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Microchip-based chemical and biochemical analysis systems

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Abstract

This review focuses on chemical and biochemical analysis systems using pressure-driven microfluidic devices or microchips. Liquid microspace in a microchip has several characteristic features, for example, short diffusion distances, high specific interfacial area and small heat capacity. These characteristics are the key to controlling micro unit operations and constructing new integrated chemical systems. By combining multiphase laminar flow and the micro unit operations, such as mixing, reaction, extraction and separation, continuous flow chemical processing systems are realized in the microchip format. By applying these concepts, several different analysis systems were successfully integrated on a microchip. In this paper, we introduce the microchip-based chemical systems for wet analysis of cobalt ion, multi-ion sensors, immunoassay, and cellular analysis.

Keywords: μ-TAS; Labs-on-a-chip; Integrated chemistry lab; Microfluidic device; Thermal lens microscope; Continuous flow chemical processing; Multiphase laminar flow; Micro unit operation

Contents

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1. Introduction

1.1. Microchip-based chemical systems

The rapid development of microdevices for chemical analysis has been greatly promoted by the progress of microfabrication techniques, and microchemical systems using these devices have attracted much attention of scientists and engineers [1–4]. This new field of chemistry is known by the name of micro total analysis systems (μ-TAS), labs-on-a-chip, or integrated chemistry lab. As expressed by the name, the concept of these microchip-based systems proposes the integration of the various chemical operations involved in conventional analytical processes done in a laboratory, such as mixing, reaction and separation, into a miniaturized flow system.

Most studies describing microchip-based analytical systems concern DNA analysis using microchip electrophoresis systems. Electrophoretic methods on a microchip have been investigated intensively and their effectiveness and usefulness have been demonstrated in some applications especially in clinical diagnosis and molecular biology fields. Although electrophoretic methods have many advantages, other methods are desirable from the viewpoint of continuous and sequential chemical processing required for various applications. To realize these complicated systems, it is necessary to utilize chemical properties and potentials of molecules. For this purpose, microfluidic systems using pressure-driven flow were suitable. In this paper, we review the microchip-based chemical and biochemical analysis systems using pressure-driven flow for continuous and sequential chemical processing.

1.2. Characteristics of liquid microspace

Liquid microspace has several characteristic features different from bulk scale, for example, short diffusion distances, high interface-to-volume ratio (specific interface area; solid/liquid or liquid/liquid), and small heat capacity. These characteristics in the microspace are key to controlling chemical unit operations, such as mixing, reaction, extraction and separation, and constructing the integrated chemical systems. Especially, to control molecular transport in the microspace such as microchips, the molecular transportation time and the specific interface area must be considered. The molecular transportation time is given by:

$$t = L^2/D \quad (1)$$

where t, L, and D are the molecular transportation time, diffusion distance and coefficient, respectively. The specific interface area, σ, can be expressed as:

$$\sigma = S/V \propto 1/L \quad (2)$$

where S and V are the interface area and the volume,

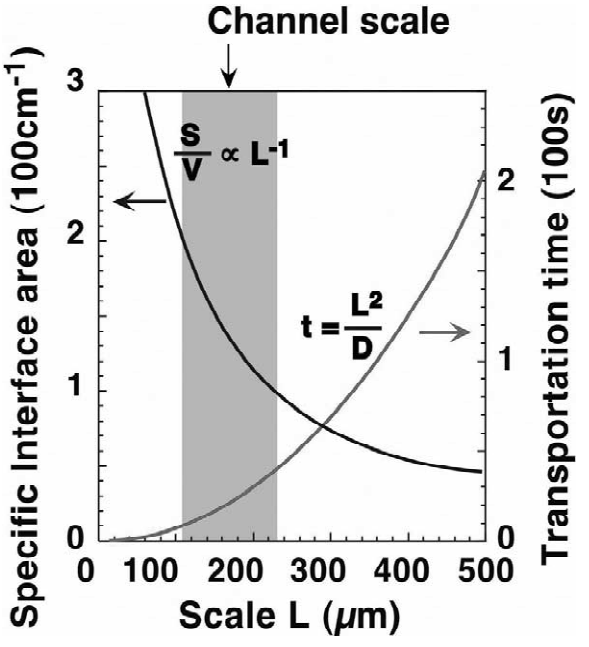

Fig. 1. Scale dependence of specific interface area and diffusion time in microchip.

respectively. Fig. 1 shows the scale dependence of the molecular transportation time and the specific interface area.

As expressed in Eq. (1), the transportation time is proportional to the square of the scale. Therefore, the transportation time takes several hours to a day when the diffusion distance is 1 cm since the diffusion coefficient of typical molecular ions is on the order of 10^{-5} cm^2/s. In contrast with that case, it takes only several tens of seconds when the diffusion distance is 100 μm. The specific interface area of the 100-μm scale microspace is equivalent to that provided by using a separatory funnel with rather vigorous mechanical shaking. These kinds of scale merits become remarkable below the scale of about 250 μm.

1.3. Fundamental concepts of continuous flow chemical processing

Focusing primarily on high applicability, we have developed pressure-driven continuous flow chemical processing (CFCP) on microchips. In order to realize CFCP, a combination of micro unit operation (MUO) and multiphase laminar flow was proposed. Components of MUO are shown in Fig. 2. By combining these MUO components, various kinds of chemical processing can be integrated onto the microchips. We have demonstrated the integration of fundamental MUOs, such as mixing and reactions [5–7], two- and three-phase formations [8,9], solvent extraction [10–14], solid-phase extraction [15,16], heating [17,18], and cell culture [19]. Moreover, formation of a stable multiphase laminar flow network in microchannels has also been reported [8,9,20].

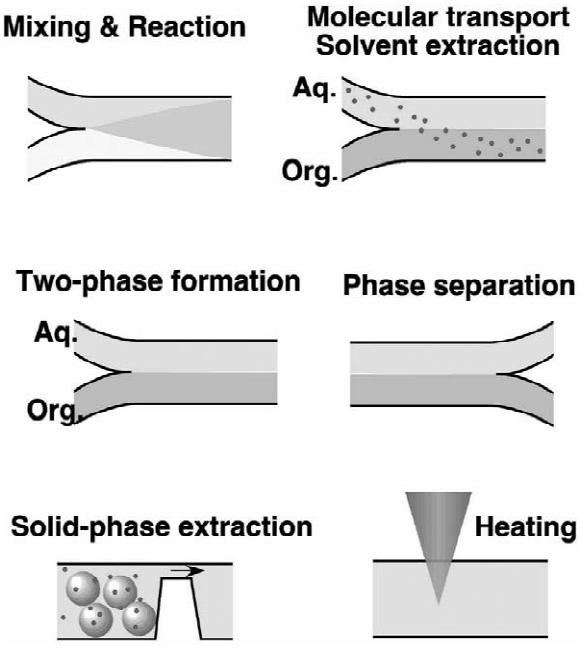

Fig. 2. Major components of micro unit operation (MUO).

As shown in Fig. 2, MUOs utilizing aqueous–organic multiphase laminar flow such as solvent extraction are key technology in CFCP. In the following sections, recent advances in stabilization method of multiphase laminar flow and application of CFCP were described.

2. Stabilization of multiphase laminar flow in microchannels

2.1. Fabrication of microchips

Microchips were fabricated using a photolithographic wet etching method [8]. Mechanically polished 0.7-mm thick Pyrex glass plates were used (top and bottom plates). Inlet and outlet holes were drilled by ultrasonic sandblasting on the top plate. For good contact between the substrates and the photoresist and protection of the substrates during glass etching, 20-nm thick Cr and 100-nm thick Au layers were evaporatively deposited on the substrates under a vacuum. Two-μm thick positive photoresist was spin-coated on the Au metal layer and baked at 90 °C for 30 min. UV light was exposed through a photomask by using a mask aligner to transfer the microchannel pattern onto the photoresist. The photoresist was developed and a pattern with 10-μm wide lines was obtained. The Au and Cr layers were etched with I_2/NH_4I and $Ce(NH_4)_2(NO_3)_6$ solutions. The bare glass surface with the microchannel pattern was etched with a 50% HF solution at an etching rate of 13 μm/min. After glass etching, the remaining photoresist was removed in acetone and metals were removed in I_2/NH_4I and $Ce(NH_4)_2(NO_3)_6$ solutions.

2.2. Guide structure

A specially designed photomask pattern was used in order to fabricate the microchannels with guide structures to form the stable liquid–liquid interface [21]. Since the photomask had three independent channel patterns for one microchannel, the three channels were etched independently on the glass substrate initially. The depth of the microchannels grew as they remained with etching HF solution as time passed. The three independent microchannels become one microchannel with guide structures at the bottom after a few minutes.

A schematic cross-sectional view of the guide structures is depicted in Fig. 3a. Fig. 3b shows a 3-D image of the etched microchannel structures as observed with a color laser confocal microscope. The three-lobed guide structures at the bottom of the microchannel are clearly seen. The guide height above the bottom of the microchannel was 5 μm. The guide shape and height were controlled arbitrarily using etching time and the gap between lines of the mask pattern.

Photographs of the liquid–liquid interface formed in the microchannels are shown in Fig. 4. The expected interfaces are formed throughout the microchannels. Although the reason for the interface stability was not analyzed using hydrodynamics,

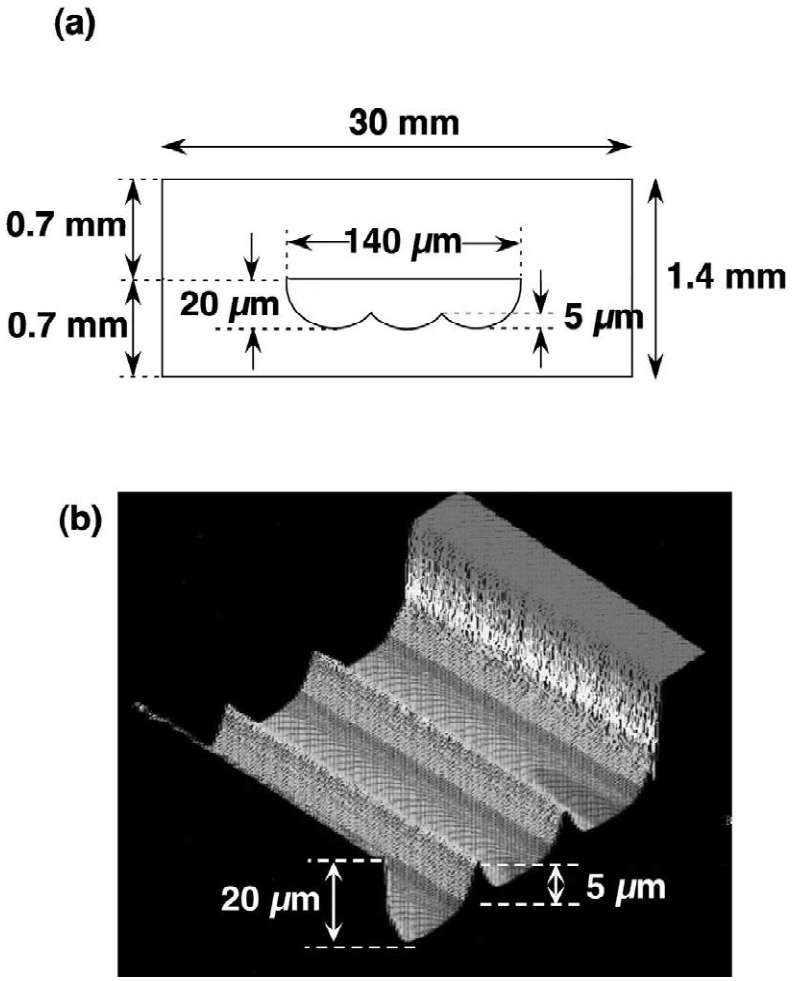

Fig. 3. (a) Cross-sectional view of the guide structures fabricated in a microchip. (b) 3-D image of the structures.

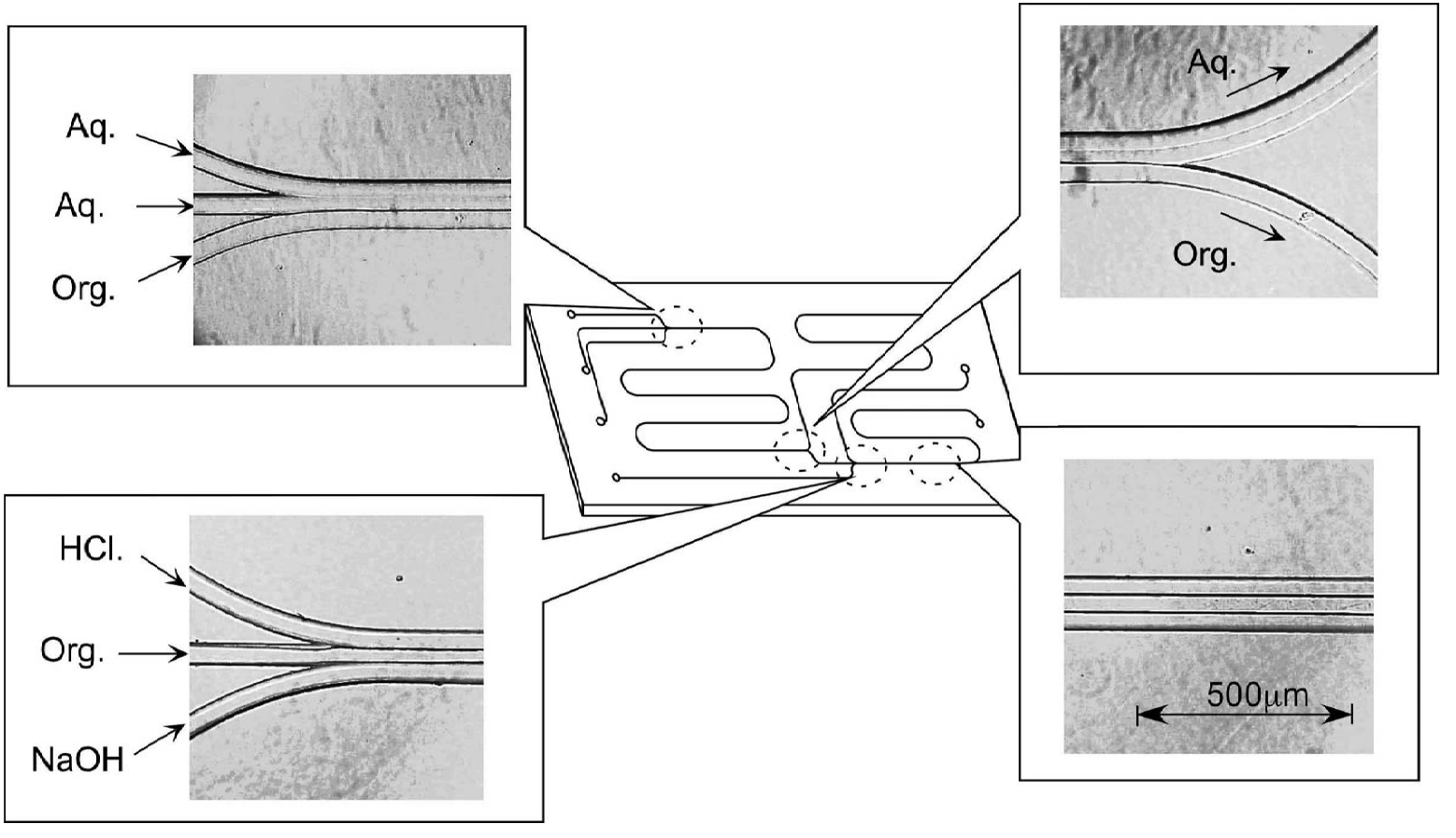

Fig. 4. Photographs of the liquid–liquid interface formed in the microchannels. (a) Confluence of water and *m*-xylene. (b) Phase separation at branching part. (c) Confluence of HCl, organic phase, and NaOH. (d) Stable three-phase flow.

parameters influencing stability of interfaces, such as surface tension, contact angle, etc., may be favorably affected by the guide structures. Experimentally, it is impossible to form interfaces inside the microchannels over a long distance without the guide structures. Phase separation between aqueous and organic phases is also not possible without them.

2.3. Surface chemical modification

We have investigated stabilization of the liquid interface and control of the liquid confluence and separation by utilizing chemical modification of the microchannel wall [22]. In this method, the microchannel for organic solvent flow was modified by surface coupling of octadecylsilane (ODS) group while the microchannel for aqueous flow had a bare glass surface. In order to demonstrate effectiveness of this method, two-phase crossing flow was performed. The microchannels were designed to cross with an angle of 30°. In order to characterize the surfaces, we measured contact angles of interfaces of air/water/plate and nitrobenzene/water/plate. The relationship between the contact angle and surface tensions are written by Young' equation as follows:

$$\gamma_{AB} - \gamma_{AC} = \gamma_{BC} \cos\theta \equiv \Gamma_{BC} \quad (3)$$

where θ is the contact angle and γ_{AB}, γ_{AC} and γ_{BC} are surface tensions between AB, AC and BC interfaces, respectively. In our experiments, B corresponds to air or nitrobenzene and A and C denote water and substrate plate, respectively. To compare the ODS-modified and non-modified plates, the relative surface tension is defined as Γ_{BC}, where a larger Γ_{BC} corresponds to a hydrophilic surface. The results are summarized in Table 1. The surface tensions between water/air and water/nitrobenzene are given in the literature as 72.3×10^{-3} and 25.7×10^{-3} N/m at 296 K. As shown in Table 1, Γ_{BC} values for the ODS modified glass plate are negative while those for the bare glass are positive. As defined in Eq. (3), a negative Γ_{BC} value means that the surface tension between water/plate is larger than that between air (or nitrobenzene)/plate. As long as pressure difference and fluctuation are less than the surface tension difference, the immiscible liquid flow interface should be maintained and the two-phase confluence and separation should work as designed. Usually, the top and bottom glass plates are thermally bonded at 650 °C. The ODS group on the glass surface might be damaged by this high temperature. Therefore, we developed a non-bonded sealing method as shown in Fig. 5. In this method, 2.1-mm holes were fabricated at the center of the

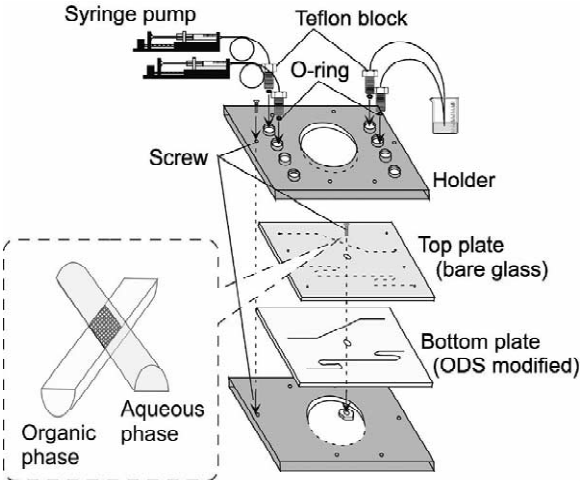

Fig. 5. Schematic illustration of the sealing and connection method. The top and bottom plates are pressed by a screw and holders.

Table 1
Summary of contact angle measurements at air/water/plate and nitrobenzene/water/plate interfaces [22]

Plate	B	Contact angle (°)	Γ_{BC} ($\times10^{-3}$ N/m)
Bare glass	Air	16	69
ODS modified glass	Air	108	−22
Bare glass	Nitrobenzene	37	21
ODS modified glass	Nitrobenzene	143	−21

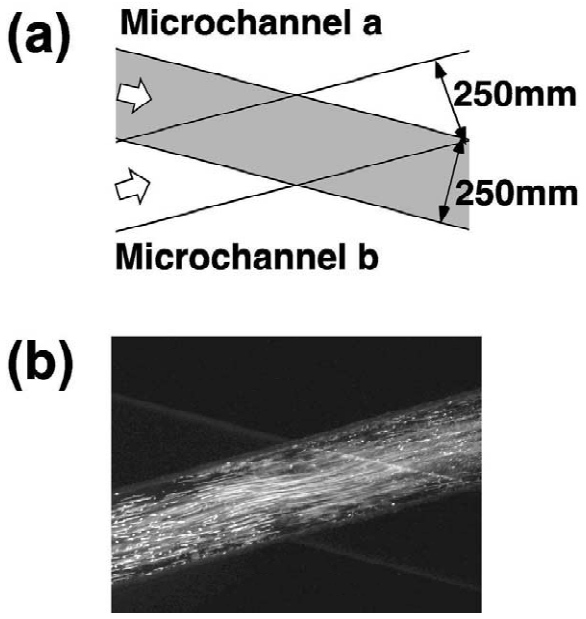

Fig. 6. (a) Illustration of crossing region. (b) Fluorescent image of probe particles dispersed in aqueous phase.

plates and the two plates were fixed by a screw (2 mm diameter) and a nut. Furthermore, the screw-fixed plates were sandwiched by aluminum holders. By pressing both the center and edge of the plates, sufficient non-bonded sealing was realized. To visualize the aqueous phase flow at the water/nitrobenzene interface, loci of fluorescent polystyrene particles in the aqueous flow were observed. As shown in Fig. 6, the aqueous phase flow bent slightly at the crossing region and then went nearly straight. In order to demonstrate the effectiveness of our method, gas phase (air) instead of nitrobenzene was introduced into the ODS-modified channel and a stable air/water crossing flow was observed. Although density of air is much less than that of water, surface tensions are dominant rather than gravity in the micro space and interfaces can be formed independently of the density difference. The result showed that the method could be applied not only to liquid/liquid contact, but also to gas/liquid contact.

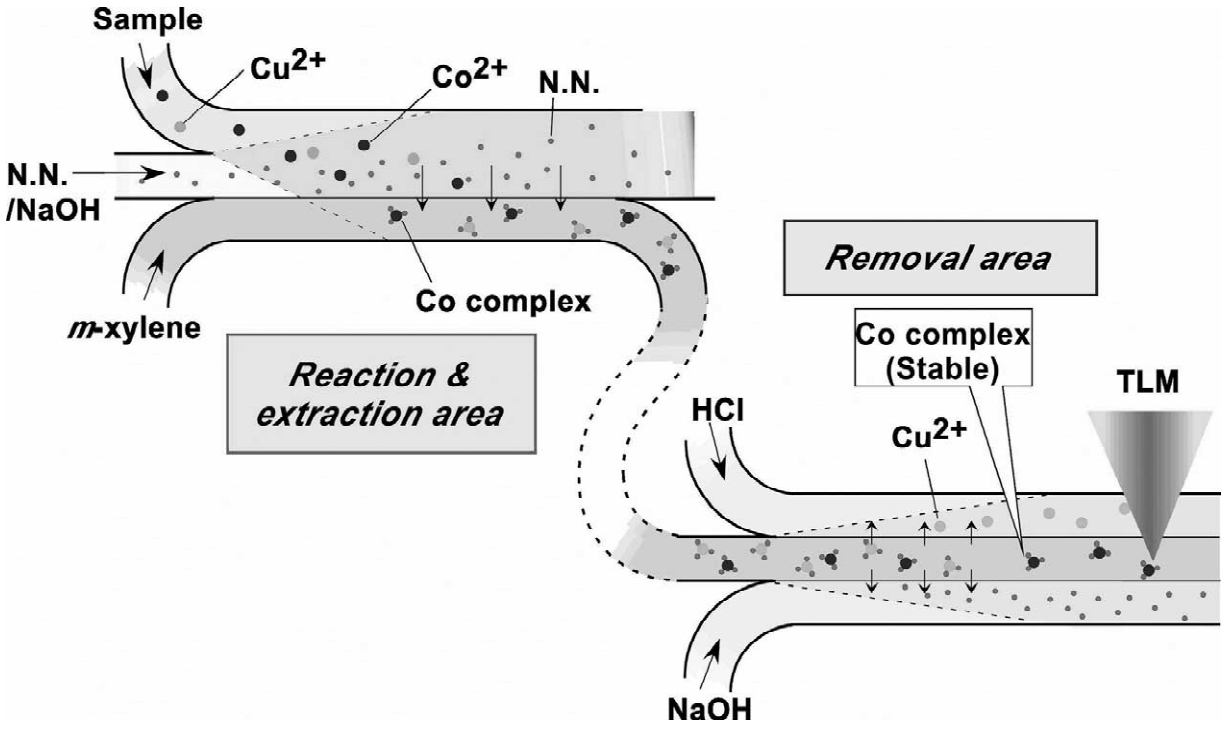

Fig. 7. Schematic illustration of cobalt ion determination by combining MUOs.

3. Applications

3.1. Co(II) wet analysis

A schematic illustration of Co(II) wet analysis by using CFCP is shown in Fig. 7 [21]. The microchip consists of two different areas: the former is the reaction and extraction area and the latter is the washing, i.e. decomposition and removal, area. In the former area, the sample solution containing Co(II) ions, the 2-nitroso-1-naphthol (NN) solution and *m*-xylene are introduced at a constant flow rate through three inlets using microsyringe pumps. These three liquids meet at the intersection point, and a parallel two-phase flow, consisting of an organic/aqueous interface, forms in the microchannel. The chelating reaction of Co(II) and NN and extraction of the Co(II) chelates both proceed as the reacting mixture flows along the microchannel. Since the NN reacts with coexisting metal ions such as Cu(II), Ni(II) and Fe(II), these coexisting metal chelates are also extracted into the *m*-xylene. Therefore, a washing process is needed after extraction for the decomposition and removal of coexisting metal chelates.

The coexisting metal chelates decompose when they make contact with hydrochloric acid and the metal ions are dissolved in HCl solution. The decomposed chelating reagent, NN, is dissolved in sodium hydroxide solution. In contrast to the coexisting metal chelates, the Co chelate is stable in HCl and NaOH solutions and remains.

In the latter (washing) area, the *m*-xylene phase containing the Co chelates and the coexisting metal chelates from the former (reaction and extraction) area is interposed between the HCl and NaOH solutions, which were introduced through the other two inlets at a constant flow rate. Then, the three-phase flow, HCl/*m*-xylene/NaOH, forms in the microchannel. The decomposition and removal of the coexisting metal chelates proceed along the mi-

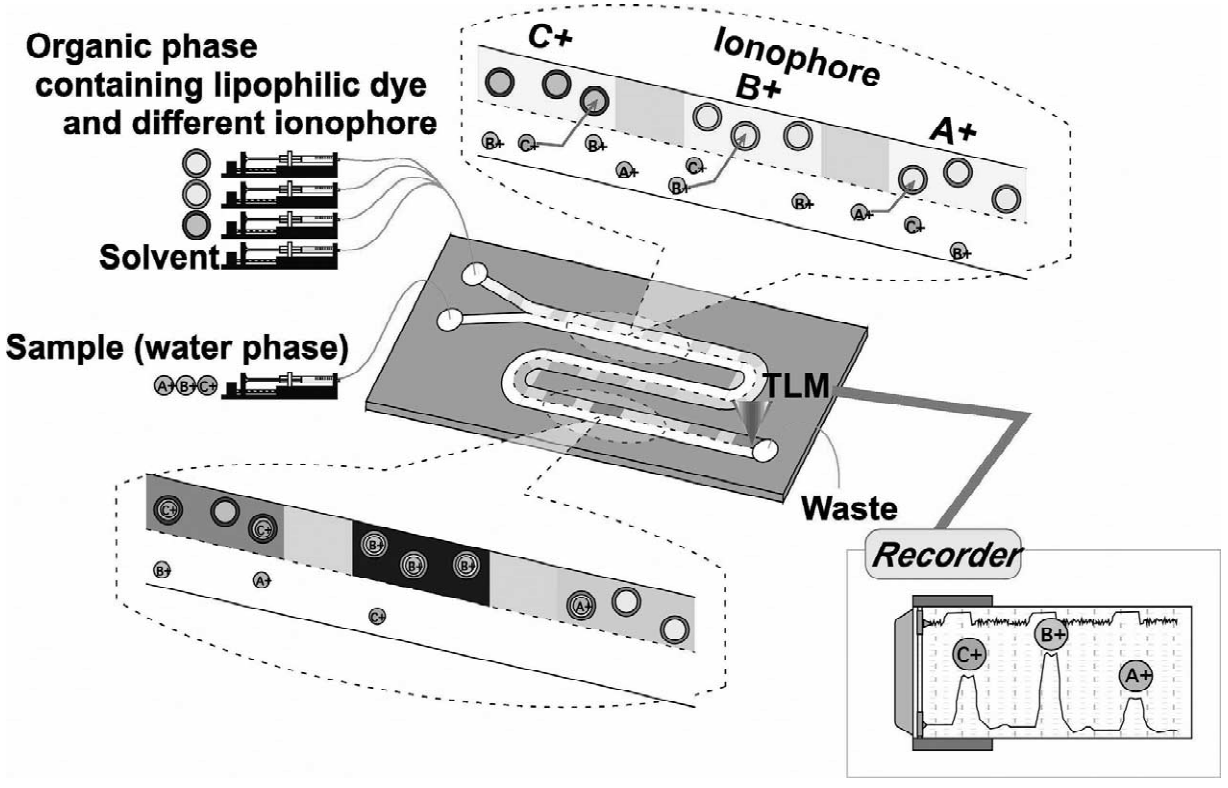

Fig. 8. Concept of sequential ion-sensing system using single microchip.

crochannel in a similar manner as described above. Finally, the target chelates in *m*-xylene are detected downstream by a thermal lens microscope (TLM) [23–25].

Admixture samples of Co(II) and Cu(II) were analyzed in the system. In the reaction and extraction area, the intensity of the TLM signal gradually increased with the microchannel length since both Co(II) and Cu(II) ions reacted with NN and were extracted into *m*-xylene. In the washing area, the Cu chelates were decomposed and removed. In contrast to the Cu chelates, the Co chelates still remained in the *m*-xylene phase. Therefore, the intensity of the TLM signal gradually decreased with the microchannel length and became constant about 2 mm downstream from the confluence point. From the TLM signal 3 mm downstream from the confluence point of HCl, *m*-xylene and NaOH, we could obtain linear relationship between Co(II) concentration and TLM signal.

3.2. Multi-ion sensing

In order to perform multi-ion sensing using a single microchip, a new methodology involving neutral ionophore-based ion pair extraction combined with intermittent pumping of multiple organic phases, and formation of laminar two-phase flow with an aqueous sample solution was proposed [10,14].

Fig. 8 shows the basic concept of the multi-ion sensing using a microchip. Different organic phases containing the same lipophilic pH indicator dyes, but different ionophores, are introduced sequentially into the microchannel by on–off switching of syringe pumps. In this case, organic phase without ionophore is introduced in between the two organic phases containing different ionophores, in order to avoid contamination. Aqueous sample solutions containing different ions are introduced from the other inlet to form laminar two-phase flow with the intermittently pumped organic phases. The selective ion pair extraction reaction proceeds during flow, thus, different ions can be selectively extracted into different organic phases depending on the selectivity of neutral ionophores contained in the respective organic phases. Downstream in the flow, the ion pair extraction reaction equilibrates, thus, downstream detection of the color change of organic phase allows sequential and selective multi-ion sensing in the single aqueous sample solution containing multiple ions.

In this case, valinomycin and DD16C5, which are known to exhibit high selectivity when used in conventional ion sensors, were selected as highly selective potassium and sodium ionophores, respectively. Three types of aqueous sample solutions were investigated at the system: buffer solution containing 10^{-2} M K^+, buffer solution containing 10^{-2} M Na^+, and buffer solution containing both ions. When the aqueous phases containing a single type of ion were used, selective extractions occurred in each case, i.e. potassium ions were extracted only for organic phase segment containing valinomycin, and sodium ions were extracted only for that containing DD16C5. By using ionophores for Na^+, K^+, Ca^{2+} and other ions, the system will become a useful tool for ion analysis of biological fluid.

3.3. Immunoassay

Immunoassay is one of the most important analytical methods and it is widely used in clinical diagnoses, environmental analyses and biochemical studies because of its extremely high selectivity and sensitivity. Enzyme-linked immunosorbent assay (ELISA) or other immunosorbent assay systems, in which antigen and antibodies are fixed on a solid surface, are applicable to many analytes with high sensitivity and are used practically in many fields including clinical diagnoses.

The conventional heterogeneous immunoassay, however, requires a relatively long assay time, and involves troublesome liquid-handling procedures and large quantities of expensive antibody reagents. Moreover realization of point-of-care (POC) testing is difficult with conventional immunoassay, since rather large devices are necessary for automated practical diagnosis systems. To overcome these drawbacks, a microchip-based system seems to be effective. Integration of analytical systems into a microchip should bring about enhanced reaction efficiency, simplified procedures, reduced assay time, and lowered consumption of samples, reagents, and energy.

Recently, several papers about integration of

heterogeneous immunoassay systems into microscale devices were published [15,16,26,27]. In our previous paper [15], we reported the possibility of immunosorbent assay on a microchip. In the system antigen–antibody reactions were performed on surfaces of microbeads packed in a microchannel with a dam structure (Fig. 9). We showed that the reaction time necessary for an antigen–antibody reaction was reduced to 1/90 in the integrated system because of size effects of the liquid microspace.

We expanded this system into a microchip-based clinical diagnosis system [16]. Human carcinoembryonic antigen (CEA), one of the most widely used tumor markers for serodiagnosis of colon cancer, was assayed with this system. An ultratrace amount of CEA dissolved in serum samples was successfully determined in a short time with this system.

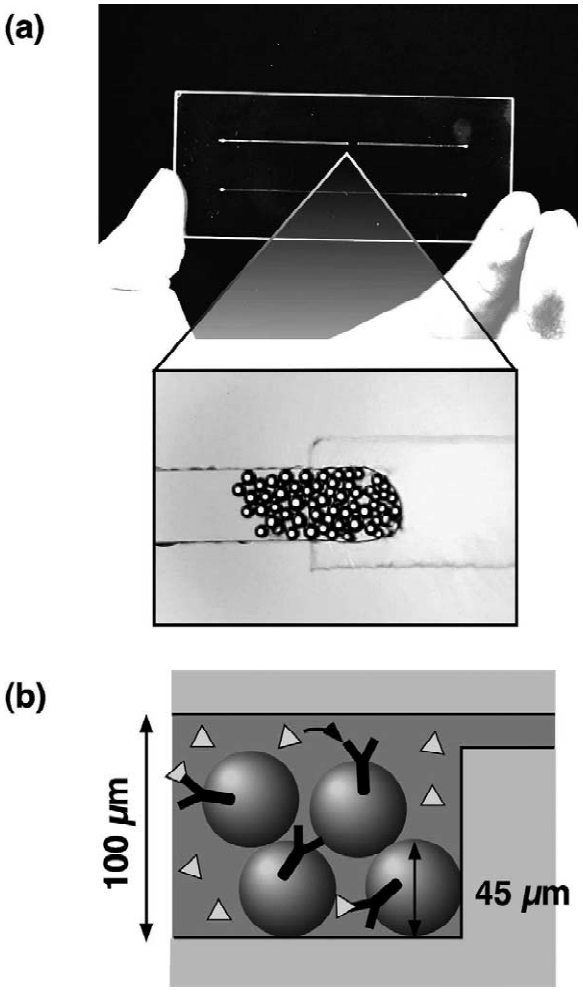

Fig. 9. (a) Immunoassay microchip with microbeads. (b) Cross-section image of the reaction area.

Polystyrene beads pre-coated with anti-CEA antibody were introduced into a microchannel, and then a serum sample containing CEA, the first antibody, and the second antibody conjugated with colloidal gold were reacted successively. The resulting antigen–antibodies complex, fixed on the bead surface, was detected using a TLM. A highly selective and sensitive determination of an ultratrace amount of CEA in human sera was made possible by a sandwich immunoassay system that requires three antibodies for an assay. A detection limit dozens of times lower than the conventional ELISA was achieved. Moreover, when serum samples for 13 patients were assayed with this system, there was a high correlation ($r = 0.917$) with the conventional ELISA. The integration reduced the time necessary for the antigen–antibody reaction to ~1%, thus shortening the overall analysis time from 45 h to 35 min. Moreover, troublesome operations required for conventional heterogeneous immunoassays could be substantially simplified. This microchip-based diagnostic system was the first μ-TAS shown practical usefulness for clinical diagnoses with short analysis times, high sensitivity, and easy procedures.

In these microchip systems, higher integration is thought to be easily realized using multichannels. To realize higher throughput analyses, a microchip system, which can process several samples simultaneously, was reported recently [27]. In this integrated system, the chip had branching multichannels and four reaction and detection regions; thus the system could process four samples at a time with only one pump unit (Fig. 10). Interferon γ was assayed by a three-step sandwich immunoassay with the system coupled to a TLM as a detector. The biases of the signal intensities obtained from each channel were within 10%, and CVs were almost the same level as the single straight channel assay. The assay time for four samples was 50 min instead of 35 min for one sample in the single channel assay; hence, higher throughput was realized with the branching structure chip.

Simultaneous assay of many samples may be achieved by simply arraying many channels in

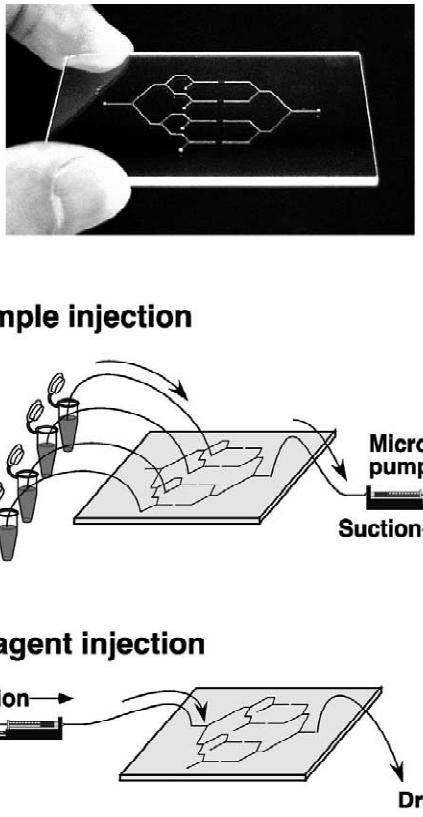

Fig. 10. Simultaneous determination system with branching multichannel immunoassay chip.

parallel on a chip also. This approach, however, needs many pumps and capillary connections and high integration seems to be difficult. On the other hand, a microchip with branching microchannels seems to be suitable for simultaneous assay. By branching multichannels, the numbers of pumps and capillary connections required for the system should be reduced.

3.4. Cell analysis

The microchip techniques seemed to provide some advantages for cellular biochemical analysis systems because the scale of a liquid microspace inside the microchip is fitted to the size of cells. For instance, by using a microflask fabricated in a microchip, rapid and secure exchange of media or reagents will be achieved by simple operations under continuous measurements. The rapid and secure exchange of media is very advantageous to time-resolved analysis. Moreover, a glass microchip is favorable for optical detection under a microscope because the chip can be fabricated transparently with flat surfaces. It seems to be useful to develop a microchip-based culture flask in which all procedures for cell analysis, i.e. cell culture, chemical stimulation and measurement, can be performed. However, very few papers report biochemical analysis of a cell cultured in a microchip to date.

Recently, we developed a novel cell analysis system consisting of a scanning TLM detection system and a cell culture microchip (Fig. 11) [19]. A microflask ($1\times10\times0.1$ mm; 1 μl) was fabricated in a glass microchip. A cell suspension was introduced into the microflask, and then the chip was incubated at 37 °C in a CO_2 incubator. After cultivation, the microchip with capillaries connected to syringe pumps was mounted on the TLM stage, and TLM signals were measured with scanning of the stage to

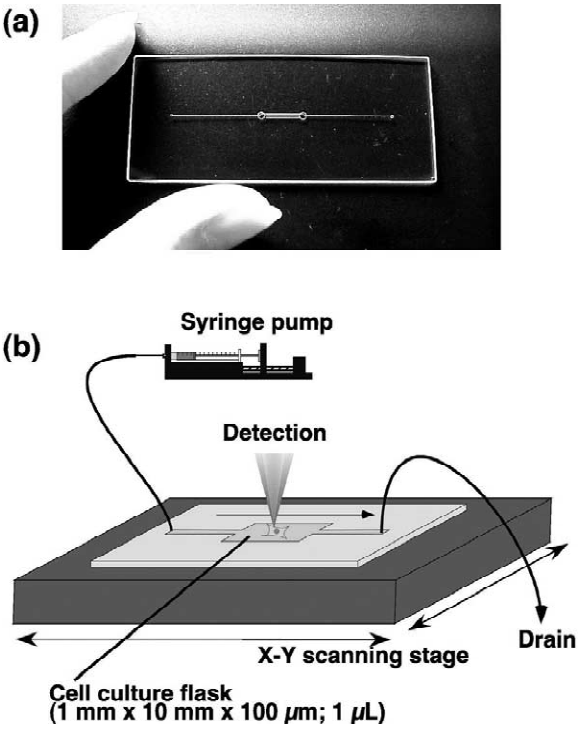

Fig. 11. (a) Cell culture chip. (b) Scanning thermal lens microscope system for single cell imaging.

obtain a 2D-image. The system could detect non-fluorescent biological substances with extremely high sensitivity without any labeling materials and had high spatial resolution of ~1 μm. The microchip system was good for liquid control and simplifies troublesome procedures. This system was applied to monitoring of cytochrome *c* distribution in a neuroblastoma–glioma hybrid cell cultured in the microflask. Cytochrome *c* release from mitochondria to cytosol during the apoptosis process was successfully monitored with this system (Fig. 12). The system seems to be applicable to the monitoring systems of cellular released compounds in combination with some analytical microchips.

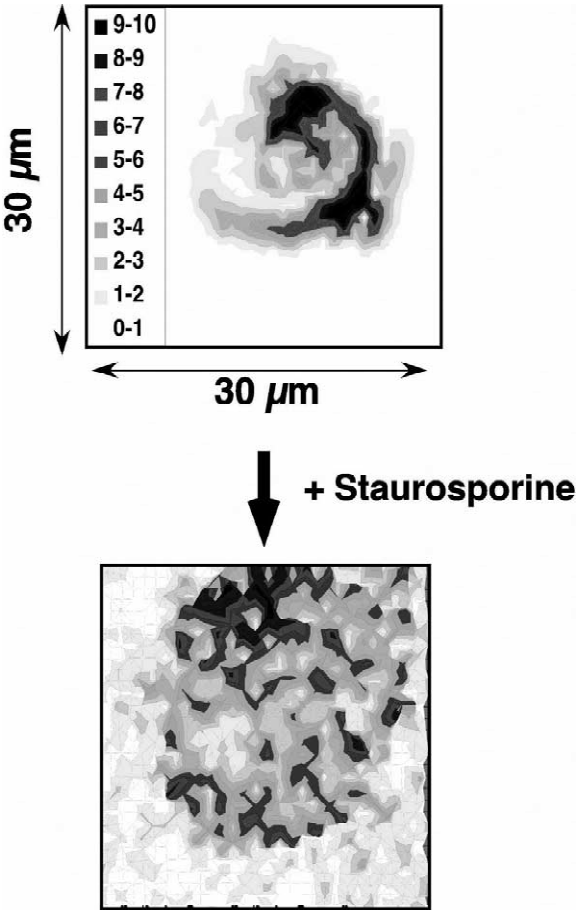

Fig. 12. Direct imaging of cytochrome *c* in a cell and its distribution change during the apoptosis process.

4. Conclusions

In this review, concept of the microchip-based chemical systems, i.e. continuous flow chemical processing (CFCP), multiphase laminar flow and micro unit operation (MUO) were introduced. Since stabilization of the laminar flow was very important in the system, flow stabilization methods utilizing guide structure and surface chemical modification were also demonstrated. In addition, usefulness and effectiveness of our method were demonstrated in some applications, Co^{2+} wet analysis, multi-ion sensor, immunoassay and cell analysis.

The continuous flow chemical processing can be applied not only to analytical chemistry but also to general chemical operation such as organic synthesis [28], combinatorial chemistry and physical chemistry.

Acknowledgements

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