

[19] P. Abgrall, V. Conedera, H. Camon, A. M. Gue, N. T. Nguyen, SU-8 as a structural material for labs-on-chips and microelecromechanical systems, Electrophoresis 28 (2007) 4539-4551.

[20] B. Jankovicova, S. Rosnerova, M. Slovakova, Z. Zverinova, M. Hubalek, L. Hernychova, P. Rehulka, J. L. Viovy, Z. Bilkova, Epitope mapping of allergen ovalbumin using biofunctionalized magnetic beads packed in microfluidic channels The first step towards epitope-based vaccines, J. Chromatogr. A 1206 (2008) 64-71.

[21] Y. Sun, Y. C. Kwok, N. T. Nguyen, A circular ferrofluid driven microchip for rapid polymerase chain reaction, Lab Chip 7 (2007) 1012-1017.

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Figure Legends

Fig. 1. Schematic representation of ATP amplification reaction and its detection. (i) Sample ATP reacts with excess AMP catalyzed by adenylate kinase (ADK) to produce twice mole of ADP. (ii) ADP reacts with excess phosphoenolpyruvate (PEP) catalyzed by pyruvate kinase

(PK) to produce equivalent mole of ATP, and (i) and (ii) makes chain-reaction. (iii) Amplified ATP is detected by luciferin-luciferase reaction.

Fig. 2. Reaction chamber chips with a flow channel and a dam structure. (a) Top view of AA07. AA07 consisted of a flow channel (Width, 1 mm; depth, 0.3 mm; length, 85 mm) and a dam structure (Width, 1 mm; depth, 0.025 mm; length, 5 mm). (b) Cross-section of the joint. The sepharose beads were packed inside the flow channel by the dam structure.

Fig. 3. Photos of the PDMS chips. (a) The column was fully packed with normal sepharose beads AA07. (b) The normal sepharose beads and dyed sepharose beads were alternately packed to the AA07 column. (c) Folded pattern (AA05) to reduce the chip size. Leakage of the beads was observed in AA05. (d) Wide column pattern (AA09) to enhance cross sectional area, and the packing condition of the beads in AA09 was worse than in AA07.

Fig. 4. ATP concentration gradients in 100-nM ATP sample. Concentrations of ATP in the fractions from the chip were indicated. ATP concentration in each fraction was assayed using luciferase, as described in Experimental.

Fig. 5. Conversion rates of ADK and PK with various layer lengths. Conversion rates were calculated by measuring the amounts of ADP and ATP. (a) Reactants reached equilibrium with a length >5.5 mm at a flow rate of 0.03 in the ADK layer. (b) Reactants reached equilibrium with a length >2 mm at a flow rate of 0.03 in the PK layer.

Fig. 6. Correlation between peak ATP concentrations and number of ADK-PK layers. ATP concentrations in peak top areas were obtained by averaging 5 fractions in each peak.

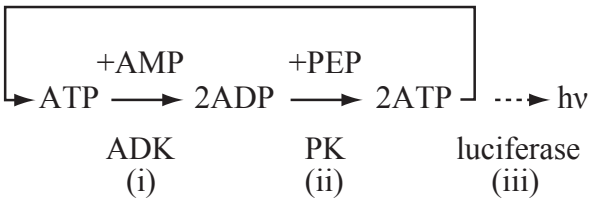

FIG 1

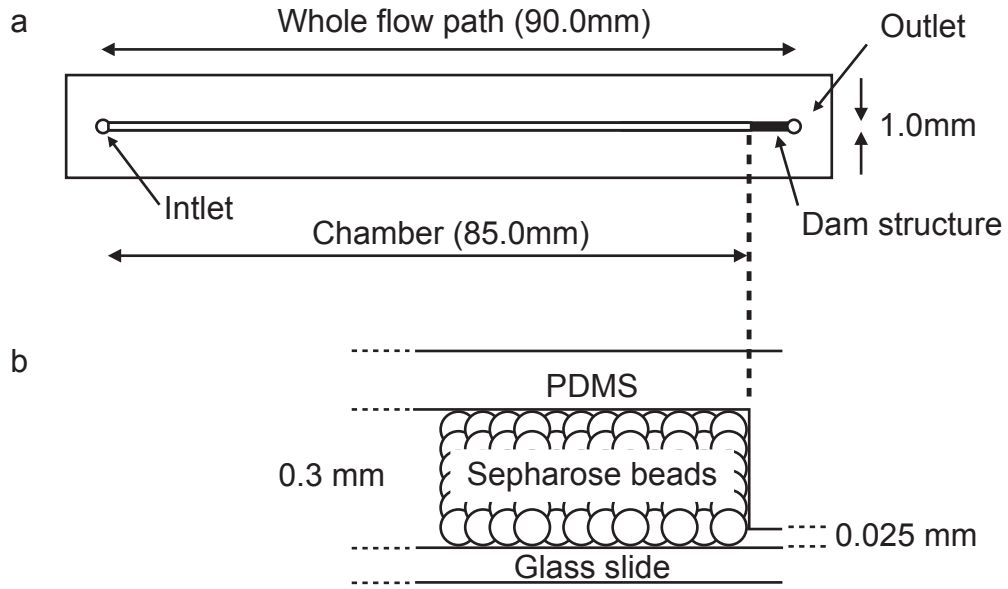
a
Whole flow path (90.0mm)
Outlet
1.0mm
Intlet
Chamber (85.0mm)
Dam structure
b
PDMS
0.3 mm
Sepharose beads
0.025 mm
Glass slide

FIG 2

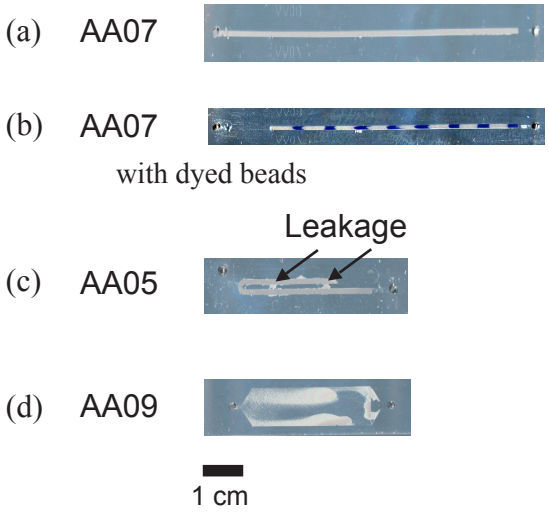

FIG 3

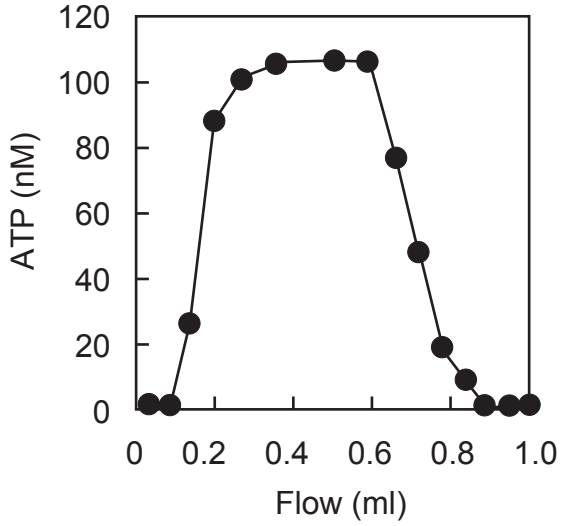

FIG 4

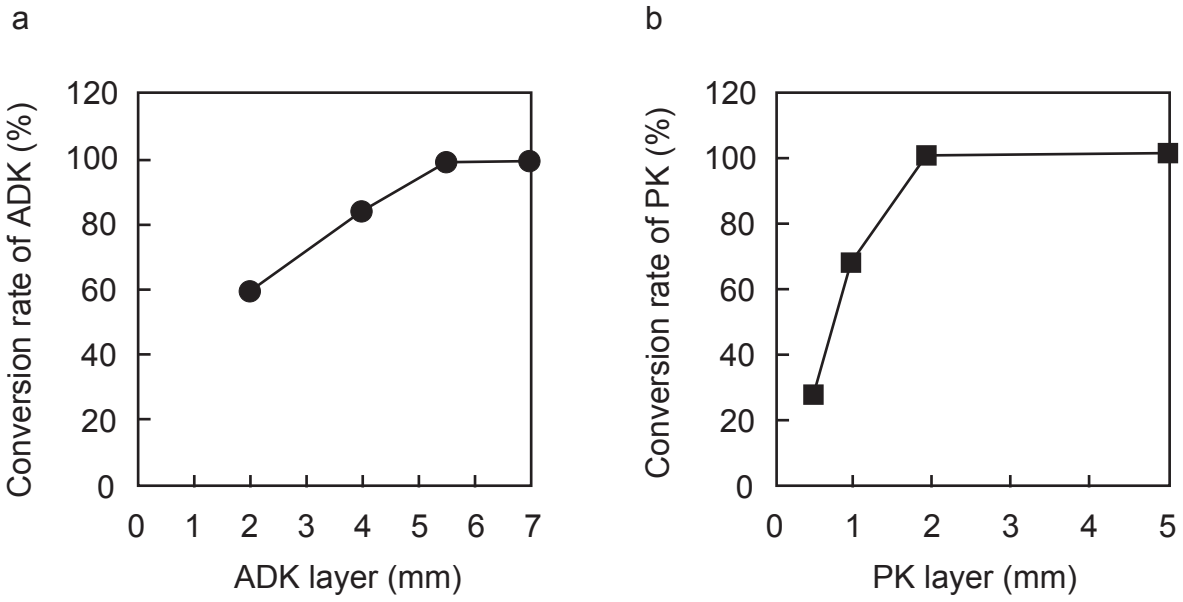
a
Conversion rate of ADK (%)
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7
ADK layer (mm)
b
Conversion rate of PK (%)
120
100
80
60
40
20
0
0 1 2 3 4 5
PK layer (mm)

FIG 5

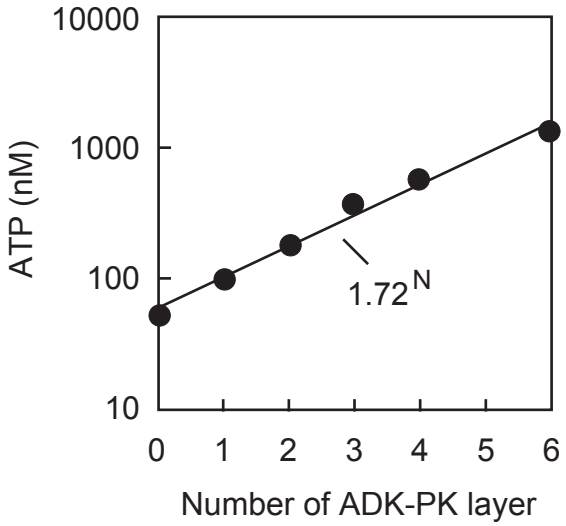

FIG 6