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A Bio-fluidic Device for Adaptive Sample Pretreatment and Its Application to Measurements of *Escherichia coli* Concentrations

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Abstract In this paper, we describe a bio-fluidic device for adaptive sample pretreatment, in order to optimize the conditions under which absorbance assays can be conducted. This device can be successfully applied to the measurement of *Escherichia coli (E. coli)* concentrations using adaptive dilution, with which the dilution ratio can be adjusted during the dilution. Although many attempts have been previously made to miniaturize complex biochemical analyses at the chip scale, very few sample pretreatment processes have actually been miniaturized or automated at this point. Due to the lack of currently available on-chip pretreatments, analytical instruments tend to suffer from a limited range of analysis. This occasionally hinders the direct and quantitative analysis of specific analytes obtained from real samples. In order to overcome these issues, we exploit two novel strategies: dilution with a programmable ratio, and to-and-fro mixing. The bio-fluidic device consists of a rectangular chamber constructed of poly(dimethylsiloxane) (PDMS). This chamber has four openings, an inlet, an outlet, an air control, and an air vent. Each of the dilution cycles is comprised of four steps: detection, liquid drain, buffer injection, and to-and-fro mixing. When using adaptive sample pretreatment, the range in which *E. coli* concentrations can be measured is broadened, to an optical density (O.D.) range of 0.3~30. This device may prove useful in the on-line monitoring of cell concentrations, in both fermenter and aqueous environments.

Keywords: absorbance, adaptive dilution, bio-fluidic device, *E. coli* concentration, sample pretreatment, to-and-fro mixing

INTRODUCTION

Recently, a variety of studies have been conducted on analytical systems, in the hope of developing a miniaturized system in which both biological and chemical analyses can be conducted [1-4]. These studies have met with varying degrees of success, but have resulted in the development of several small devices that are usable in a wide variety of applications, including clinical diagnostics [5], immunoassays [6], protein chips [7], and cell separation [8]. However, the majority of previous works have focused on downsizing, as it has been determined that analytical performance is best improved at the chip scale [4,9,10]. The previously developed devices, though, retain several limitations, as they tend to be applicable only to pre-determined analytical procedures, without proper sample pretreatment.

The generic analytical procedures associated with the acquisition of information from a sample should always include a sample pretreatment step, a process involving the appropriate preparation of samples, in order to achieve optimal conditions for analysis [11]. For example, biochemical reactions require a variety of specialized conditions, including proper pH and salt concentrations, as these reactions take place only under a specific and restrictive set of conditions. Electrophoretic separation requires a sample treatment which causes each component to move at a different rate. As most sensors have only a limited detection range, proper sample pretreatment is required in order for accurate and automatic measurements to be made. Therefore, measurement procedures can, occasionally, suffer from a lower degree of precision when the real samples are outside the optimal measurement range for sensor operation. In order to circumvent these disadvantages, various flow injection analysis techniques [12], and sample handling procedures predicated on fluidics [13], have been developed. For the practical and generalized use of a μTAS (micro total analysis system) device, it is important that the sample pretreatment is integrated onto chips [14,15].

In this paper, we describe a bio-fluidic device for adaptive sample pretreatment, and also apply this system toward the detection of *Escherichia coli (E. coli)* concentrations, in a broad range. We automated and min-

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iaturized the sample pretreatment step, using two strategies for adaptive dilution: dilution with a programmable ratio, and to-and-fro mixing. The term, "adaptive dilution" refers to a technique with which the measurement range can be broadened via a feedback control. For on-chip fluid manipulation, we employed droplet manipulation techniques within the bio-fluidic device, as mixing and dilution within the device were easier to achieve than in a continuous flow situation. In order to change the dilution ratio with the feedback signal, all of the analytical procedures were conducted using programmable sequences: reading, drain, injection, and mixing. Also, in order to maintain a constant sample volume during the several steps of the procedure, the injection volume was set equal to the amount of drained liquid. This allowed us to circumvent the limitations inherent to the limited volume of the dilution chamber within the bio-fluidic device.

The second strategy used with the bio-fluidic device in this study was "to-and-fro mixing". Micro-scale fluids seldom mix well without vortexing or shaking, and so many microfluidic researchers have tried to solve this problem. Several techniques have been employed in service of this goal, including the use of a complex channel network [16-18], turbulence-creating structures [19] and active mixers [20]. However, these approaches still require a specific flow-through structure for mixing. Recently, it was reported that the droplet movement in two or more directions is a reasonable way for mixing [21-24]. Therefore, we utilized a bi-directional mixing technique, in which the liquid in the bio-fluidic device is caused to move to-and-fro. After an appropriate duration of this bi-directional movement, the liquid should be uniformly mixed. The details of this technique are reported herein.

MATERIALS AND METHODS

Sample Preparation

E. coli was used as a model cell for this experiment. *E. coli* (DM5α(DE3)) cells were cultured at 37°C overnight in Super Broth medium. One liter of this medium contained 35 g of trypton, 20 g of yeast extract, 5 g of NaCl, 5 mL of 0.1 N NaOH, and deionized water. After the bacterial cells had been harvested via centrifugation, the medium was removed. The cell pellets were then washed twice with 1 mL of water, after which 1 mL of the cell suspension (more than 10^9 cells per mL) was collected. The samples, with six different concentrations, were prepared via dilution to arbitrary ratios. Red food dye (Roha Dyechem Limited, India) solution was employed in order to visualize the degree of mixing, and viscous samples were prepared using an aqueous glycerol solution (Sigma, St. Louis, MO, USA). The seven samples were shown to exhibit different viscosities, as the volume ratios of glycerol were 0, 5, 10, 25, 50, 60, and 70% (v/v). Each of the samples contained an identical quantity of red food dye.

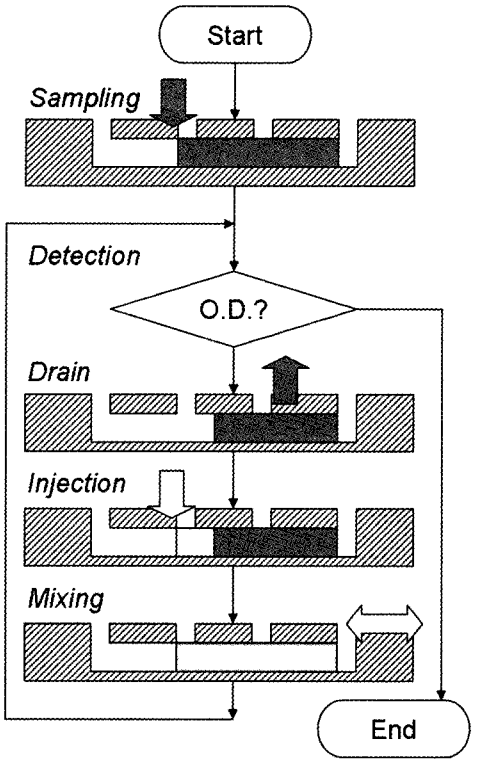

Fig. 1. The control logic of adaptive dilution in the bio-fluidic device.

Design of Control Logic and the Bio-fluidic Device

Fig. 1 shows the adaptive dilution logic, which encompassed both dilution with a programmable ratio and to-and-fro mixing. Measurements were conducted after sampling. The decision as to whether more dilution cycles were required was determined in accordance with the measurement signals. For one cycle of dilution, the bio-fluidic device drained some of the fluid, injected the buffer, and then mixed the two. This cycle was repeated until a set point, rather than by a predefined number of repetitions. Although no information regarding initial dilution ratios was provided, this adaptive dilution was intended to create a suitable measurement condition, via the adjustment of the concentration of the sample.

This logic was implemented by a simple device structure, and realized via pneumatic fluidic actuation. The bio-fluidic device was basically composed of one rectangular chamber, equipped with four connection ports (Fig. 2A). All fluidic actions, including injection, draining, and mixing, were carried out within the chamber. Each of the connection ports also had its own specific function, either injection, draining, air control, or air venting. The sample and dilution buffers were transported into the injection port, and the mixture was exported from the draining port. The control port and air vent were required for the carrying out of the pneumatic fluidic actuation. The control port at the right end of the chamber was connected to an external source of compressed air. The left end was

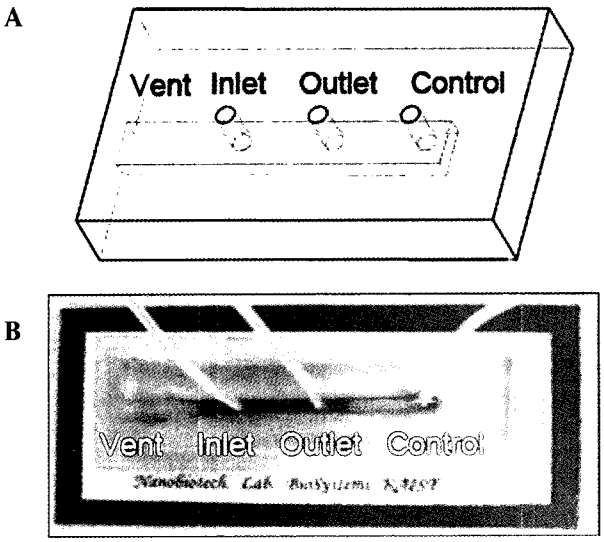

Fig. 2. Configuration of the bio-fluidic device: (A) the layout and (B) photograph. The overall size of the bio-fluidic device was 75 × 25 × 6 mm, where the dimensions of the chamber in the device were 50 × 2.5 × 2 mm.

occupied by an air vent. As a consequence, the air pressure pushed from the left and pulled to the right, in order to mix the liquid droplets within the bio-fluidic device.

Fabrication of the Bio-fluidic Device

The bio-fluidic device used in this study was constructed via replica molding, using the poly(dimethylsiloxane) (PDMS), which is an elastic, transparent polymer [25]. The chamber mold was constructed by attaching a piece of chamber shape film to the slide glass. The PDMS pre-polymer and curing agent (Sylgard 184; Dow Corning, Midland, MI, USA) were mixed at a 10:1 ratio, and stirred sufficiently. The mixture was then degassed under vacuum, until all bubbles had disappeared. The mixed PDMS was poured into the mold, and cured in a convection oven for 1 h at 80°C. The mold was then removed, and 3 mm diameter holes were punched for each of the port connections. In order to bond the PDMS with another slide glass, the surfaces of the PDMS replica and the slide glass were activated by air plasma at 200 mTorr and 200 W for 10 sec, using an expanded plasma cleaner (PDC-002; Harrick Science, Ossing, NY, USA). The injection, drain, and control ports were then connected to the pumps, using 1/16-inch Teflon tubes. A photograph of the fabricated device is provided in Fig. 2B. The overall size of the bio-fluidic device was 75 × 25 × 6 mm, and the dimensions of the chamber within the device were 50 × 2.5 × 2 mm.

Experimental Setup

The experimental setup involved a lamp, a spectrophotometer, syringe pumps, and a personal computer (PC) (Fig. 3). An optical fiber (core diameter 600 μm) was

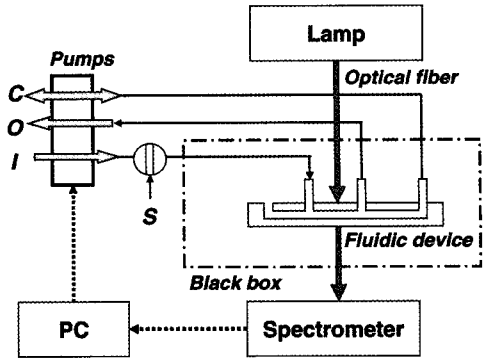

Fig. 3. Schematic of the experimental setup. The thick line arrow represents the path of the light, while the thin line arrow represents the flow of fluids. S: sampling, I: inlet of the buffer, O: outlet of the fluid, C: air control.

fixed at the top of the device, in order to transmit the light generated by a tungsten halogen lamp (LS-1; Ocean Optics, Dunedin, FL, USA). Another optical fiber was installed at the bottom of the device, and transmitted the light to a spectrophotometer (S2000-UV-VIS; Ocean Optics). A plastic mockup (100 × 55 × 32 mm) was constructed, and used to block the light. The syringe pumps (XL3000; Cavro, San Jose, CA, USA) were used for bio-fluidic actuation. Each pump was capable of pushing or pulling one of 24,000 steps, in a total range of 60 mm. We used 10 mL syringes, so the unit volume for each push or pull manipulation was 0.417 μL, with a coefficient of variation (CV) of 0.05%. Unless otherwise noted, a 50 μL sample volume was initially injected into the chamber inlet. The drain and injection volumes varied according to the dilution ratios. The spectrophotometer was interfaced using a USB (universal serial bus) connection, and the pumps were actuated via RS-232 serial communication. A control program was coded to realize the logic, using Visual C++ 6.0.

RESULTS AND DISCUSSION

Effect of Sample on "to-and-fro Mixing"

Prior to the sample concentration measurements, we assessed the degree to which to-and-fro movements (number and amplitude) had been achieved, in order to confirm sufficient fluid mixing. Because a drain of non-uniform droplets would result in an inaccurate dilution, full mixing is required for adequate dilution. We utilized red food dye solutions in order to determine the sufficiency of mixing achieved by three types of to-and-fro movement. The optimal mixing time for each of the different types of movement was monitored in accordance with the stabilization of the optical density signal. As the fluid became more thoroughly mixed, the signal gradually exhibits a reduction in its rate of fluctuation (Fig. 4). Although thorough mixing could be achieved by a diffusion-driven process with no to-and-fro movement, this required over

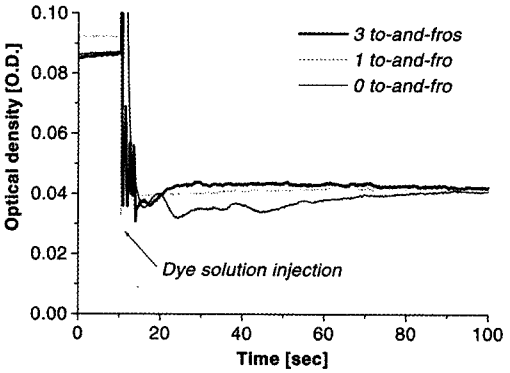

Fig. 4. The absorbance profiles after "to-and-fro mixing" of the red food dye. To-and-fro mixing was initiated at 10 sec, by injection dye solution. The absorbance was measured at 550 nm.

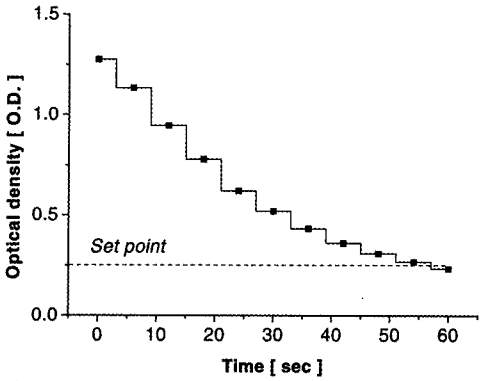

Fig. 5. The absorbance change after each dilution cycle at 550 nm. The set point was 0.25, the dilution ratio of each cycle was 0.845 ± 0.027(mean ± standard deviation).

Table 1. Effect of the sample viscosity on "to-and-fro mixing"

Glycerol (v/v, %)	Viscosity[a] (cps)	"to-and-fro number" for sufficient mixing
0	1.000	~5
5	1.143	10~15
10	1.311	10~15
25	2.095	15~20
50	6.050	30~
60	10.960	30~
70	22.940	30~

[a]The viscosity value was obtained from the supplier [27].

100 sec to realize. With one to-and-fro movement, thorough mixing was achieved in only 50 sec. With three to-and-fro movements, the mixing time was attenuated to only 10 sec, indicating that repetitive to-and-fro actuation was capable of increase mixing by a factor of almost 10. Therefore, in the following experiments, we utilized the "3 to-and-fro" movement setting, for 10 sec.

The mechanism by which thorough mixing of the sample occurred was believed to rely on the turbulence inherent to the circulation of the droplets [26]. When a liquid droplet moves in one direction, its velocity is fast in the center, and slower in the periphery. Each change of direction results in a difference in the velocity gradient, which elicits turbulence. Thus, the mixing effect tends to increase with repeated to-and-fro movements.

In order to characterize the effects of viscosity in the sample solution, we also analyzed a group of glycerol samples (Table 1). We attempted to determine the numbers of turn-around movements necessary for sufficient mixing in each of these samples. When the sample contained no glycerol, five to-and-fro movements were sufficient for optimal mixing. In samples containing 5 and 10% glycerol, 10 to 15 to-and-fro movements were required, at minimum, for efficient mixing. 20 to-and-fro movements were required to thoroughly mix a 25% glycerol solution. However, when a solution contained in excess of 50% glycerol, the sample could not be reliably mixed even with 30 to-and-fro movements. According to these results, samples with viscosity of less than 2.0 cps were considered to be mixable using to-and-fro movements. As the majority of biological samples range in viscosity from 0.3 to 1.7 cps, (for example, 1.0 and 1.2~1.4 cps for water at 20°C and plasma at 37°C, respectively), the mixing of most biological samples appeared to be rather feasible [28,29]. This result indicates that a to-and-fro strategy might prove quite useful in the mixing of viscous solutions, in a simple, passive, and monolithic structure.

Validation of Adaptive Dilution in the Bio-fluidic Device

Adaptive dilution was demonstrated in the bio-fluidic device using red food dye solutions. The sample volume was maintained at 50 μL, whereas the drain and injection volumes were both set at 25 μL (60 of 24,000 units with 10 mL syringe actuation). All steps in this procedure, including draining, injection, and mixing, were successfully conducted in the bio-fluidic device, coupled with instant monitoring and detection. The draining and injection steps required 1.5 sec each, and mixing took 3 sec. The sample returned to its original position again at 6 sec, during which the next cycle began. As we were dealing with microfluids, some gas bubbles problems occurred [3], but were resolved by careful pneumatic actuation.

Fig. 5 shows the results of the adaptive dilutions. We injected the red dye solution, and entered a command of "dilute until an absorbance of 0.25." The sample volume was maintained at 60 μL, whereas the draining and injection volumes were both 8.34 μL (20 of 24,000 units). The activity of the device was discontinued after 10 cycles. The initial absorbance was 1.27, and the final absorbance was 0.23. The dilution factors, which were calculated according to the formula: [present absorbance]/[past absorbance], were 0.845 ± 0.027 (mean ± standard deviation). The absorbance decrement profile was similar to a log graph, and a constant dilution ratio was maintained throughout the procedures. This finding indicates that our bio-fluidic device did, indeed, successfully carry out adaptive dilutions.

Table 2. The reproducibility of automatic dilutions

Sample[a]	Initial absorbance	Number of dilution cycles	Dilution ratio	Coefficient of variance (%)
1	1.202	3	0.661 ± 0.005	0.76
2	1.270	3	0.598 ± 0.021	3.51
3	1.218	3	0.561 ± 0.039	6.95
4	1.335	3	0.643 ± 0.007	1.09
5	1.247	3	0.661 ± 0.005	7.56
6	1.297	3	0.652 ± 0.015	2.30
7	1.252	3	0.673 ± 0.029	4.31
8	1.348	4	0.703 ± 0.019	2.70
9	1.343	4	0.681 ± 0.005	0.73
10	1.382	4	0.730 ± 0.034	4.66
11	1.056	2	0.614 ± 0.011	1.79
12	1.136	2	0.630 ± 0.017	2.70
13	1.239	3	0.580 ± 0.041	7.06
14	1.113	3	0.595 ± 0.053	8.91
15	1.120	3	0.630 ± 0.051	8.10
16	1.025	3	0.644 ± 0.016	2.48
17	1.068	3	0.661 ± 0.066	9.98

[a]The absorbance of sample 1~10 (dye solution) were measured at 482 nm while the sample 11~17 (*E. coli*) were measured at 600 nm.

Reproducibility of Automatic Dilution

Table 2 displays the reproducibility data acquired in the automatic dilution experiments. Samples 1~10 were analyzed with dye solution, with absorbance values being measured at 482 nm. Samples 11~17 were analyzed using *E. coli* solution at an absorbance of 600 nm. After injecting 50 μL of the sample, dilution was repeated, with pop and push cycles of 20.9 μL, and then mixed together. As samples with absorbance values in excess of 1.5 yielded more inaccurate measurements, the initial absorbance was set within a range between 1.0 and 1.4. Two to four dilution cycles were performed when the dilution ratio was set at approximately 0.6 and the command entered was "dilute until an absorbance of 0.4." The coefficients of variances from many of the samples were less than 10%. However, the dilution ratios determined in the repetitive experiments were in a range between 0.561~0.730, even though identical samples were employed. The poor reproducibility of the dilution ratio can be attributed to the following. Other than the electronic noise of the spectrophotometer, light intensity would tend to vary during the measurements, as the result of the mismatch (albeit small) between the fluidic device and the optical fiber. Another possible explanation for this discrepancy is the inaccuracy of fluidic actuation as the result of the differing push and pop volumes. This may be the result of our use of continuous sampling and drain modes in the current device format, which requires a linkage of all tube connections for sampling, dilution, and draining. We are currently attempting to improve the configuration of the device for better volume control, in order to rectify the inaccurate volume actuation associated with the carry-over between samples.

Measurement of *E. coli* Concentrations

The absorbance method is extensively used in the measurement of cell concentrations, because it is simple, fast, and moderately accurate. However, this method also carries the disadvantage of limited sensing range; it cannot be measured at above the saturation value, due to the characteristics of the optical sensor. When it transduces light intensity into an electrical signal, the sensor output is saturated at values over 3 O.D. Because, in many cases optical density values of cell suspensions increase over 10 O.D., dilution into a measurable range is essential for the precise determination of the concentration of the analyte [30]. The cell concentration in a fed-batch culture frequently rises to more than 10^{11} cells/mL. Also, conventional high-density cell cultures require manual dilution in order for cell concentrations to be measured. Thus, in order to accurately measure cell concentrations in high-density cultures, the samples must be manually diluted to adequate concentrations, due to the limited sensing ranges of the measurement systems. This manual dilution is associated with several drawbacks, including the general inconvenience of the repeated dilution process, and the sizeable inaccuracy associated with accumulated errors. In addition to saturation, optical density, also called absorbance (A), has a vary narrow range [31,32]. Using the conventional method, the optical density has been measurable only in a small O.D. range, of less than 1.0.

Table 3. The adaptiveness of dilutions for *E. coli* samples

Sample absorbance	Final diluted absorbance	Number of dilution cycles
0.44	0.445	1
1.16	0.375	3
2.82	0.461	5
3.12	0.511	5
4.16	0.498	6
31.20	0.608	11

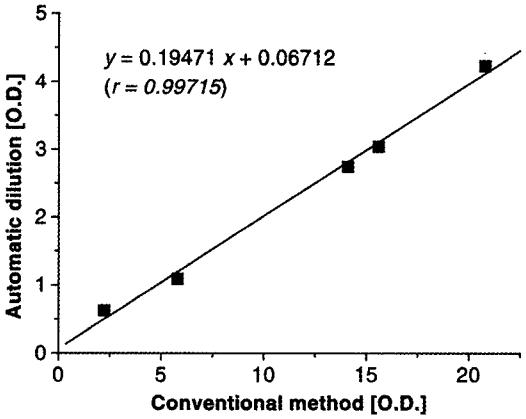

Fig. 6. Comparison of absorbance measurements between the adaptive dilution method and the conventional method. Experiments were conducted with *E. coli* samples at a wavelength of 600 nm.

Therefore, samples have had to be manually diluted in a step-by-step fashion, using a pipette.

In this study, we measured the concentrations of five *E. coli* samples, in order to conduct a comparison between our device and a conventional device. As shown in Fig. 6, our method appears to be a viable alternative to manual dilution. The absorbance value from our device was comparable to that achieved by the conventional method, with a 0.19471 ratio. This ratio matches the theoretical value of 1/5, because the light path of our device is only 2 mm, rather than 10 mm, as is the case with the conventional method.

We also conducted adaptive dilution, in order to enlarge the detectable range, as our device measured a broadened range of up to 30 O.D. Table 3 displays the numbers of dilution cycles, which indicate the different overall dilution ratios in accordance with the initial concentrations. The final measurement settings were fixed at 0.35~0.70 O.D., with a 0.5 dilution ratio. The dilution was accompanied by differing numbers of cycles, until the predefined region was achieved. We determined that the numbers of dilutions necessary increased directly with the concentrations of the samples.

The precision of a measurement relies on the photometric error associated with the measurement of light. Therefore, the precision of concentration differs, over the absorbance value range. At low concentrations, such as $A<0.1$, and at relatively high concentrations, such as $A>1.0$, the relative standard deviation (σ_A/A) under these conditions is greater than 10 times the standard deviation of transmittance, σ_T, which indicates that the measurement is less precise than at other ranges. The range in which the results produce the smallest relative error occurs at log(e) (= 0.434). In order to achieve optimal precision, the concentration of the sample, or the pathlength through the sample, must be adjusted so that the output of the instrument is in a range between 0.4~0.7 O.D., if possible. In other words, the adjustment of the concentration by appropriate dilution is one method by which the sensing range can be made wider and/or more precise than would be the case without adjustment. It should be emphasized that, because of the saturation, the initial concentration is not measurable, and the dilution ratio cannot be accurately calculated. However, the adaptive dilution conducted in this study provides a method by which the measurement range can be effectively broadened, because it adjusts the sample concentration into the precise region in which the instrument can measure it, via the activity of a feedback control (around 0.434 O.D.).

CONCLUSION

In this study, we developed a bio-fluidic device for the adaptive pretreatment of a sample to achieve optimal analytic conditions, using two novel strategies: dilution with a programmable ratio, and to-and-fro mixing. The device developed in this study was associated with several advantages. First, the simplicity of the device made it easy to integrate several functions into a single device. Second, the programmable-sequence aspect of the device made it possible to carry out many tasks sequentially within the same device.

Adaptive sample pretreatment appears to be applicable to other bio-fluidic devices and systems. Typical examples might be the on-line monitoring of cell mass within a fermenter, or a portable field instrument for environmental water testing. In high-density cell cultures, with O.D. values in excess of 10, conventional dilution is often associated with several disadvantages, including the general inconvenience from the repeated process, and the inherent inaccuracy caused by the accumulation of human errors. Our device, then, may prove useful in the automation of high-density cell cultures, in order to provide on-line cell concentration monitoring. This general technique could also be applied to water testing kits. In order to analyze various species in water, including phosphorus, ozone, and metal ions, as well as COD (chemical oxygen demand), colorimeters are frequently used by environmental researchers. In this kind of testing, however, because each type of measurement requires specific sensor cartridge with narrow detection ranges, it is often necessary to change the sensor cartridge in accordance with chemical concentration ranges. The adaptive dilution method may solve this problem.

The optimization of the to-and-fro mixing method, on

the other hand, will require more research into the mixing effect, with regard to to-and-fro actuation amounts, dilution controls, *etc*. An issue also remains with respect to the current lack of experimental verification of this method in real situations. One such verification could be achieved by the performance of real-time sampling, using a working fermenter. Such a study is currently underway, as part of our ongoing effort to optimize the bio-fluidic device and its attendant protocols.

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