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Integration of Flow Injection Analysis and Zeptomole-Level Detection of the Fe(II)-*o*-Phenanthroline Complex

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Microchannels having a 150×100 μm cross section were fabricated in a quartz glass chip as a component in an integrated flow injection analysis (FIA) system. They were put to use for flow, mixing, reaction, and detection. The reaction system was a chelating reaction of divalent iron ion with *o*-phenanthroline (*o*-phen), and a photothermal microscope was applied for the ultra-sensitive detection of the non-fluorescent reaction product. Nano liter levels of solutions were introduced and transported by capillary action and mixed by molecular diffusion. Zeptomole levels of the reaction product were detected quantitatively. This was the first demonstration of an on-chip chemical determination device which integrates the primitive FIA system without using electroosmotic liquid control or fluorometric determination.

Keywords On-chip integration, flow injection analysis, photothermal microscope, molecular diffusion

The miniaturizations of chemical systems have been proposed and developed from various viewpoints of mechanical engineering and analytical chemistry.[1-5] A miniaturized gas-chromatographic air analyzer, micropump, and micromixer were fabricated using micromachining techniques. In these micro systems, the basic operating functions involved in chemical systems, such as liquid transport, mixing, reaction, separation, and detection were miniaturized in a small box or on a planar glass substrate. More recently, miniaturization of analytical systems has become of major interest. One example is a micro total analysis system (μ-TAS)[6], in which sample treatment, handling, and detection are performed with three-dimensional micro-flow manifolds.[7] A new attempt to realize analytical chemistry in a liquid micro space has been studied using capillary electrophoresis or capillary electrochromatography.[8-18] Many studies dealing with electrically driven separation techniques on a chip have been reported, such as capillary electrophoresis[8-12], free-flow electrophoresis[13], open-channel electrochromatography[14], micellar electrokinetic capillary chromatography[15], capillary gel electrophoresis[16], sizing of DNA restriction fragments[17,18] and so on. The miniaturization of these analytical systems has attracted much interest as a substitute for ordinary capillaries. One reason for this trend is that electroosmotic flow and electrophoresis are very suitable means for fluid control and separation in a micro space, such as micro channels. Samples handled in this line of study are necessarily aqueous solutions, and biological substances are the main analytes. For example, DNA fragments or amino acids solutions were transported and pumped in micro channels by electroosmotic force, and mixed with a dye for labeling, separated by electrophoresis, and then detected by a laser-induced fluorescence method (LIF). This method provides not only miniaturization of instrumentation, but also great improvements in the separation performance and shorter analysis times.

However, there are restrictions, in principle, for wider applications in general chemistry if combinations of these electroosmotic fluid control, electrophoretic separation, and fluorometric detection methods are employed. First, the electroosmotic driving force can be applied only to aqueous solutions or to a few polar solvents.[19-21] Moreover, applying an electric field causes spatial inhomogenization of the components, owing to a difference in the mobility; this could lead to a spatial change in the sample composition during transportation under a high electric field, although this effect is necessary for electrophoresis analyses. There are usually multi-components in a sample solution, and each component has a characteristic mobility. Therefore, an electroosmotic driving force is surely suitable for separation, but not for transportation. Secondary, although LIF is surely ultra sensitive, of course it can only be applied to fluorescent species. Therefore, a more suitable method for driving samples and detection is desired for the integration of more general chemical and analytical systems.

Thus, in this study, we examined the capillary action for liquid transport, molecular diffusion for mixing, and

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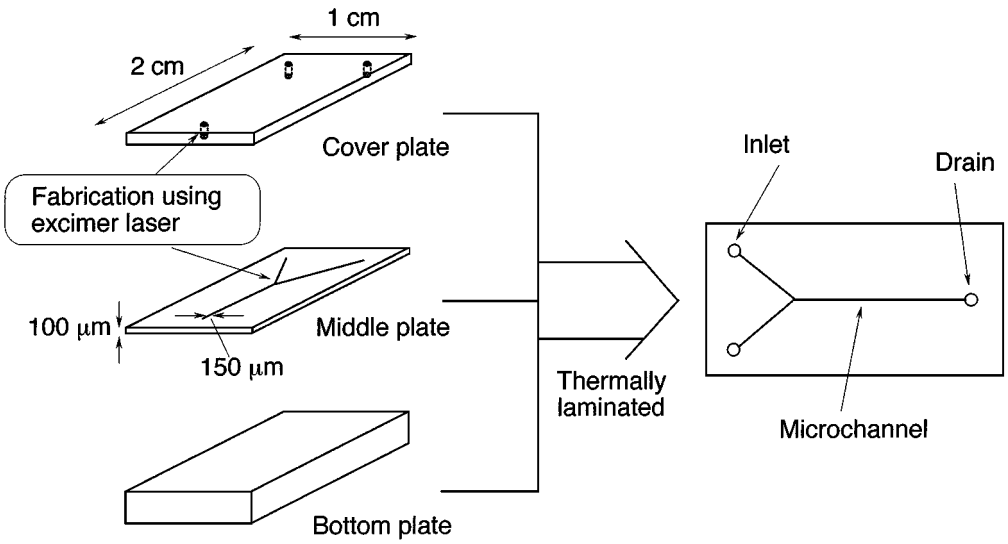

Fig. 1 Assembly and layout of the glass chip. Fabrication pattern of the quartz glass plates is shown on the left-side figure.

the thermal lens microscope for ultra-sensitive detection of non-fluorescent species. We have developed a thermal-lens microscope, and have shown that it can be applied to the ultra-sensitive detection of non-fluorescent substances.[22] In this study, we tried to integrate a flow-injection analysis (FIA) system on a glass chip, and applied it to the ultra micro determination of divalent iron ions.

Experimental

Quartz glass chip

The assembly and layout of the glass chip used are shown in Fig. 1. The chip comprised three pieces of quartz glass plate, *i.e.*, the cover, middle, and bottom plates having thicknesses of 350, 100, and 500 μm, respectively. Each plate was a 1×2 cm rectangle. A highly focused and intensified excimer laser beam illuminated the middle plate to pierce the channel part; the beam was then scanned to inscribe the channel pattern. The micro channels were made inside the glass chip by sandwiching the middle plate between the top and bottom plates. Three small holes (1 mm in diameter) were mechanically bored on the top glass for two inlets and an outlet (a drain). These three plates were laminated by using an optical contact; that is, the plates were polished to an optically smooth and flat (λ/10) finish, and then laminated together in an oven at 800°C without any adhesive. The microchannels formed in the glass chip were 150 μm wide and 100 μm deep.

Thermal lens measurement in a microchannel

The measurement principle for the thermal-lens microscope was given previously[22], as well as details of the detection of a sample in a microspace. Therefore, only a brief explanation is given here. The excitation and probe beams were introduced coaxially into an optical microscope and focused into the sample in the microchannel after passing through an objective lens. The glass chip was mounted on a 3-D stage which could be controlled in 1 μm steps in each direction; the step was precise enough for positioning the foci of the laser beams. The numerical aperture (NA) of the objective lens was 0.65, and the spot size of the coaxial beams was about 1 μm. The thermal-lens effect arose within the region of the confocal volume, and the confocal length was calculated to be 1.6 μm for this NA value and excitation wavelength mentioned below. Therefore, the volume contributing to signal generation was estimated to be about 3 fl.

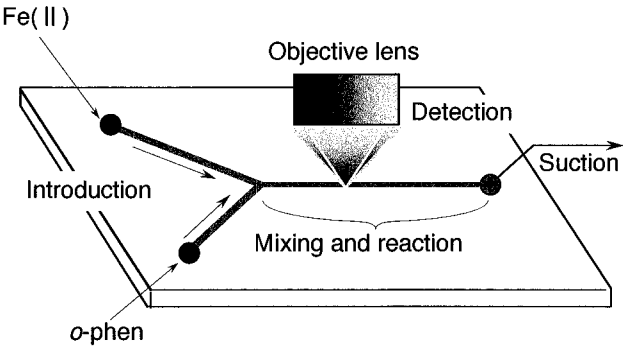

Fig. 2 Schematic illustration of the integrated FIA system.

The experimental setup was the same as mentioned in our previous report.[22] The excitation beam was the 514.5 nm emission line of an argon ion laser which was mechanically chopped by a light chopper at 1.69 kHz. The probe beam was a He-Ne laser of 632.8 nm. The excitation beam passing through the sample was filtered using an optical narrow band-pass filter for separating from the probe beam, and was then further spa-

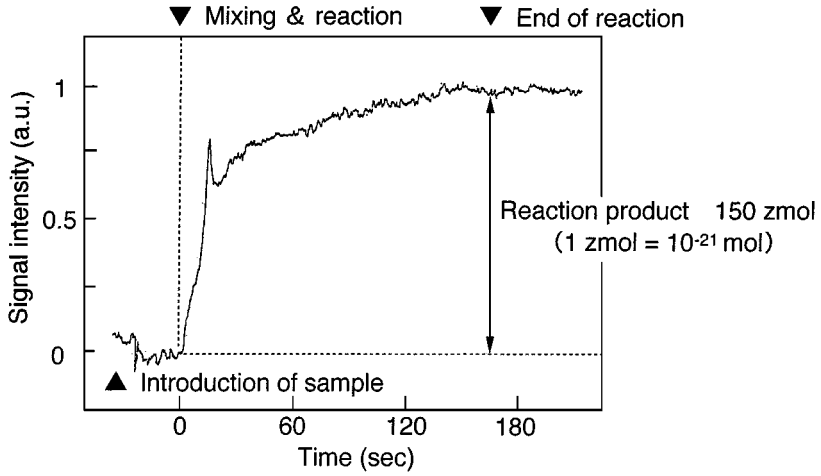

Fig. 3 Temporal behavior of the thermal-lens signal showing the production of the Fe(II)-phen chelate. The concentration of Fe(II) solution used here was 8.0 μM.

tially separated by a diffraction grating. Finally, it was introduced into a photodiode after passing through a pinhole, and the thermal lens effect was detected from the change in the detected probe beam intensity.

Samples and reagents

Ammonium iron(II) sulfate hexahydrate (Mohl's salt) was used to prepare an aqueous standard stock solution of 1.0 mM Fe(II). The sample solutions of Fe(II) were prepared at concentrations of 4 – 20 μM by stepwise dilution with ultra purified water. Hydroxyl ammonium sulfate was added (0.028 w/w) to each sample solution as a reductant. A solution of *o*-phenanthroline (*o*-phen) was prepared as follows: first *o*-phen was dissolved in a small amount of ethanol and diluted with water to give concentrations of 1 mM and 40 μM. The pH of these solutions was adjusted to 4.7 by sodium acetate/acetic acid buffer (1 mM).

Results and Discussion

A schematic illustration of the integrated FIA system is shown in Fig. 2. First, every microchannel was filled with pure water. Then, Fe(II) and *o*-phen solutions were injected into each reservoir. When water in the drain hole was suctioned out, the two solutions were introduced simultaneously into the mixing and reaction microchannel by capillary action. The sample solutions were mixed by molecular diffusion, and the chelating reaction occurred. The volume of the mixing and reaction channel was 200 nl, and therefore the absolute amounts of Fe(II) and *o*-phen introduced were calculated to be 0.4 – 2 pmol and 100 pmol, respectively. The coaxial laser beams were irradiated into the channel 4 mm downstream from the intersection point and the reaction product was detected there.

Liquid transport, control, mixing and stirring in the integrated FIA system by capillary action and molecular diffusion were examined at first. In the integrated FIA system, microfluid flow in the microchannel was visualized using two kinds of concentrated dye solutions (Methylene Blue and Sunset Yellow aqueous solutions), and the behavior of liquids in the microchannel was observed under an optical microscope. These dye solutions were introduced from the two reservoirs at the top of the Y-shaped microchannel shown in Fig. 2. In the drain reservoir at the opposite end of the mixing and reaction channel, we dipped in a thin piece of yarn and suctioned out the solutions by capillary action. The two solutions formed a parallel laminar flow in the microchannel, and did not mix with each other during the flow. This may have been because hardly any shear flow diffusion occurred. However, once the yarn was pulled out of the drain and the flow was stopped, they began to diffuse, gradually mixing. The mixing was sufficient and no mechanical stirring was done. These behaviors of the liquid are considered to be characteristic in a narrow channel, and are of great convenience for integration of the chemical operations on a chip. The details are discussed more quantitatively in the following experiment along with a theoretical consideration.

Fe(II) and *o*-phen solutions were mixed with each other, after which a chelating reaction occurred in the microchannel. The reaction product had a light absorption peak at 510 nm, and the molar absorptivity at this wavelength was 11400 (M^{-1} cm^{-1}). Though the excitation wavelength 514.5 nm was not exactly at the absorption peak, almost the same molar absorptivity was provided. Therefore, we monitored the progress of the reaction by a thermal-lens microscope. The temporal behavior of the thermal-lens signal is shown in Fig. 3. The time origin was when the flow was stopped. The signal began to rise at t=0 and increased steeply until t=20 s. The signal kept increasing slowly until it

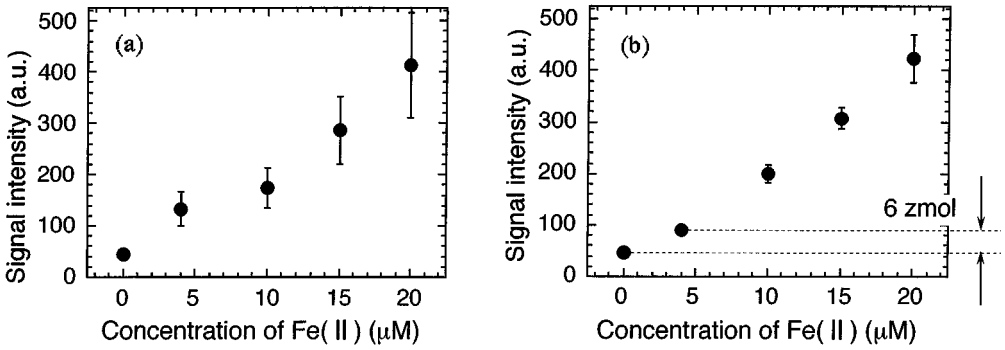

Fig. 4 Calibration curve of the Fe(II) solution and the absolute amount of chelating reaction product. The results of the reaction in microchannel are given in (a), and those of the reaction in bulk quantity with detection in the chip are in (b). The concentration of the *o*-phen solution was 1 mM.

reached a steady state at around t=160 s. Because the difference in the signal intensity between these stationary and background levels exhibited a linear response to the concentration of Fe(II) solution, we could conclude that the monitored signal originated from the chelating reaction product.

The time required for molecular diffusion in the microchannel is roughly estimated on the basis of the relation between diffusion time and diffusion length,

$$l=(Dt)^{1/2}, \quad (1)$$

where l, t and D are the diffusion length, diffusion time and diffusion coefficient, respectively. When the diffusion length is assumed to be the channel size (150 μm) for sufficient mixing, and D is *ca.* 10^{-5} cm^2/s,[23] the mixing time is estimated to be *ca.* 20 s from Eq. (1). This value corresponds well to the signal rising time in Fig. 3. The diffusion time given by Eq. (1) represents only a rough estimation of the time required for a uniform concentration distribution, and it required, actually, more time for completely uniform mixing, as can be seen in Fig. 3. Therefore, at least, we could confirm that the rapid mixing of two solutions was possible by molecular diffusion, without mechanical stirring in the microchannel. Further, the time required for diffusion mixing could be roughly estimated by Eq. (1); the equation showed that the mixing time could be shortened effectively by using a narrower channel size. The mixing time amounted to 100 – 200 ms for a 10 μm channel size, and a virtually instantaneous mixing would be possible for a narrower channel.

Next, Fe(II) was determined in this on-chip integrated FIA system. After Fe(II) and *o*-phen solutions were introduced into the microchannels, they were uniformly mixed and the chelating reaction product was detected by the thermal-lens microscope. The concentration of the Fe(II) solution was 4 – 20 μM, which corresponded to the absolute amount of introduction, 0.4 – 2 pmol Fe(II). For a comparison, Fe(II)-phen chelate, prepared in a separate bulk reaction, was introduced and detected in the same microchannel. The results are plotted in Figs. 4(a) and (b). They showed good linear calibration lines. Considering that the absolute amount of Fe(II) in the microchannel was 0.4 – 2 pmol and the detection volume was 3 fl, 6 – 30 zmol of Fe(II)-phen chelate was detected in this integrated FIA system. From Fig. 4(a), the lower limit of detection was estimated to be 1.9 zmol by doubling the standard deviation. Though the system was very primitive, we could integrate the FIA system on a chip. Using this system, we succeeded in carrying out a reaction and detection at the zeptomole level.

There are some points which we still need to mention concerning this system. First, the signal intensity of the Fe(II)-phen chelate produced in the microchannel suffered a greater variation in each measurement, as shown in Fig. 4(a), compared with the case of the reaction in the bulk (Fig. 4(b)). The standard deviation in the former case was three-times as large as that in the latter. The ratio of the volume of each solution introduced into the reaction microchannel was considered to be not well controlled, and that may have lead to the reduced reproducibility of the amount Fe(II)-phen chelate produced. A more precise fluid control system in an on-chip microchannel network is required for more exact determinations. Second, there was some deviation from the linear relationship between the signal intensity and the concentration of Fe(II) solution (1 – 10 μM) in the microchannel reaction when the concentration of *o*-phen was kept at 40 μM, as shown in Fig. 5(a). The results obtained for the bulk quantity under the same conditions are shown in Fig. 5(b). This concentration (40 μM) is still sufficiently more than that required for this reaction to proceed stoichiometrically. The signal intensity shown in Fig. 5(a) gradually deviated downward from linearity and dropped sharply at 10 μM, while good linearity was held, as shown in Fig. 5(b). As mentioned earlier, because the mixing time by diffusion seemed to be sufficient for the reaction to go to completion, the drop in the complex formation should not have been due to insufficient mixing.

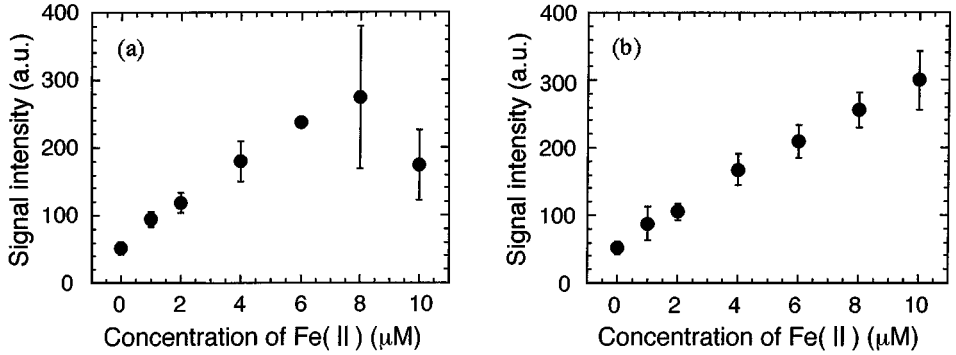

Fig. 5 Calibration curve of Fe(II). The results of the reaction in the microchannel are given in (a), and those of the reaction in bulk quantity with detection in the chip are in (b). The concentration of the *o*-phen solution was 40 μM.

Although the origin of this result is not clear, the following explanation may be conceivable. The ratio of the wall area to the volume in the microchannel is much larger than that in an ordinary flask, which might have had some effect on the sample mixing within the microchannel.

Though the system is very primitive, we could integrate the FIA system without resorting to electroosmotic fluid control and LIF detection. We demonstrated integration of several basic chemical operations, *i.e.*, liquid transport, mixing, stirring, reaction and detection, although there are still some problems. For the present, most of the studies on μ-TAS have dealt with separation by electrophoresis, fluid control by electroosmosis, and detection by LIF. However, our non-electroosmotic and non-florescent system can be applied not only to aqueous samples, but also to organic samples, and the detection method can be adopted to a greater variety of chemical species. In addition, it is not necessary to worry about the localization of chemical species and a change in the sample composition due to an electric field. Therefore, it is expected that more complicated systems, including extraction, purification, and so on, may be integrated; that is, integration of an entire chemical laboratory system might be possible.

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