Preliminary Study of Orthogonal Electrochromatography for Simultaneous Two-Dimensional Separation Using a Monolithic Polymer Layer

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Abstract
Fabrication of a monolithic polymer layer, as the stationary phase, equipped with two electrodes was studied for simultaneous two-dimensional separation, combining high performance liquid chromatography and orthogonal electrophoresis. The monolithic polymer layer was prepared between a pair of glass plates by a two-step UV-initiated photo-polymerization. Prior to preparation of the monolithic polymer layer for separation, high-density monolith bars were prepared along the electrodes to suppress the penetration of bubbles formed at the electrodes into the separation monolith. Using the monolithic polymer layer, 2D separation of colored-dyes was successfully achieved by combining gradient HPLC and orthogonal electrophoresis. The 2D separation of proteins was also demonstrated.

Keywords: Electrophoresis; Gradient elution; HPLC; Monolithic polymer layer; Two-dimensional separation

1. Introduction
Recently, studies based on a comprehensive analysis of various compounds in biogenic systems, such as proteomics and metabolomics, have attracted much attention [1-3]. In these omics studies, numerous compounds have to be analyzed and the importance of separation methods increases for such studies. Since the properties of analytes are diverse, it is generally difficult to separate all compounds using only a single separation method. Therefore, two or more separation methods (or modes) are often used for comprehensive analyses.

Two-dimensional gel electrophoresis (2D-GE) is one of the best known 2D separation methods for proteins and peptides, in which analytes are first separated based on the difference in isocratic points, and second by molecular size [4,5]. Two-dimensional HPLC (2D-HPLC) is also frequently utilized for the separation of biological, medical, and pharmaceutical samples [6]. In 2D-HPLC, analytes fractions eluted from the first-dimension column are sequentially introduced into a second-dimension column for further separation. Other 2D-separation methods, including 2D-gas chromatography (2D-GC) [7], 2D-capillary electrophoresis (2D-CE) [8], HPLC-GC [9,10], and LC-CE [8], have also been developed. In all cases, these separations are performed sequentially, i.e., the analytes were separated via a second mode following a first separation mode. Since sequential separation is often time consuming, the development of a rapid simultaneous 2D separation method is required.

Capillary electrochromatography (CEC) is a separation method combining HPLC and CE [11-13]. However, CEC is a method for one-dimensional separation, wherein the two separation modes are used simultaneously. Alternatively, the combination of chromatographic and electrophoretic separations has been utilized for 2D separation. Haugaard et al. reported paper chromatography combined with orthogonal electrophoresis in 1948 [14], and the same combination of the methods was reported by Strain and Sullivan in 1951 for the separation of metal ions [15]. Van Ooij developed apparatus for simultaneous thin layer chromatography (TLC) and orthogonal electrophoresis in 1973 [16]. More recently, in 2013, Stevenson et al. reported simultaneous 2D separation (TLC and electrophoresis) using dual solvent reservoirs [17].
In previous simultaneous 2D separations combining chromatography and electrophoresis, the stationary phases were usually paper or TLC plate. Therefore, the flow of chromatographic separation was provided by capillary action or gravitational force. Although TLC separation using electroosmotic flow (EOF) has been reported [18], the combination of EOF-driven TLC with orthogonal electrophoresis has not been reported yet. Moreover, to the best of our knowledge, HPLC using pressurized flow has not been combined with orthogonal electrophoresis.

In this study, we developed a simultaneous 2D separation method combining HPLC using pressurized flow and electrophoresis, or orthogonal electrochromatography (OPEC). In the proposed method, the mobile phase was supplied to the stationary phase layer by pumps, in similar fashion to conventional HPLC, and a voltage was applied orthogonally to the mobile phase stream. A monolithic polymer layer was prepared between a pair of glass plates and used as the stationary phase for OPEC, because monolithic stationary phases have been used frequently as highly permeable chromatographic supports [19-22]. The fabrication of a monolithic polymer layer for simultaneous 2D separation and the basic performance of this proposed method were investigated.

2. Experimental

2.1. Chemicals

Reagents were purchased from Wako Pure Chemicals (Osaka, Japan), unless otherwise specified. All chemicals were used as received. Butyl methacrylate (BMA), dodecyl methacrylate (DMA), ethylene dimethacrylate (EDMA), 3-methacryloxypropyltrimethoxysilane (MAPS, Shin-Etsu Chemicals, Tokyo, Japan), 1,4-butanediol (PrOH), 1-butanol (BuOH), 1-pentanol (PeOH), methanol, and 2,2-dimethoxyphenyl-2-acetophenone (DMPA), were used for preparation of monolith. 2-Aminoethanethiol, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC-HCl), and acrylic acid were used for modification of electrode surface. Acetonitrile (ACN) and trifluoroacetic acid (TFA) were used as the mobile phase. Rhodamine 6G, acid red 52, quinine sulfate, pyrene, perylene, and coronene, were used as analytes of small compounds. Borate, borax, 4-fluoro-7-sulfamoylbenzofurazan (ABD-F, Dojindo, Tokyo, Japan), ethylenediaminetetraacetic acid (EDTA), and hydrogen chloride were used for labeling of proteins (bovine serum albumin (BSA), ovalbumin, and α-lactalbumin).

2.2. Preparation of monolithic polymer layer equipped with electrodes for simultaneous 2D separation

The preparation of monolithic polymer layer for 2D separation was summarized in Fig. 1, and the procedure is as follows. Gold coated copper electrodes for electrophoresis (0.05 mm thickness, see Fig. 1A) were immersed in a mixture of saturated aqueous NaOH solution and ethanol (1:3) for 3h to clean the surface. The electrodes

![Fig. 1.](image-url)
were then reacted with a 10 mM solution of 2-aminoethanethiol for 3 h and then washed with distilled water. The electrodes were then immersed in a mixture of 5 mM EDC-HCl and 10 mM acrylic acid aqueous solutions for 1 day to introduce acrylic functional groups to the electrode surface, and then washed with distilled water. The modification procedure for the glass surface was almost identical to our previous method for fused-silica capillaries [21,22]. A pair of glass plates (40 × 50 × 1 mm) was immersed in 1 M NaOH for 1 h at 65 ºC and then washed sequentially with distilled water, 1 M HCl, and distilled water again. After drying the glass plates at room temperature, a mixture of MAPS and acetone (1:2) was dropped on the center of one glass plate and the other glass plate was placed on the top. The pair of glass plates sandwiching the MAPS solution was kept at 65 ºC for 3 h to modify the glass surface. After the washing and drying the plates, the center of the glass plate was masked (masked area: 50 × 30 mm) and Ag paste (Type-XA-874, Fujikura Kasei, Tokyo, Japan) was spin coated (7200 rpm) on both edges of the plate (5 × 50 mm for each area). Two electrodes were set on the glass plate with Ag paste, and then another glass plate was set on the electrode. The electrodes and glass plates were fixed by thermal treatment at 150 ºC for 30 min. The dimensions of the space between the two glass plates was 28 × 50 × 0.06 mm.

Preparation of the monolith was achieved using a method similar to that previously used for fused-silica capillaries [21,22]. A mixture of DMA, EDMA, porogens and DMPA was used to fill the space between the plates. The glass plate was irradiated with a UV lamp at 0ºC for 10-20 min. If necessary, a mask was used to limit the irradiation, as described later. After polymerization, the monolith was washed with methanol to remove unreacted monomer and porogens. The modification procedure for the glass surface was modified the glass surface. After the washing and drying the glass plates, the center of the glass plate was masked (masked area: 50 × 50 mm), and the glass plate with Ag paste (Type-XA-874, Fujikura Kasei, Tokyo, Japan) was spin coated (7200 rpm) on both edges of the plate (5 × 50 mm for each area). Two electrodes were set on the glass plate with Ag paste, and then another glass plate was set on the electrode. The electrodes and glass plates were fixed by thermal treatment at 150 ºC for 30 min. The dimensions of the space between the two glass plates was 28 × 50 × 0.06 mm.

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### 2.3. Apparatus

The monolithic polymer layer equipped with electrodes, shown in Fig. 1C, was connected with two pumps for gradient elution (LC-10ADvp, Shimadzu, Kyoto, Japan). A 10 µL microsyringe was connected to the injection port via a fused-silica capillary. The electrodes beside the monolithic polymer layer were connected with a power supply (Model-3810m, Anatech, Tokyo, Japan). In this study, sample spots were observed using a CCD-camera (WAT-902H ULTIMATE or WAT-250D2, Watec, Yamagata, Japan) with irradiation (white light or LED of 405 nm).

### 3. Results and discussion

#### 3.1. Preparation of monolithic polymer layer

The composition of the reaction solution for the monolith was investigated first without the optical mask shown in Fig. 1B, i.e., the monolith was prepared entirely between the glass plates. The compositions of the reaction solutions are summarized in Table 1. Solution #1 failed to form a homogeneous monolith between the glass plates, forming a monolith in only 1/3 of the whole area, with the rest of the area being void. Solution #2 formed the monolith over the whole area. However, small vacant areas (about 1 mm in diameter) without monolith were found as spots in the monolithic polymer layer. Thus, the combination of Bu(OH)2 and PrOH was not suitable for monolith preparation. Next, combinations of Bu(OH)2/PrOH and Bu(OH)2/PeOH as the porogens (#3 and #4 in Table 1) were attempted. After polymerization, these two solutions fabricated the monolith, without defects or vacant areas, successfully. However, a heterogeneous area was slightly found near the electrodes in the case of #3. Therefore, reaction solution #4 was used in this study.

When a voltage was applied to the monolithic polymer layer via two electrodes, the electric current passing through the monolith was successfully measured using an ammeter. In addition, orthogonal electrophoresis of the ionized analytes was also observed (data not shown). However, with the application of a higher voltage, the bubbles formed at the electrodes penetrated into the monolith layer, as shown in Fig. 2A (200 V, 1.3-1.5 mA). The formation of gas bubbles at the electrode was theoretically unavoidable with the application of high voltage. Therefore, we investigated

![Fig. 2. Images of the monolithic polymer layer prepared by (A) single step preparation and (B) two-step preparation. In (A), bubbles generated by the application of voltage penetrated into the center of the monolith. In (B), high density bars were fabricated into the electrodes.](image-url)

#### Table 1. Compositions of reaction solutions used for preparing monolithic polymer layers (wt%).

<table>
<thead>
<tr>
<th>DMA</th>
<th>EDMA</th>
<th>Bu(OH)2</th>
<th>PrOH</th>
<th>BuOH</th>
<th>PeOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>34</td>
<td>6</td>
<td>26</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>#2</td>
<td>32</td>
<td>8</td>
<td>26</td>
<td>34</td>
<td></td>
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<tr>
<td>#3</td>
<td>32</td>
<td>8</td>
<td>26</td>
<td>34</td>
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</tr>
<tr>
<td>#4</td>
<td>32</td>
<td>8</td>
<td>26</td>
<td>34</td>
<td></td>
</tr>
</tbody>
</table>

* 1 wt% DPMA respect to monomers was added as photoinitiator.
methods to inhibit the penetration of bubbles into the separation area. For this purpose, the walls of the high density monolith were prepared at the electrodes using the following procedure.

First, the monolith was polymerized by photo-initiated polymerization with an optical mask, as shown in Fig. 1B, for 20 min. By this polymerization, the high-density monolithic walls were prepared partially along both electrodes. The unreacted solution in the masked area was flushed with methanol. Then, the reaction solution was again used to fill between the glass plates. The solution was irradiated again by UV for 10 min without the mask to prepare the monolith for separation. The two-step preparation resulted in a monolithic polymer layer designed with both high and low density, as shown in Fig. 2B. The SEM image of high and low density monoliths are shown in Fig. 3.

The preparation of high density bars resulted in the decrease in electric current under the application of voltage (200 V, 0.4-0.5 mA). When further high voltage was applied to the designed monolithic polymer layer, bubbles formed at the electrodes escaped from the outlet end though the narrow zone between the electrode and the wall (high density bars). Although the bubbles sometimes penetrated into the wall, they did not enter the monolith used for the separation.

3.2. 2D separation on monolithic polymer layer

As shown in Fig. 1, the CCD-observation was employed for detecting the analytes. Therefore, colored analytes, for the CCD-observation, were separated using the monolithic polymer layer. Figure 4 shows a series of images of the monolithic polymer layer during separation without the application of voltage. The time, superimposed in each image, represented the time passed after injection (1 µL, 1 mM each). The capillary in the images was used for sample injection (see Fig. 1C).

As shown in Fig. 4, separation without application of the orthogonal electric field resulted in a linear, or one-dimensional, separation of the analytes. The analytes were separated by only reversed phase liquid chromatography and eluted in the order; quinine, rhodamine 6G, acid red 52, pyrene, perylene, and coronene. The linear migration of analytes indicated that the pressurized flow in the monolithic polymer layer was not oblique and crooked during the gradient procedure. That is, the density (or flow resistance) of the monolith in the separation field was approximately constant and independent the position of the monolith.

With the application of voltage, analytes were separated in two-dimensionally due to orthogonal electrophoretic migration, as shown in Fig. 5. That is, cationic quinine and rhodamine 6G migrated toward the negative electrode.
(lower side of the electrode in Fig. 5) electrophoretically and to the outlet end with chromatographic migration. In contrast, the negatively charged compound of acid red 52 migrated to the positive electrode. Therefore, these three analytes were separated two dimensionally. The neutral analytes (pyrene, perylene, and coronene) migrated to the outlet end directory without electrophoretic migration. The straight movement suggested that the acidic mobile phase suppressed the generation of electroosmotic flow (EOF). When the mobile phase of neutral pH was used, EOF generated in the layer provided unstable separation. Therefore, the suppression of EOF would be necessary in this study. The two-dimensional separation shown in Fig. 5 is the first report of combining HPLC using pumps with orthogonal electrophoresis.

The effect of orthogonal electrophoresis on the band broadening was investigated. The theoretical plate number of quinine, rhodamine 6G, and perylene in Fig. 4 were 8800, 3000, and 5100 N/m, respectively. In Fig. 5, these values were reduced to 5500, 2400, and 4300, respectively. Although the proposed method will be effective to enhance the separation in electrophoretic direction, the separation efficiency in chromatographic direction would be suppressed.

3.3. 2D separation of proteins

Proteins are often separated using two-dimensional separation methods, such as 2D-GE, and 2D-HPLC. The proposed method, or orthogonal electrochromatography using monolithic polymer layer as stationary phase, was applied to the two-dimensional separation of proteins. For the optical observation of proteins, the analytes were labeled with ABD-F by reaction with thiols in the molecules (amino and carboxyl groups are not reacted) [23].

As shown in Fig. 6A1-3, the labeled-proteins were separated without the application of an orthogonal electric field. In this 1D-chromatography, BSA and ovalbumin were not separated. In the case of 2D-separation, positively charged proteins in the acidic mobile phase migrated towards the negative electrode, and BSA and ovalbumin were completely separated by orthogonal electrophoresis (vertical direction in Fig. 6B3) whereas their chromatographic behaviors were the same (their horizontal positions were the same). The combination of two different separation modes was effective in enhancing the separation of proteins.

4. Conclusion

The monolithic polymer layer equipped with electrodes was successfully fabricated via two-step polymerization. Using the monolithic polymer layer, 2D-simultaneous separation, a combination of gradient HPLC and orthogonal electrophoresis, was achieved. Enhancements in separation efficiency in 2D mode were demonstrated in the separation of model proteins. The combination of the proposed method and a simultaneous detection method, such as that used in a capillary array DNA sequencer, would contribute to enhancing the performance in comprehensive analysis.

Acknowledgement

This work was partially supported by JSPS Intellectual Property Utilization Promotion Highway.

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