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Integrated chemical systems on microchips for analysis and assay. Potential future, mobile high-performance detection system for chemical weapons*

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Abstract: By analogy to unit operations (e.g., mixers, reactors, etc.) used in conventional chemical engineering, the concept of *microunit operations* permits the integration of complicated chemical systems onto a small microchip. A protocol for fabrication of such microchips is described, and its use is illustrated in several examples. In addition, the thermal lens microscope, which determines nonfluorescent species at the single-molecule level, is indispensable as an ultrasensitive detector for general use. Applications of microchip technology are given for chemical analysis, immunoassay, and full bioassay. Microchip analysis can provide very large enhancements in sensitivity and substantial reductions in measurement time as compared with conventional analytical methods.

INTRODUCTION

High-performance analytical technology may serve as one of the most effective tools to alleviate the menace of chemical weapons (CW), which, in the worst cases, can affect humans from tens of kilometers away, are invisible and unscented, and do not reveal their symptoms until a few days afterward.

Recent development in microchemical technology enables us to integrate even a complicated chemical system onto a small microchip [1–9]. Microchemical technology is applicable to a wide range of chemical processes, including analyses [2–5], bioassays [2,3,6], and chemical syntheses [7–9]. The merit of the microintegration is that we can use rapid, efficient, and sophisticated chemical technologies anytime and anywhere as the occasion demands. That is, mobile and high-performance analytical systems will be realized in the near future. We cannot deal with CW and biological weapons (BW) attacks by conventional huge and complicated analyzers in analytical centers, because samples must be transferred from the actual spot to laboratories and it usually takes hours or days for these machines to show the results of analyses. Microchemical chips are expected to make analyses in minutes or even shorter periods in the future.

Now, on-chip integration of electrophoresis, mostly for genome technology, is the most popular topic in microchemical process research (Fig. 1a) [10]. Highly sensitive detection methods for extremely small amounts of samples are necessary for microchemical technology in general. In the on-

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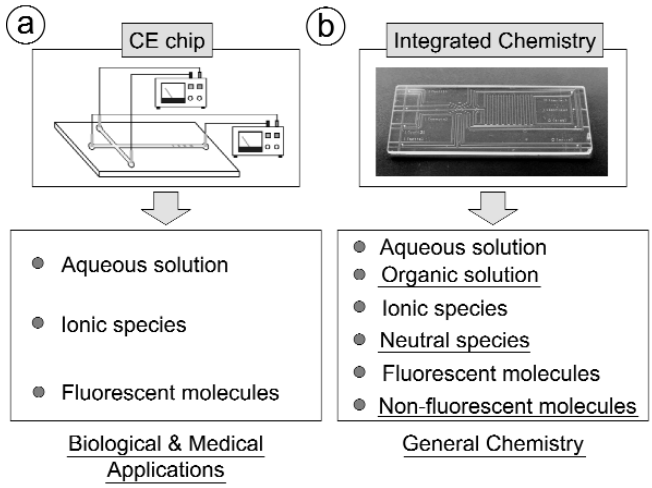

Fig. 1 Comparison between (a) on-chip electrophoresis and (b) integrated chemistry.

chip electrophoresis experiments, fluorescent labeling and laser-induced fluorescence (LIF) detection are used in a highly sensitive detection method [11]. However, the variety of chemical and biological weapons is so wide that combinations of electrophoretic separation and LIF are incapable of covering all of them. The analytical methods for chemical and biological weapons must cover even neutral and nonfluorescent species. An integration methodology that can be applied to more diverse analytical and biosystems is demanded.

Our research group has developed a more general methodology for microintegration of analytical systems (Fig. 1b) [12]. For this subject, microunit operation (MUO), continuous-flow chemical process (CFCP), multiphase laminar flow (MPLF), and thermal lens microscopy (TLM) are key elements of our methodology. MUO and CFCP are basic concepts for the integration of general chemical processes onto microchips [12]. Micro fluid control based on MPLF [13] and photothermal determination of nonfluorescent species using TLM [14] are fundamental technologies to realize MUO and CFCP.

In this review article, together with a brief recipe for fabricating microchemical chips [13], MUO, CFCP, TLM, and some of their applications to chemical analysis and biological assays are introduced.

FABRICATION

A variety of microfabrication methods, such as photolithography/wet etching, laser fabrication, sandblasting, reactive ion etching, and fast atomic beam fabrication were applied for preparing microchips depending on the microchannel sizes [1]. However, the most commonly used fabrication method for making glass microchips is the photolithography/wet etching method. An outline of our protocol is shown in Fig. 2 [13].

A mechanically polished 0.7-mm-thick Pyrex glass plate was the substrate (bottom plate). The substrate plates were annealed at 570 °C for 5 h before use. 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. A 2-μm-thick positive photoresist was spin-coated on the metal 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 $Ce(NH_4)_2(NO_3)_6$ solu-

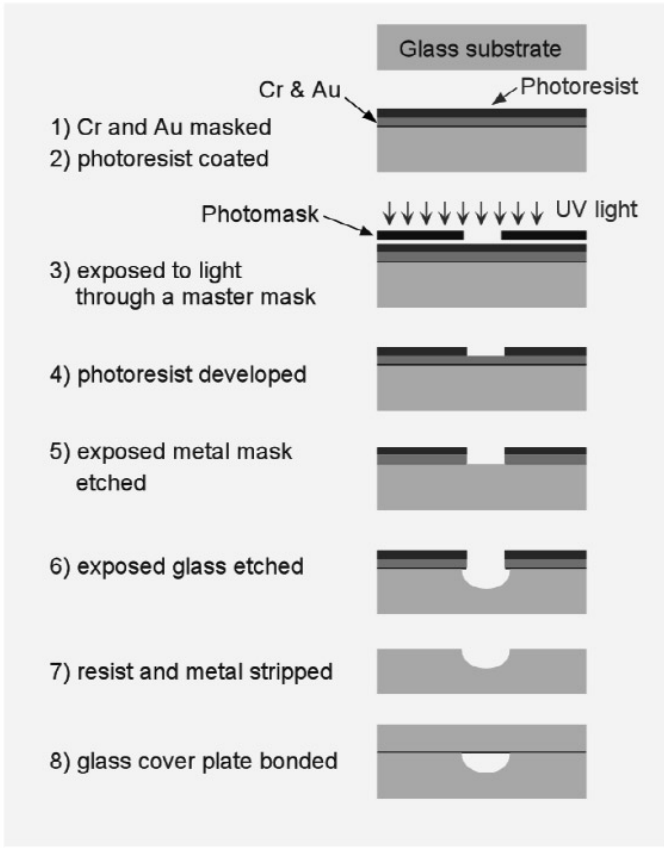

Fig. 2 Protocol of glass microchip fabrication by photolithography/wet etching method.

tions. Inlet and outlet holes were drilled by ultrasonic sand-blasting on another Pyrex glass substrate (cover plate). The cover and etched plates were thermally laminated in a furnace at 650 °C for 5 h after washing in an ethanol and NaOH solution.

MICROCHEMICAL PROCESS

When one constructs a conventional macro-scale chemical plant based on chemical engineering, chemical processing required for the chemical plant is designed by combining conventional macro-scale unit operations [15,16]. From the analogy of unit operations, such as mixers, reactors, etc., in chemical engineering, we introduced the concept of MUO, which has led to integration of complicated chemical systems on a microchip [12]. In previous studies, we demonstrated the integration of fundamental MUOs, such as mixing and reaction [17–19], two- and three-phase formations [13,20–22], solvent extraction [13,20–26], solid-phase extraction [27,28], optical or electrical heating [29,30], and cell culture [31].

Some examples of the fundamental MUOs are illustrated in Fig. 3 [12]. These MUOs basically utilize the physical characteristics of liquid microspace. The outstanding characteristics are large specific interface area (S/V), short molecular and energy transport time by diffusion, and small volume and heat capacity [7,32]. These physical characteristics are in proportion to inverse, second, and third power of the scale, respectively. Therefore, several orders of rapid molecular and energy migration in and between liquid phases comparing to the macro-scale chemical processes is available in the microspace. And con-

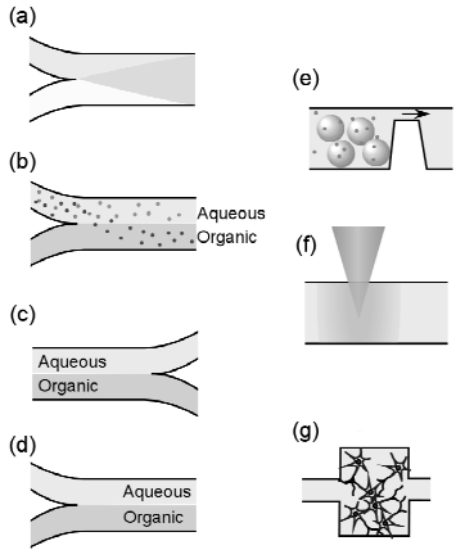

Fig. 3 Schematic illustrations of MUOs: (a) mixing and reaction, (b) solvent extraction, (c) phase separation, (d) two-phase formation, (e) solid-phase extraction, (f) heating, and (g) cell culture.

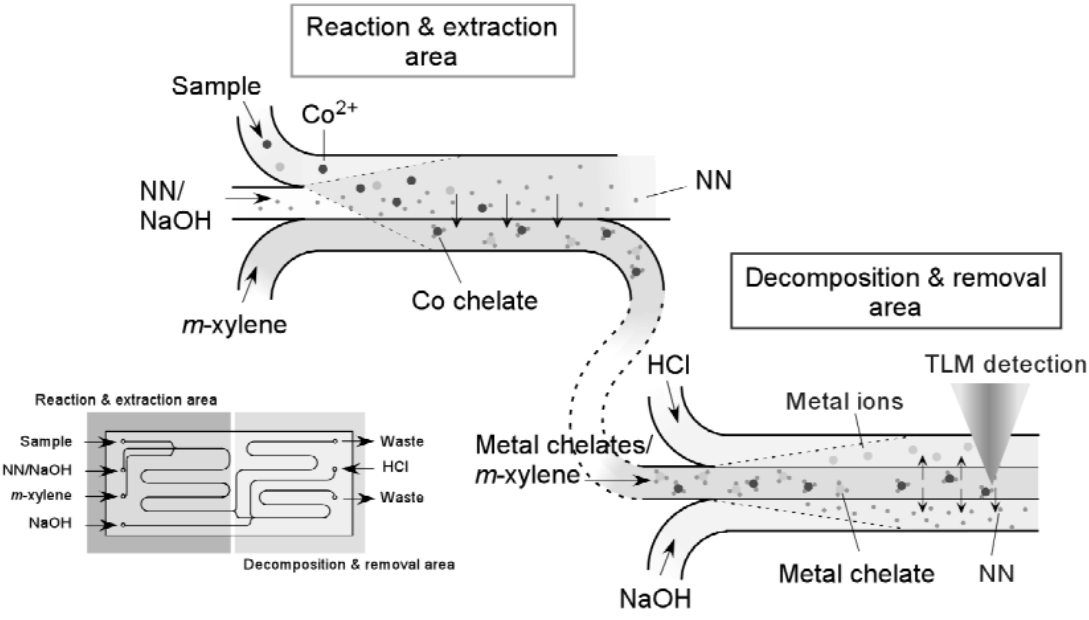

Fig. 4 Schematic illustration of Co(II) determination by combining MUOs (NN: 2-nitroso-1-naphtol).

sequently, mixing, extraction, surface reaction, and optical temperature control become very fast, efficient, and homogeneous without using any mechanical actions but spontaneous motion of molecules.

In addition to development of the MUOs, we demonstrated that a stable multiphase flow network can be formed in microchannels [13]. By combining MUOs in a multiphase flow network, we thought that a complicated chemical system including chemical processing could be integrated on a microchip. We call this methodology CFCP [12].

As the MUOs are operated in y-shaped or straight microchannels, they can be easily combined with each other. Various kinds of MUOs are combined in serial or in parallel, and almost every kind of chemical processes can be realized on microchips. A chemical process of chelating and extraction on microchip is shown in Fig. 4 as an example [12]. A microchemical process for chelating, extraction, and purification is composed of MUOs. The y-shaped channels for mixing and extractions can be put together, and the one channel is common for these two MUOs. Therefore, the chelating and extraction part of Fig. 4 becomes a w-shaped channel. All of the chemical processes are carried out within the continuous flow, so the method is called CFCP.

As these processes are pressure-driven and the molecule handling is based on diffusion, neutral species and nonaqueous solvents can be treated on microchips. Thus, general chemical processes and systems can be integrated on microchips by using MUO and CFCP. This is the most outstanding merit of the methodology we developed.

FLUID CONTROL AND ULTRASENSITIVE DETECTION

To realize the CFCP illustrated in Fig. 4, fluid control and ultrasensitive detection for nonfluorescent species at a single-molecule level were indispensable.

Fortunately, the Reynolds number of micro flowing liquids is usually smaller than ten, and flow inside a microchannel tends to be laminar [33–35]. In addition, gravitational force is two or three orders smaller than liquid–liquid or liquid–solid interface tension, and gravity is negligible compared to surface tension [13]. Consequently, MPLF as shown in Fig. 5 is naturally formed in microchannels [12].

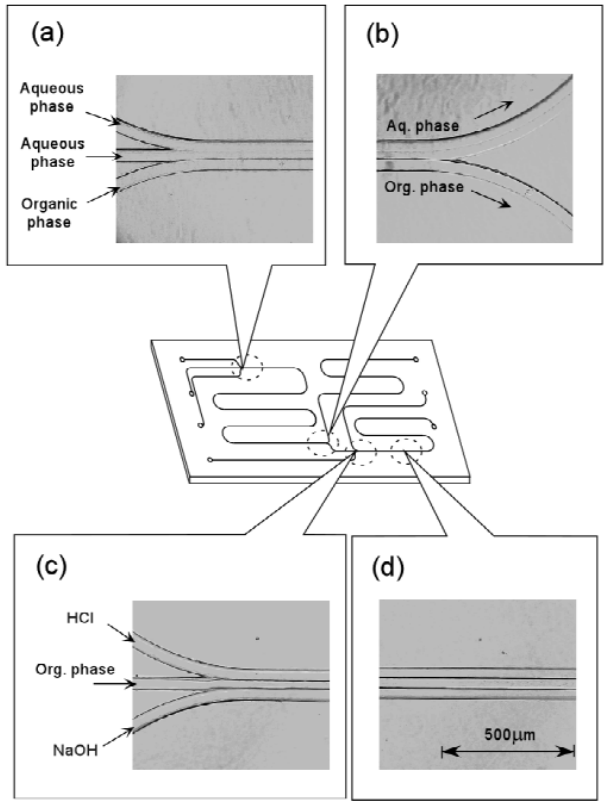

Fig. 5 Photographs of the liquid–liquid interface formed in the microchannels: (a) confluence of water and *m*-xylene; (b) phase separation part; (c) confluence of HCl, organic phase, and NaOH; and (d) three-phase flow.

Note that organic and aqueous phases flowed side by side, in spite of density difference between the two phases.

In addition, we fabricated a guide structure at the bottom of microchannel [12] to further stabilize the MPLF. By means of the guide structure, control of MPLF necessary for CFCP illustrated in Fig. 4, which was stabilized during the whole analytical procedure.

Another indispensable factor is ultrasensitive detection of nonfluorescent molecules, because analytes or products of general chemical process are usually nonfluorescent. We have developed our original ultrasensitive detection method, a TLM for nonfluorescent species [14]. The author must omit the detailed explanation of the principle of TLM for lack of space. In short, the TLM detects the laser-induced thermal lens effects under the optical microscope. A photograph of a commercially available desktop TLM (Institute of Microchemical Technology, Inc., Japan) is shown in Fig. 6. Generally, it is difficult to detect the thermal lens effect for optical microscopes of which chromatic aberration is compensated. In our TLM optics, the chromatic aberration between excitation and probe laser beams are adjusted to an almost optimal distance. This desktop TLM was proved to have the ability of determination at half-molecule levels in a liquid sample [36,37].

Fig. 6 A desktop TLM (Institute of Microchemical Technology, Inc., Japan).

ANALYTICAL APPLICATIONS

As described above, the design, fabrication, control, and detection methods were established for integrated microchemical systems. We have applied the techniques to analytical, synthetic, biochemical, cell-biological, and physicochemical applications. Typical examples for analytical applications among them are introduced in this review.

Heavy metal analysis

The first example is trace heavy metal analysis in environmental water, made possible with the microchip, the operational principle of which was described in Fig. 4 [12]. This example is for trace cobalt analysis. The structure and photo of the chip is shown in Fig. 7. A total chemical process for wet analysis was decomposed into 7 kinds and 13 steps of unit operations, such as chelating, extraction, washing, etc., and translated into MUOs. The CFCP design in Fig. 4 was reconstructed from those

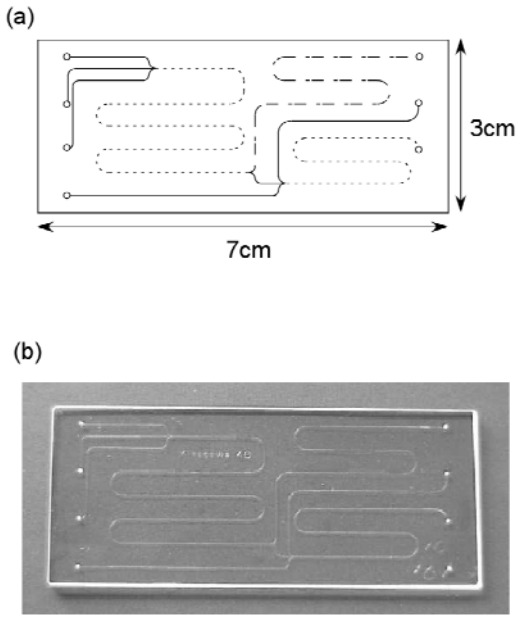

Fig. 7 Microchip. (a) Schematic illustration. The solid, dotted, and chain lines are 50 μm wide and 20 μm deep, 140 μm wide and 20 μm deep, and 90 μm wide and 20 μm deep, respectively. (b) Photograph.

MUOs. The microchip consists of two different areas: the former is the reaction and extraction area and the latter is the washing, that is, decomposition and removal, area. In the former area, the sample solution containing Co(II) ions, the 2-nitroso-1-naphtol (NN) solution and *m*-xylene are introduced at a constant flow rate through three inlets using the 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 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, washing 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 the HCl solution. The decomposed chelating reagent, NN, is dissolved in the sodium hydroxide solution [38] in contrast to the coexisting metal chelates, the Co chelate is stable in HCl and NaOH solutions and remains [38].

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 microchannel in a similar manner as described above. Finally, the target chelates in *m*-xylene are detected downstream by TLM.

The advantages of our approach compared with a conventional method are simplicity and omission of troublesome operations. The acid and alkali solutions cannot be used simultaneously in the conventional washing method, but this becomes possible by using a three-phase flow in the microchannel. This chemical processing corresponds to the integration of eight MUOs on a microchip: two-phase formation, mixing and reaction, extraction, phase separation, three-phase formation, decomposition of coexisting metal chelates, removal of metal ions, and removal of reagents.

Cobalt in aqueous solution containing admixture was successfully determined. Even the zmol (10^{-21} mol) levels of cobalt could be extracted and determined. The calibration line showed good linearity, and the determination limit obtained from 2σ reached 0.13 zmol, that is, 78 chelate molecules. What is more important is that the analytical time was reduced from 2–3 h to only 50 s. This kind of drastic reduction of analytical time and system size, even for the complicated chemical procedure like this, anticipates the future application to mobile advanced analytical equipment.

Immunoassay system

The second example is the microintegrated immunoassay system [27,28,39]. Immunoassay utilizes highly selective and sensitive antigen–antibody reactions. Therefore, immunoassay is very suitable for detecting biological substances such as disease marker proteins, drugs, biologically harmful substances, and maybe chemical weapons. But the demerit of immunoassay is analytical time, complicated procedure, and very expensive reagents. The microchip technology is a promising method for overcoming these demerits of immunoassay [27,28,39–45].

The microchip for immunoassay is shown in Fig. 8 [39]. Polystyrene beads of 45 μm in diameter were introduced into a 100-μm microchannel fabricated on a glass chip. This situation in the microchannel filled with polystyrene beads is similar to a peripheral blood vessel containing blood cells. Some important chemical reactions in a living body, such as antigen–antibody reaction, proceed quite efficiently on the surface of blood cells. We anticipate that one of the reasons for the high reaction efficiency is the very large specific surface area of the blood cells and short diffusion distance in the clearance space in the blood vessel. And then, we have applied biomimetic microchannel to immunoassay. The schematic illustration is shown in Fig. 9. A microtiter plate is usually used in the conventional immunoassay. The field of the antigen–antibody reaction is localized on the wall in the well. Therefore, a labeled antibody must move to the wall in order to react an antigen on the wall. On the other hand, the liquid phase of the microchannel filled with polystyrene beads was much smaller than that of the microtiter plate. The longest distance from an antibody molecule to the reaction-solid surface may be less than 20 μm. Since diffusion time is reduced dramatically by using our system, the time necessary for the antigen–antibody reaction can be reduced. Secretory human immunoglobulin A (s-IgA), which is well known as a stress indicator, was assayed with this system, and assay time was dramatically reduced from 24 h to about 20 min. Furthermore, a sandwich method was introduced to this integrated immunoassay system, and a more specific and sensitive immunoassay was realized.

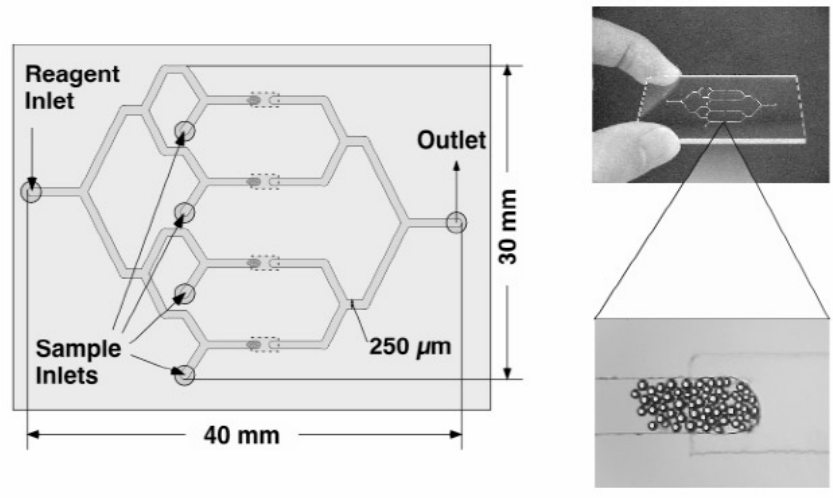

Fig. 8 Microchip for immunoassay.

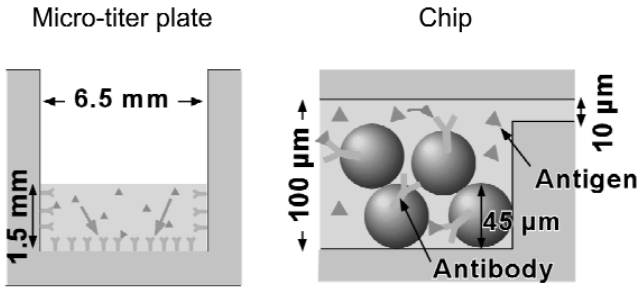

Fig. 9 Schematic illustration of the immunoassay in microtiter plate (bulk) and immunoassay chip (micro).

Human carcinoembryonic antigen (CEA), which is one of the most widely used tumor markers for serodiagnosis of colon cancer, was assayed. The time for assay was reduced from a few days to less than 1 h, and its determination limit, several 10 pg/ml, was also at least one order superior to the conventional enzyme-linked immunosorbent assay (ELISA) method.

This micro immunoassay system has already been applied to practical samples. Trace CEA was determined from patient sera. The assay results corresponded to the values obtained from conventional ELISA methods, and successfully detected abnormal values against the colon cancer patients.

Bioassay system

The last example is integration of a more complicated system. We have integrated a full bioassay system on a microchip as shown in Fig. 10. Bioassay systems require cell cultivation, stimulation, and various kinds of enzymatic and chemical reactions. These individual processes were miniaturized on respective microchips, and then were put together on one microchip by CFCP. These bioassay microchips were applied to the direct detection of a retrograde neurotransmitter emitted by the stimulation of glutamate, in vivo direct detection of cytochrome-C during apoptosis, and the monitoring of a NO release process by carcinogen stimulation.

These bioassay chips may be useful not only for the study of chemical toxicity or basic cell biochemistry, but also the rapid detection of trace biohazardous chemicals in the future.

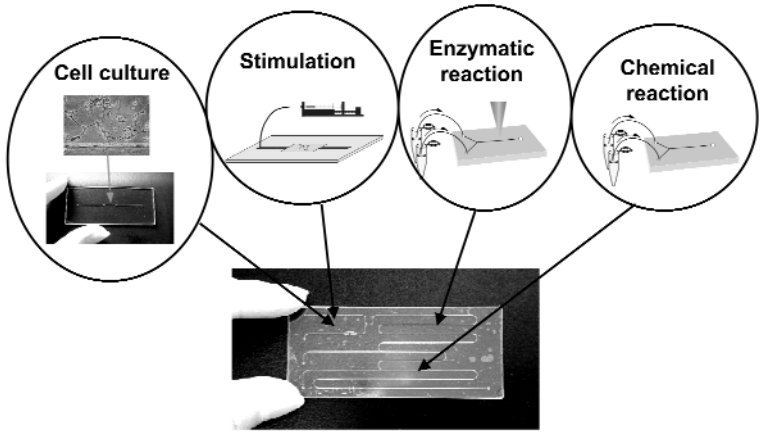

Fig. 10 Schematic illustration of bioassay system on a microchip.

CONCLUSION

We have developed an integration method of general chemical processes and systems on a microchip. These design, fabrication, control, and detection methods are applicable for various kinds of chemical and biological systems. The merit of the integrated microchemical systems are drastic process time reduction, sample and reagent saving, waste reduction, and miniaturization. This technology makes the complicated, delicate, and huge chemical systems simple and compact for mobile use.

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