Reviews

Integration of Chemical and Biochemical Analysis Systems into a Glass Microchip

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This review focuses on the integration of chemical and biochemical analysis systems into glass microchips for general use. By combining multiphase laminar flow driven by pressure and micro unit operations, such as mixing, reaction, extraction and separation, continuous-flow chemical processing systems can be realized in the microchip format, while the application of electrophoresis-based chip technology is limited. The performances of several analysis systems were greatly improved by microchip integration because of some characteristics of microspace, *i.e*., a large specific interface area, a short molecular diffusion time, a small heat capacity and so on. By applying these concepts, several different analysis systems, *i.e*., wet analysis of cobalt ion, multi-ion sensor, immunoassay, and cellular analysis, were successfully integrated on a microchip. These microchip technologies are promising for meeting the future demands of high-throughput chemical processing.

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1 Introduction

It is predicted that an enormous number of analyses will be required in our future daily life, especially in clinical and biochemical fields. Rapid progress of molecular biology and biochemistry brings a lot of useful and important information. From these achievements, a huge number of genetic mutations, like SNPs and biological markers, like disease markers have been established. These indices reveal the risk or degree of diseases or constitution to individuals. According to a simple calculation, the number of required diagnostic analyses is thought to be the product of the population by number of indices. To perform all of these analyses, it is clear that large amounts of money, time, and resources will be required, which is very difficult to realize by conventional analytical methods. To solve this problem, microchip technology is thought to be useful.

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Microchips or microfluidic devices for chemical and biochemical analyses have been greatly developed owing to the progress of microfabrication techniques. Microchemical systems using these devices have attracted much attention of scientists and engineers. 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.[1-4] As expressed by the name, the concept of these microchip-based systems proposes the integration of 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 have concerned DNA analysis by microchip electrophoresis with laser-induced fluorescence detection. These microchip-based electrophoretic systems have great advantages in some applications, especially in clinical diagnosis and molecular biology fields. Although they are very useful in some specific fields, other analytical methods are required for various applications which involve several chemical processes. To realize these complicated systems, it is necessary to utilize the

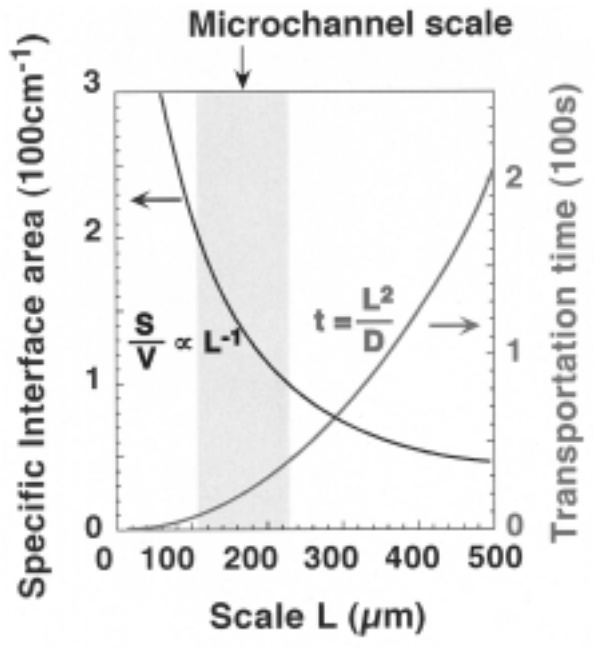

Fig. 1 Scale dependence of a specific interface area and diffusion time in a microchip. S, interface area; V, volume; L, diffusion distance; t, molecular transportation time; D, coefficient.

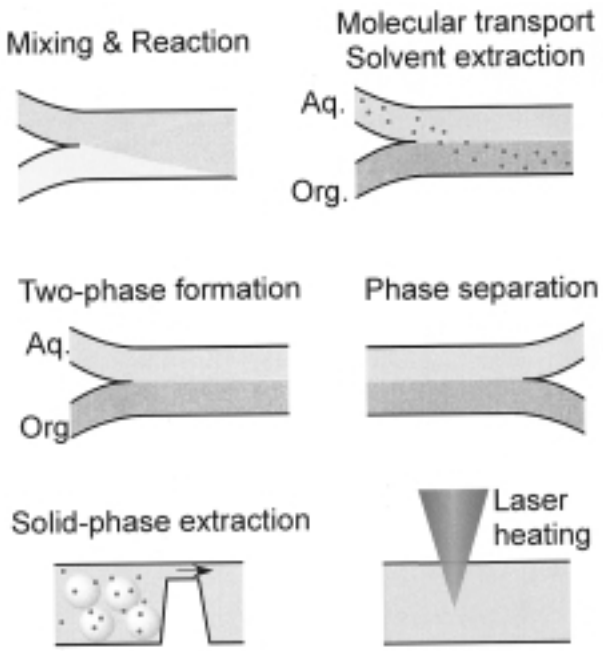

Fig. 2 Typical components of micro unit operation (MUO).

chemical properties and potentials of molecules effectively. For this purpose, microfluidic systems using pressure-driven flow are suitable. To realize highly organized and useful microfluidic systems, we proposed "continuous flow chemical processing (CFCP)", which is composed of several "micro unit operations (MUO)". Moreover, we developed a thermal lens microscope (TLM) which can detect non-fluorescent molecules with extremely high sensitivity in micro space. In this paper, we review the microchip-based chemical and biochemical analysis systems using pressure-driven flow for continuous and sequential chemical processing.

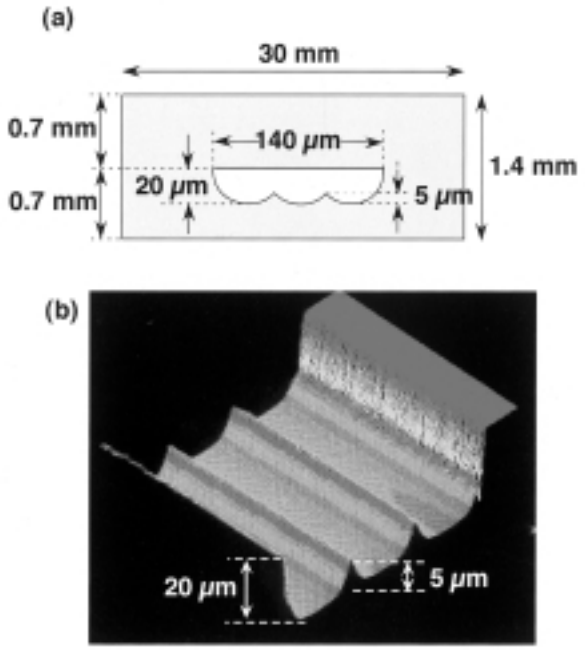

Fig. 3 (a) Cross-sectional view of guide structures fabricated in a microchip. (b) 3-D image of the structures.

2 Fundamentals of Microchip Chemistry

2·1 Characteristics of liquid microspace

A liquid microspace has several characteristic features different from the 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 a microspace, such as microchips, the molecular transportation time and the specific interface area must be considered. Figure 1 shows the scale dependence of the molecular transport time and the specific interface area.

The transport time is proportional to the square of the scale. Therefore, the transport time takes from several hours to one 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 a scale of about 250 μm.

2·2 Concepts of micro unit operation and 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. The components of MUO are shown in Fig. 2. By combining these MUO components, various kinds of chemical processing can be integrated into the microchips. We have demonstrated the integration of fundamental MUOs, such as

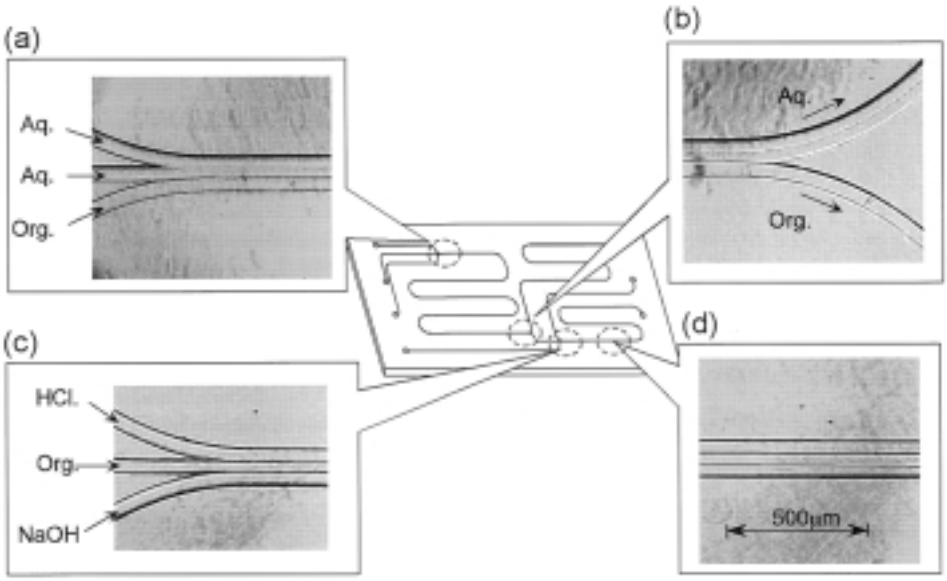

Fig. 4 Photographs of a liquid-liquid interface formed in microchannels. (a) Confluence of water and *m*-xylene. (b) Phase separation at the branching part. (c) Confluence of HCl, organic phase, and NaOH. (d) Stable three-phase flow.

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, the formation of a stable multiphase laminar flow network in microchannels has also been reported.[8,9,20] As shown in Fig. 2, MUOs utilizing aqueous–organic multiphase laminar flow, such as solvent extraction, are key technology in CFCP.

2·3 Fabrication of microchips

In general, a microchip is made from a glass plate, a silicon wafer, polydimethylsiloxane (PDMS), polymethylmethacrylate (PMMA), other polymers, or their combination. Because of the chemical and physical stability and optical transparency for detection, a glass microchip is suitable for various applications.

Glass microchips were fabricated using a photolithographic wet etching method.[8] Mechanically polished 0.7-mm-thick Pyrex glass plates were used for top and bottom plates. Inlet and outlet holes were drilled by a diamond-coated drill on the top plate. Twenty-nm-thick Cr and 100-nm-thick Au layers were evaporatively deposited on the bottom plate under a vacuum for good contact between the substrates and the photoresist and protection of the substrates during glass etching. 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 tranfer 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. The etched bottom plate and the top plate with inlet and outlet holes were thermally bonded without any adhesive in an oven at ~650˚C.

2·4 Stable multiphase laminar flow using guide structure

The confluence of two solutions or, in some cases, even the two-phase formation of an organic solvent and aqueous solution is easily realized by simple introducing two solutions into a Y- or T-shape microchannel. It is, however, very difficult to keep a long liquid-liquid interface or stable multiphase flow in a normal microchannel. To stabilize the interfaces, a guide structure is very effective (Fig. 3).

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

A cross-sectional view of the channel with guide structures is shown in Fig. 3(a). Figure 3(b) is a 3-D image of the etched microchannel structures which was observed with a laser confocal microscope. 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 arbitrarily controlled using the 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, the parameters influencing the stability of interfaces, such as the surface tension, and contact angle 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 the aqueous and organic phases is also not possible without them.

2·5 Surface chemical modification

Chemical modifications of the microchannel surface is also effective to stabilize the liquid-liquid interface. We have investigated the stabilization of the interface and control of the liquid confluence and separation by utilizing a chemical modification of the microchannel wall.[22] In this method, the microchannel for organic solvent flow was modified by surface coupling of the octadecylsilane (ODS) group, while the microchannel for aqueous flow had a bare glass surface. In

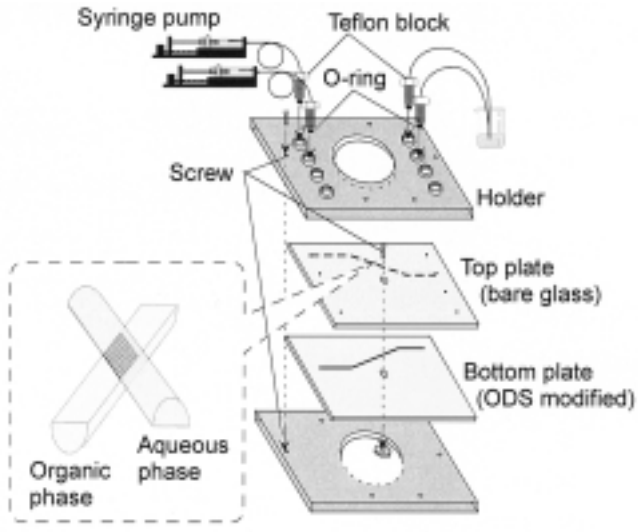

Fig. 5 Schematic illustration of the sealing and connection method for a chemically modified glass microchip. The top and bottom plates are pressed by a screw and holders.

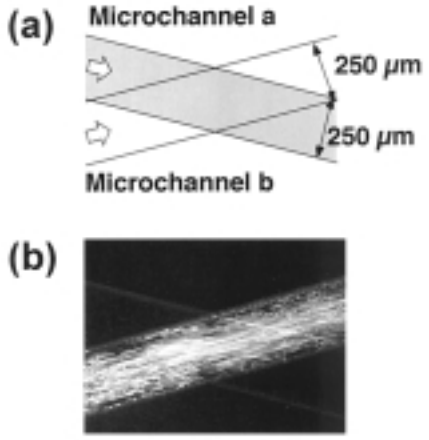

Fig. 6 (a) Illustration of the crossing region. (b) Fluorescent image of probe particles dispersed in the aqueous phase.

order to demonstrate the effectiveness of this method, two-phase crossing flow was performed.

A microchannel was fabricated on both the top and bottom plates, and these two channels were designed to cross at an angle of 30° by laminating the two plates (Fig. 5). The surface of only the bottom plate was modified with the ODS group after etching of the channel. 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 plates and the two plates were fixed by a screw (2 mm diameter) and a nut. Furthermore, 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 was slightly bent at the crossing region, and then went nearly straight. In order to demonstrate the effectiveness of our method, a gas phase (air) instead of nitrobenzene was introduced into the ODS-modified channel and a stable air/water crossing flow was observed. Although the density of air is much less than that of water, surface tensions are dominant rather than gravity in the microspace, and interfaces can be formed independently of the density difference. The result showed that the method could be applied not only to a liquid/liquid contact, but also to a gas/liquid contact.

3 Applications

3·1 Wet analysis of Co(II)

A schematic illustration of a Co(II) wet analysis 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, a sample solution containing Co(II) ions, a 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 resulting Co(II) chelate proceed as the reacting mixture flows along the microchannel. Since the NN reacts with the coexisting metal ions, such as Cu(II), Ni(II) and Fe(II), these coexisting metal chelates are also extracted into *m*-xylene. Therefore, a washing process is necessary 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 moved into an HCl solution. The decomposed chelating reagent, NN, is dissolved in a 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 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 microchannel 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 the 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 a linear relationship between the Co(II) concentration and the TLM signal.

3·2 Multi-ion optical sensor

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

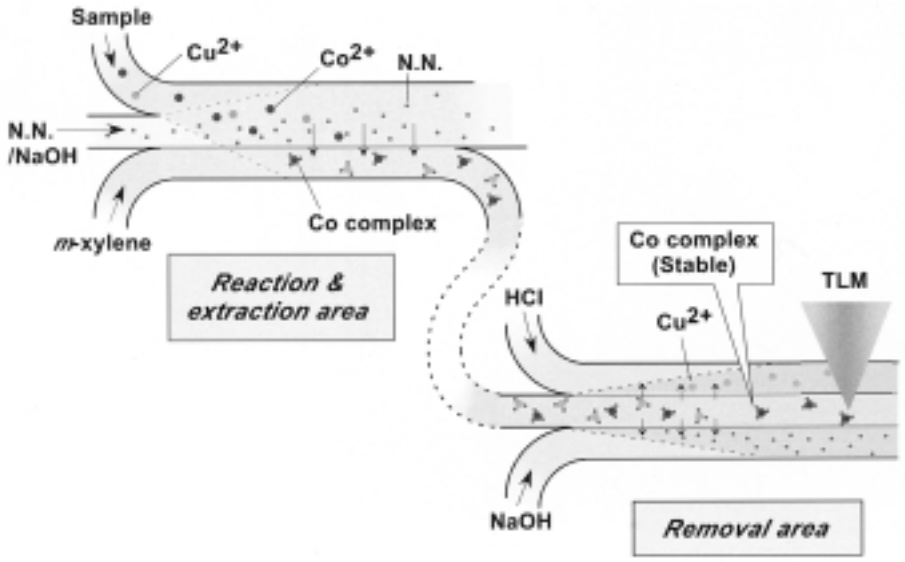

Fig. 7 Schematic illustration of cobalt ion determination by combining MUOs.

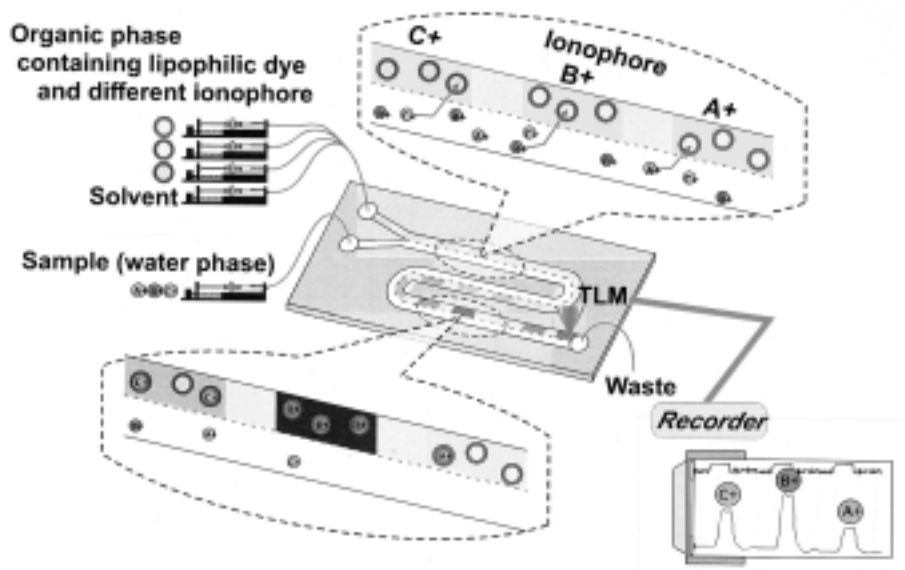

Fig. 8 Concept of a sequential ion-sensing system using a single microchip.

of multiple organic phases, and the formation of laminar two-phase flow with an aqueous sample solution was proposed.[10,14]

Figure 8 shows the basic concept of 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 the on-off switching of syringe pumps. In this case, an organic phase without an ionophore is introduced in between the two organic phases containing different ionophores, in order to avoid contamination. Aqueous sample solutions containing one or several kinds of 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 comes to equilibrate, and then detection of the color change of organic phase at the downstream area allows sequential and selective multi-ion sensing in a 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 analyzed with the system: a buffer solution containing 10^{-2} M K^+, 10^{-2} M Na^+, or 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 an 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 the ion analysis of a biological fluid by future optimization.

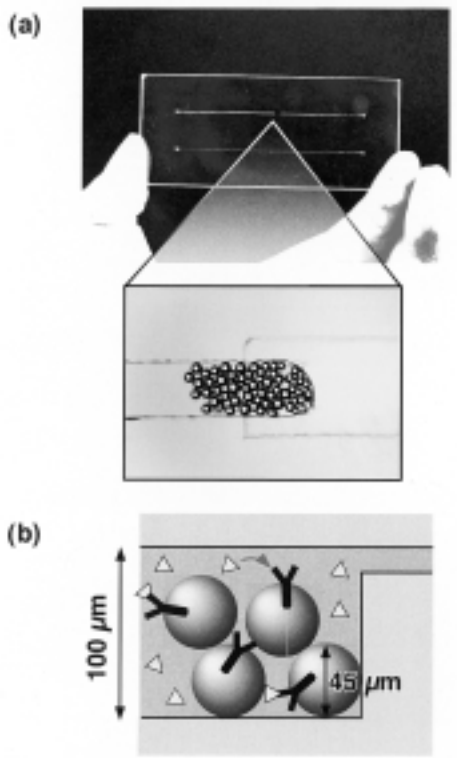

Fig. 9 (a) Immunoassay microchip with microbeads. (b) Cross-section image of the reaction area.

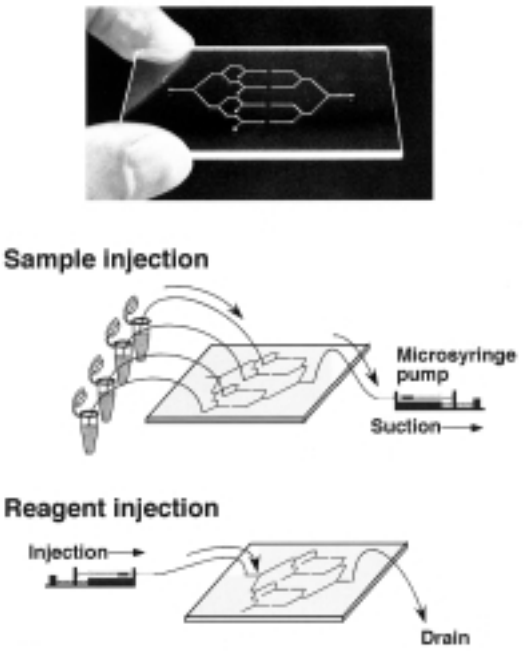

Fig. 10 Simultaneous determination system with a branching multichannel immunoassay chip.

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 the 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. The integration of analytical systems into a microchip should bring about an enhanced reaction efficiency, simplified procedures, a reduced assay time, and a lowered consumption of samples, reagents, and energy.

Recently, several papers concerning the integration of heterogeneous immunoassay systems into microscale devices were published.[15,16,26,27] In our previous paper,[15] we reported on the possibility of an immunosorbent assay on a microchip. In this 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.

This effect is brought about by an increase in the specific interface area and a reduction of the diffusion distance. An increase in the specific interface area means an increase in the reaction field. The specific interface area of a 50-μL solution in a microtiter plate well (0.65 mm in diameter) was estimated to be 13 cm^{-1}, whereas that of the microchannel (11 beads in 100 μm × 100 μm × 200 μm channel space) was 480 cm^{-1}. Therefore, that of the microchannel was 37-times larger than that of the microtiter plate, and the reaction rate may be increased by this larger reaction field.

In the case of the conventional microtiter plate assay, a 1.5-mm movement would be necessary for the most distantly located antibody molecule to react with an antigen fixed on the surface of the well, since the liquid depth was 1.5 mm. On the other hand, the liquid phase of the microchannel filled with polystyrene beads was much smaller. The longest distance from an antibody molecule to the reaction-solid surface may be less than 20 μm. Because the diffusion time is proportional to the squares of the diffusion distance, the diffusion time of the antibody molecule to the antigen in the microchip would be more than 5600-times shorter than in the conventional method.

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 within a short time with this system.

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

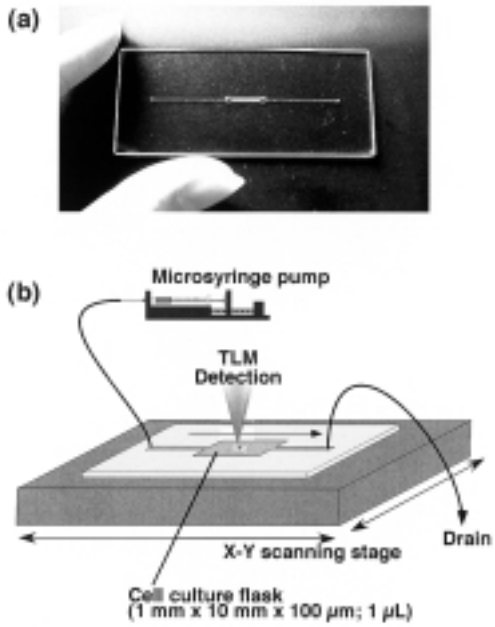

Fig. 11 (a) Cell culture chip. (b) Scanning thermal lens microscope system for single-cell imaging.

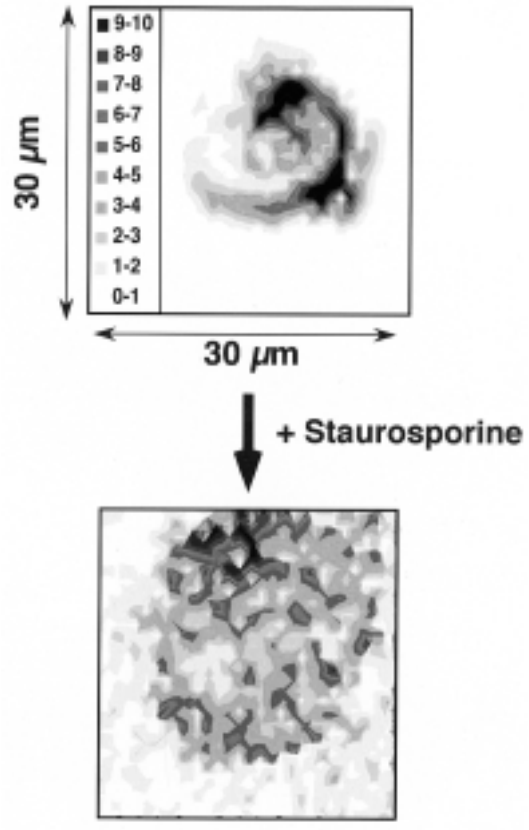

Fig. 12 Direct imaging of cytochrome *c* in a cell and its distribution change during the apoptosis process.

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 to show 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 by multichannels. To realize higher throughput analyses, a microchip system, which can process several samples simultaneously, was reported.[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 3-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, a higher throughput was realized with the branching structure chip.

The simultaneous assay of many samples may also be achieved by simply arraying many channels in parallel on a chip. This approach, however, requires 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 a simultaneous assay. By branching multichannels, the numbers of pumps and capillary connections required for the system should be reduced. An automated multichannel immunoassay system with a much higher throughput will be realized by the development of multiple fluidic control devices in the near future.

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 the cells. For instance, by using a microflask fabricated in a microchip, a rapid and secure exchange of media or reagents could 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. There are, however, very few papers about microchip-based biochemical analysis systems in which both cell culture and analyses can be performed.

Recently, we developed a novel cell analysis system consisting of a scanning TLM detection system and a cell culture microchip (Fig. 11).[11] A microflask (1 mm × 10 mm × 0.1 mm; 1 μL) was fabricated in a glass microchip. After a cell suspension was introduced into the microflask, 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 while scanning of the stage to obtain a 2D-image. The system could detect nonfluorescent biological substances with extremely high sensitivity without any labeling materials, and had a high spatial resolution of ~1 μm. The microchip system was good for liquid control, and simplified troublesome procedures. This system was applied to monitoring the 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.

4 Conclusions

In this review, the concept of 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 a guide structure and a surface chemical modification were also demonstrated. In addition, the usefulness and effectiveness of our method were demonstrated in some applications: Co^{2+} wet analysis, multi-ion sensor, immunoassay and cell analysis.

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

Many merits and uses of microchip systems with pressure-driven flow have been revealed. By utilizing several characteristics of microspace, *i.e*., a large specific interface, a short molecular diffusion distance, and so on, the performances of several analysis systems were greatly improved by microchip integration. In the near future, these microchip-based systems will be widely spread and highly beneficial to our daily life by further efforts of analytical chemists and engineers.

5 Acknowledgements

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