Absorption Spectrometry Using a Diffractive Optical Element Constructed by Gel Electrophoresis

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A novel diffractive optical element was constructed by means of gel electrophoresis. Five capillaries were filled with a methylene blue solution and inserted into a slab gel. An electrical potential was then applied to the slab gel. Methylene Blue in the capillaries underwent electrophoretic migration, forming clear blue bands in the gel. The bands functioned as a transmission grating, and diffracted a laser beam. The intensity of the diffracted light increased with increasing the concentration of methylene blue.

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Introduction

Absorption spectrometry and fluorescence spectrometry are powerful means for the detection of both organic and inorganic compounds. Absorption spectrometry is widely used in a variety of applications, but the sensitivity of the technique is poor due to the fact that it involves measuring the change in the transmittance of incident light, a major limitation. On the other hand, the sensitivity of fluorescence spectrometry is from ten to thousands of times higher than that of absorption spectrometry because no background signal is generated. However, only a limited number of organic compounds are fluorescent and, as a result, fluorescence spectrometry is not as heavily used as absorption spectrometry. Typically, analytes are derivatized by a reaction with a fluorescent compound when this technique is used. Unfortunately, in many cases, the labeling efficiency is too low to achieve highly sensitive detection. In fact, the property of the fluorescent tag used frequently determines the detection limit in practical trace analysis.¹ As a result, an analytical technique with high sensitivity and wide applicability for determining nonfluorescent compounds would be highly desirable for practical trace analysis.

Diffractive optical elements have been used for dispersing light by diffraction phenomena. Recently, the development of microfabrication techniques, for example, binary optics lithography and electron-beam lithography, has led to the fabrication of diffractive optical elements.² Accurate diffractive optical elements fabricated using such technologies have been utilized in an optical system, *e.g.* measurement of the complex refractive index of a liquid³ and the observation of protein motion.⁴ In analytical spectroscopy, beam diffraction is also used to detect changes in the refractive index. A measurement of the diffraction could potentially be used for the sensitive detection of molecules in analytical spectroscopy, since the background signal could approach zero in the same manner as in fluorescence spectrometry. An analytical method using

diffraction has recently been demonstrated in an immunoassay, in which a change in the refractive index arising from the adsorption of a specific protein based on an immunological reaction is detected by measuring the intensity of the diffracted beam.^{5,6} A diffractive optical element can also be utilized as the detector in capillary electrophoresis, in which the difference in the refractive index between a running buffer solution and a sample zone is detected.⁷ This approach would essentially function as a universal detector, which could be applied to colorless samples, although the sensitivity is poorer than a conventional absorbance detector.

In our previous work, we reported on the fabrication of a diffractive optical sensor with periodic bands comprised of thymolphthalein, and demonstrated a novel type of chemical pH sensor based on light adsorption.⁸ This sensor acts as a transmission grating, and light absorption, *e.g.* a change in the pH, can be detected by the appearance of diffracted beams. The grating was fabricated using gelatin in which thymolphthalein bands had been inserted. Unfortunately, a small background signal was observed in this sensor, since the diffraction resulted from a slight difference in the refractive index between gelatin with and without thymolphthalein. This may have been caused by the method used to prepare the gelatin film. In this case, bands of gelatin containing thymolphthalein were formed and the vacancies between the grooves of the gelatin were then filled with gelatin that contained no thymolphthalein, so that the curing time for the gelatin with thymolphthalein was different from that for the gelatin without thymolphthalein, resulting in a slightly different refractive index. In addition, the issue of whether the method is applicable to quantitative analysis or not, continues to be unclear.

In this study, we describe a novel method for fabricating a diffractive optical element that is homogeneous prior to introducing a sample. Slab gel electrophoresis was utilized to form bands of a sample in a slab gel. The sample was introduced into the slab gel by means of a capillary array filled with the sample solution. When a potential was applied, the sample electrophoretically migrated in the form of a band from each capillary to the gel. A laser beam was passed through the sample bands, and the resulting diffracted beams were measured

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using a photodiode array. To demonstrate the applicability of the technique to quantitative analysis, the relationship between the sample concentration and the intensity of the diffracted laser light was evaluated based on theoretical equations derived in a previous paper.⁸

Experimental

Chemicals

Acrylamide-HG (AA), ammonium peroxydisulfate, sodium dodecylsulfate (SDS), *N,N,N',N'*-tetramethylethylenediamine, *N,N'*-methylene-bis(acrylamide)-HG (bis-AA), and sodium hydrogen carbonate were purchased from Wako Pure Chemical Industries (Osaka, Japan). Methylene Blue was obtained from Kanto Kagaku (Tokyo, Japan). All solutions were prepared using deionized water, which was further purified using an Elix-3 purification system (Millipore, Tokyo, Japan). A polyacrylamide gel (T (weight % of AA + bis-AA for the solution before polymerization) = 10%, C (weight % of bis-AA to AA + bis-AA) = 2.67%) was used as the substrate of a diffractive optical element. A stock solution of acrylamide (T = 30%, C = 2.7%) was prepared by dissolving appropriate amounts of AA and bis-AA in deionized water, and 6 mL of the acrylamide solution, 7.5 mL of deionized water, and 4.5 mL of 50 mM carbonate buffer containing 5 mM SDS (pH 9.3) were then mixed. Subsequently, 10 μ L of *N,N,N',N'*-tetramethylethylenediamine and 40 μ L of 0.2 mg mL⁻¹ ammonium peroxydisulfate were added to the mixture to initiate polymerization. The buffer solution used for the gel electrophoresis was composed of 30 mM carbonate buffer and 5 mM SDS. Sample solutions were composed of appropriate amounts of Methylene Blue, 30 mM carbonate buffer, and 15 mM SDS, which was added to prevent the adsorption of the cationic Methylene Blue to the capillary wall.⁹

Apparatus

A gel electrophoresis apparatus (KS-8033) and a power supply (MP-7652) were obtained from Marisol, Tokyo, Japan. Fused-silica capillaries (200 μ m i.d., 375 μ m o.d.) were purchased from GL Sciences, Inc. (Tokyo, Japan). An He-Ne laser, emitting at 632.8 nm with 0.5 mW of output power (05-SRR-810, Melles Griot Inc., CA), was used as a light source for absorption spectrometry using a diffractive optical element. The diffractive optical element is fixed on an X-Z stage (Sigma Koki, Tokyo, Japan). A neutral-density filter (transmittance, 10%) was used to attenuate the laser light passing vertically through the diffractive optical element. The image of the diffractive optical element was observed by means of a CCD camera (Scopeman MS 603, Moritex, Tokyo, Japan) equipped with a video monitor (PVM 1445 MD, Sony, Tokyo, Japan) and a color printer (UP 5100, Sony, Tokyo, Japan). A photograph of the diffracted light was taken by a digital camera (Fine Pix 500, Fujifilm, Tokyo, Japan). The intensity of the diffracted light was detected by a photodiode array (C4091, Hamamatsu Photonics, Shizuoka, Japan) equipped with a digital oscilloscope (LeCroy 9360, LeCroy Japan, Tokyo, Japan). An absorption spectrophotometer, UV-2400PC (Shimadzu, Kyoto, Japan), was employed for the determination of the concentration of Methylene Blue in the stock solution.

Preparation of a diffractive optical element based on gel electrophoresis

Commercially available standard glass plates were used to form a slab gel that serves as a substrate for constructing a

Fig. 1 Protocol for the construction of a diffractive optical element. (A) A capillary array is fixed between the glass plates; (B) Polyacrylamide gel is poured into the space between the glass plates until the edge of the capillary array is surrounded by the polyacrylamide gel; (C) The gel is polymerized for 1 h; (D) The imprint of the capillary array (length: 2 cm) is formed by removing it; (E) Another set of capillaries containing a sample (Methylene Blue solution) are arranged by placing them into the imprint of the capillary array; (F) After pouring a buffer solution into a tank, a voltage, 100 V, is applied. The blue bands of Methylene Blue appear.

diffractive optical element. Figure 1 shows the protocol for constructing the slab gel used in this experiment. A capillary array was prepared by gluing five capillaries of 5 cm and five capillaries of 2 cm together in an alternate configuration with no space between them. The capillary array was inserted between the two glass plates (Fig. 1 (A)); an acrylamide solution containing a polymerization initiator and a promoter was then introduced into the space between the two plates (Fig. 1 (B)). After the completion of polymerization, the capillary array was removed from the slab gel (Fig. 1 (C) - (D)), resulting in an imprint. Five capillaries (5 cm), filled with a Methylene Blue solution, were then inserted into the imprint of the capillary array in the slab gel (Fig. 1 (E)), and the reservoirs were filled with the electrophoresis buffer so as to completely immerse the capillaries. A voltage of 100 V was applied to the slab gel for about 20 min by a power supply (Fig. 1 (F)). Consequently, blue bands were formed when the sample migrated from the capillaries into the slab gel.

Results and Discussion

In this study, Methylene Blue was used as a test sample because it has an absorption band around the emission wavelength of the He-Ne laser (the absorption maximum, ~660 nm). A photograph of a diffractive optical element prepared using this technique is shown in Fig. 2. Methylene Blue appears as a series of clear bands in the slab gel after its migration from each capillary into the gel. As noted in Fig. 2, because the apparent diameters of the bands are almost the same as the space between the capillaries, diffusion to the direction perpendicular to the capillary is efficiently suppressed in the gel. We also attempted to form bands by introducing Methylene Blue without using capillaries, *i.e.* a Methylene Blue solution was directly injected into the imprint of the capillary array in the gel. However, in this case, the bands were diffuse (data not shown). This indicates that the sample can migrate, to some extent, in the direction perpendicular to the migration direction. On the other hand, the sample migrates only in the longitude direction of the capillary when capillaries are used to introduce the sample,

Fig. 2 Photograph of a diffractive optical element with a capillary array. The bands are formed at an interval of *ca.* 375 μm . The concentration of Methylene Blue is 5 mM.

since the capillary shields the electric field in the direction perpendicular to the capillary. Therefore, the use of capillaries is important to minimize diffusion in the direction perpendicular to the capillary. The concentration of SDS was also found to be important for forming clear bands of Methylene Blue. The shape of the bands was found to change depending on the concentration of SDS in the electrophoresis buffer. In the capillary, Methylene Blue interacts with negatively charged SDS micelles by means of electrostatic and hydrophobic interactions.¹⁰ As a result, Methylene Blue migrates in the form of an anionic complex of Methylene Blue with SDS micelles during gel electrophoresis. When the concentration of SDS in the electrophoresis buffer is much lower than that in a sample solution, Methylene Blue was found to be stacked at the outlet of the capillary. This might be caused by destruction of the micelles due to the dilution of SDS in the gel. In the case where the gel contains a sufficiently large amount of SDS to prevent the destruction of the micelles, Methylene Blue migrates in the form of a complex with SDS micelles. We also used an electrophoresis buffer in which the concentration of SDS was similar to the sample solution. However, diffusion in the direction perpendicular to the capillary was still observed in this case. Consequently, 5 mM and 15 mM of SDS, respectively, was used for the electrophoresis buffer and the sample solution in this experiment. Under these conditions, the band formation was optimal, due to the suppression of diffusion.

Photographs of the diffraction pattern of the laser beam are shown in Fig. 3. Figures 3 (A) and (B) show patterns obtained using a buffer and Methylene Blue solutions (5 mM) as a sample, respectively. In Fig. 3 (A), a single spot of the laser beam is observed, and no diffraction can be seen at either side of the spot. On the other hand, several spots appear when the beam passes through a diffractive optical element made by Methylene Blue, as shown in Fig. 3 (B), suggesting that the fabricated diffractive optical element is able to efficiently reduce the background signal.

To evaluate the applicability of the present method to quantitative analysis, the relationship between the concentration of the sample and the intensity of the diffracted laser light was determined for concentrations of Methylene Blue in the range of 0.1 mM to 5.0 mM (Fig. 4). To eliminate the influence of the signal arising from light scattering of the excitation light source, measurements were performed at a distance from the

Fig. 3 Image of a diffraction pattern. (A) No sample. (B) Using the diffractive optical element shown in Fig. 2.

Fig. 4 Intensity of diffraction using different concentrations of Methylene Blue, 0, 0.25, 0.5, 1.0, 2.5, and 5.0 mM. The position corresponds to the enclosed part of the photograph in figure.

transmitted laser beam. The area measured by a photodiode array is surrounded by a white square in the photograph in Fig. 4. Even for a blank sample containing no Methylene Blue, the signal due to light scattering is still observed by the photodiode array at the region near to the transmitted laser beam. The sum of the intensity of the diffracted beam increases proportionally up to 1 mM with increasing concentrations of Methylene Blue, although the signal, which will be attributed to light scattering at the surface of the glass plates and the interface between the glass plate and gel, was measured with the diffracted beam. A complete elimination of scattered light could be achieved by increasing the resolution between the diffracted beam spots, that is, the resolution between the transmitted beam and the diffracted beam of the first order must be expanded by constructing bands narrower than that used in the present study.

A calibration curve obtained by the present method is shown in Fig. 5; the open circles are the experimental values (mean value, $n = 3$) and the solid line is a theoretical curve based on an equation previously derived by us. The intensity of the diffracted beam (I) is given by⁸

Fig. 5 Calibration curve for a diffractive optics sensor. The open circles are the mean values ($n = 3$). The solid line is based on a calculation from diffraction theory.

$$D = (1/2)I_0(1 - 10^{-A}),$$

where I_0 is the light intensity of the introduced laser beam and A is the absorbance. The relative intensity ($D/D_{\max}$) is given by

$$D/D_{\max} = 1 - 10^{-A},$$

where $D_{\max}$ is the intensity of the diffracted beams when A is infinity, so that $D_{\max}$ is given by $(1/2)I_0$. From this equation, the relative intensity ($D/D_{\max}$) can be approximated by 1 when $10^{-A} \ll 1$. Practically, the absorbance of 5 mM Methylene Blue is calculated to be 2.2, that is, $10^{-2.2} = 6.3 \times 10^{-3}$, which is much smaller than 1. Therefore, the intensity of the diffracted beams for 5 mM Methylene Blue was used as $D_{\max}$ for calculating the relative intensity of the diffracted beams. The observed values are consistent with the theoretical curve, as shown in Fig. 5. The limit of detection obtained in this study was 0.25 mM. Unfortunately, the sensitivity was too poor due to the wide space between the bands in the diffractive optical element constructed in this study, as described above. The space between the bands was limited to 375 μm in this study, which is too wide compared to the conventional diffractive optical elements for achieving an efficient diffraction of the laser beam. When the space between the bands is too wide, nearly all of the laser light passes through the diffractive optical element without diffraction. In addition, the scattering light of the transmitted beam degrades the sensitivity of the present method. The diffractive optical element with a narrow space gives a high resolution between the transmitted laser beam and the diffracted

beams, thus decreasing the background signal arising from the light scattering. Hence, a more efficient collection of the diffracted beams with no background can be achieved as the space between the bands in the diffractive optical element is decreased.

To investigate the effect of the space between bands on the resolution between the transmitted beam and the diffracted beams, a simulation using a transparency film was carried out as follows: several narrow lines were aligned at a constant interval of 50 – 100 μm and printed on a transparency film. The width of each line was the same as the interval. The resolution of the diffraction generated by the transparency film was observed in order to find an optimum interval for achieving sensitive detection. From the results of this experiment, a space of 50 μm might be sufficiently narrow to obtain good resolution of the diffracted beams from the transmitted beam. Therefore, to improve the sensitivity of the absorption spectrometry using a diffractive optical element, bands with a space of narrower than 50 μm should be constructed using the present method or another technique, such as microfabrication techniques recently employed in several fields, including analytical chemistry and chemical engineering. Further investigations are now in progress using microfabrication techniques to improve the sensitivity of the present method in our laboratory. Therefore, it appears likely that a diffractive optical element will be applicable to sensitive detection in various analytical techniques, including electrophoresis and chemical sensors.

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