Technical Note

A Simple and Easy-to-Use Capillary Isoelectric Focusing Technique Using Reagent-Release Hydrogels

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Abstract

Reagent-release hydrogels containing an acid or a base solution were prepared and employed as ion suppliers in isoelectric focusing using short capillaries. In conventional isoelectric focusing techniques using microfluidic channels, complicated liquid manipulations are required, which can decrease analytical performance such as the reproducibility of a focusing position. To develop a rapid and sensitive assay based on microfluidic isoelectric focusing, we previously prepared reagent-release capillaries retaining ampholytes by physical adsorption on the inner surface of a short capillary, which were then applied to isoelectric focusing. In this study, reagent-release hydrogels containing an acid or a base solution were developed for isoelectric focusing using short capillaries in place of the acid and base solutions used in conventional microfluidic isoelectric focusing. The prepared reagent-release hydrogels showed some advantages for isoelectric focusing, *e.g.*, high durability for at least five weeks of storage, easy handling as compared to the conventional solutions, and suppression of hydrodynamic flow in capillaries, which often decreases the reproducibility in capillary isoelectric focusing. The migration behavior of ions from the hydrogels into the capillary and the formation of a pH gradient in isoelectric focusing using the reagent-release hydrogels were investigated, which confirmed that the prepared reagent-release hydrogels could form a similar pH gradient to that obtained with conventional capillary isoelectric focusing using the solutions.

Keywords: Capillary isoelectric focusing; Reagent-release hydrogel

1. Introduction

Capillary isoelectric focusing (CIEF) that is used to separate and concentrate ampholytes such as peptides or proteins based on their isoelectric points (*pI*), was first reported by Hjerten *et al.* as a highly sensitive analytical method with high resolution (0.02 *pI* units) [1]. CIEF can be used to analyze small amounts of samples automatically with the help of a commercial apparatus [2], whereas conventional IEF analysis requires a series of tedious manual procedures. Thus, many CIEF techniques have been developed to date [3,4]. In conventional CIEF, however, the focused analytes must be transported toward a detection window, which can sometimes decrease the resolution due to a band broadening during transportation [5]. Recently, many IEF techniques using microfluidic devices (microfluidic IEF, MIEF) have been developed to overcome this drawback [6–12]. MIEF has many advantages compared to CIEF, including a short analysis time (within 5

min) due to a short separation channel (several cm) and the requirement of a minimal amount of reagents and samples (several μL). In particular, a short separation length in MIEF makes whole-column imaging possible, which enables real-time observation of an IEF process over the whole length of the microchannel without the requirement of any of the transportation processes necessary for conventional CIEF [9–12]. More recently, a microfluidic device for two-dimensional electrophoresis was independently developed by Kim and Moon and Yang *et al.* to obtain a higher resolution for analyzing a complicated mixture of biogenic samples [13,14]. Mai *et al.* combined MIEF with immunoreaction techniques for glycoprotein analysis, which showed its utility as a rapid and sensitive bioassay with minimal consumption of samples and reagents [15]. Therefore, CIEF and MIEF play an important role in biological analyses, especially for analyses of biogenic compounds having isoelectric point [16–18].

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Nevertheless, the experimental procedure of MIEF is relatively complicated. To simplify the MIEF procedure, many researchers have focused on improvements of the microfluidic device used for MIEF. Yeung *et al.* described a method for the treatment of the inner surface of the capillary to suppress electroosmotic flow [19]. Yang *et al.* [20,21] and Zhu *et al.* [22,23] reported the immobilization of carrier ampholytes (CAs) on a monolithic column in the microchannel, thereby avoiding the addition of carrier ampholytes into the sample solution to prevent current increase. In our previous study, we reported that the use of a "reagent-release capillary" (RRC) facilitated the MIEF procedure simply by introducing a sample solution into a capillary *via* capillary action, soaking the cathodic and anodic ends of the capillary into basic and acid solutions filled in reservoirs, respectively, and applying an electric field [24,25]. However, this technique still requires a step of manual application of the acid and base solutions to the reservoirs after sample introduction into the RRC. Furthermore, RRC–IEF using short capillaries (4–5 cm in length) often resulted in the flow out of focused samples because of hydrodynamic flow caused by a slight height difference between the reservoirs.

To solve these problems, in this work, hydrogels containing acid or base solution (reagent-release hydrogels, RRGs) were developed and used as the source of ion supply instead of the acid and base solutions used in conventional MIEF. Furthermore, the integration of RRGs, which are defined as hydrogels that can effectively release their contained reagents to a capillary or microfluidic channel by electrophoresis, is expected to completely eliminate the complicated liquid handling operation currently required for MIEF. Since the hydrogel is solid, in contrast to the solutions used in conventional MIEF, the focusing instability caused by the hydrodynamic flow is also expected to be prevented. To confirm the applicability of the proposed RRGs to CIEF, the migration behavior of ions in a hydrogel was experimentally observed using an ionic fluorescent dye. The focusing behavior in CIEF with RRGs, and effect of five-week storage of RRGs on IEF analysis were also investigated to evaluate the analytical performance of the CIEF with RRGs.

2. Experimental

2.1. Materials

A square fused silica capillary with a 300- μm outer width and 100- μm inner width flat-to-flat was purchased from Polymicro (Phoenix, AZ, USA). Sodium hydroxide, acrylamide, *N,N*-methylenebisacrylamide, methanol, phosphoric acid, hydrochloric acid and 2-propanol were obtained from Wako (Osaka, Japan). Fluorescein and 2-hydroxy-2-methyl-propiophenone (HOMPP) were obtained from Tokyo Chemical Industry (Tokyo, Japan). Poly(dimethylsiloxane)

(PDMS) pre-polymer (SILPOT 184) and curing agent (SILPOT 184 CAT), were from Dow Corning Toray Co., Ltd (Tokyo, Japan). Sulforhodamine B, 3-(trimethoxysilyl)propylmethacrylate, *N,N*-dimethylacrylamide, tetramethylethylenediamine, ammonium persulfate, fluorescent IEF markers (*pI* = 5.1, 5.5, 7.2, 7.6, 8.1, 9.0) were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Biolyte3-10 (BIO-RAD; Hercules, CA, USA) was used as carrier ampholytes (CAs). All reagents were used without further purification. The distilled and deionized water used had a resistivity of more than $1.7 \times 10^7 \Omega \text{ cm}^{-1}$ at 25 °C.

2.2. Instrumentation

Fluorescence images of the CIEF analysis were obtained from a fluorescence microscope (Keyence Multi-Viewer System VB-S20; Keyence Corp.; Osaka, Japan). Photographs were captured with a cooled CCD color camera (VB-7010; Keyence Corp.; Osaka, Japan) installed at the front port of the microscope. Fluorescence images were collected using a 120-W mercury lamp as a light source and a filter pair (for fluorescein, excitation and emission filters at 470/40 nm and 510 nm, respectively; for sulforhodamine B, excitation and emission filters at 540/25 nm and 572 nm, respectively; for *pI* markers, excitation and emission filters at 387/28 nm and 430 nm, respectively; VB-L11; Keyence Corp.; Osaka, Japan). Fluorescence images were converted to numerical responses using ImageJ software. The ultraviolet (UV) lamp was purchased from Nanometric Technology Inc. (Tokyo, Japan).

2.3. Preparation of hydrogels containing reagents

Acrylamide monomer solution was prepared by dissolving a 20 wt% mixture of acrylamide and *N,N*-methylenebisacrylamide (weight ratio 36:1) in deionized water. Then, the monomer solutions of RRGs containing acid or base solution were prepared by mixing equal volumes of the 20 wt% acrylamide monomer solution and 200 mM phosphoric acid or sodium hydroxide solution. After adding HOMPP as photo-initiator to the mixed solutions, two dimpled-molds made from PDMS (well size: 1.5 cm length and width, and 0.6 cm height) were filled with each monomer solution containing an acid or base solution, and then UV-irradiated for 2 min. After photo-polymerization, the surfaces of the prepared RRGs were washed with deionized water. For easy-to-use CIEF experiments, a PDMS chip with two wells (1.5 cm length and width, and 0.6 cm height) was used to install the prepared RRGs, and a rectangular hall (4 cm length and 1.5 cm width) between the wells for fluorescence detection, which were prepared by simple molding.

2.4. Suppression of hydrodynamic flow in the capillary by using hydrogels

To evaluate the effect of the use of hydrogels on the suppression of the hydrodynamic flow resulting from the height difference between the reservoirs, a capillary filled with a fluorescein solution was set on the PDMS chip and both ends of the capillary were in contact with reservoirs that were filled with acid and base solutions or hydrogels. After applying a height difference of 300 μm between the reservoirs, the ends of the capillaries were observed under a fluorescence microscope.

2.5. Estimation of the electrophoretic migration of ions from the hydrogel

To evaluate the electrophoretic migration of ions from RRGs to a capillary, the anionic fluorescent dye was injected electrokinetically employing the developed microfluidic device. As model cases, solutions and hydrogels containing 10^{-4} M sulforhodamine B (anionic fluorescent dye) were prepared with deionized water and acrylamide monomer solution, respectively. To prevent the electroosmotic flow during the electrokinetic injection, the inner surface of the capillary was modified with poly-(dimethylacrylamide) (PDMA) using the same procedure as reported previously [24]. Both ends of the PDMA-coated capillary filled with a buffer solution were in contact with the solutions or hydrogels. Then, platinum electrodes were in contact with the solutions or hydrogels. By applying an electric field (450 V/cm), sulforhodamine B was introduced into the capillary by its own electrophoresis. The migration behavior of the dye toward the anode was observed under a fluorescence microscope at the anodic side of the capillary. The fluorescence intensity of a $100 \times 100\text{-}\mu\text{m}$ solution area located 1 mm from the end of capillary was analyzed using ImageJ software.

2.6. Formation of pH gradient by using a hydrogel ion supplier

The values of the formed pH gradient in CIEF during the solutions or RRGs were calculated to evaluate the applicability of the RRGs as an ion supplier. The 2% (v/v) CAs solution containing 0.5% (v/v) pI markers (pI = 5.1, 5.5, 7.6, 9.0) was introduced into the PDMA-coated capillary. Then, both ends of the PDMA capillary were contacted with the solutions or RRGs. An electric field (450 V/cm) was then applied *via* platinum electrodes inserted in the hydrogels.

2.7. Storage stability of RRGs containing the acid or base solution

To evaluate the storage stability of hydrogels containing the acid or base solution, 5 pairs of the RRGs were prepared. The RRGs were rolled up with poly(vinylidene chloride) film to prevent drying up and stored at room temperature. CIEF analyses with one pair of the stored RRGs were

carried out over five weeks every week.

3. Results and discussion

3.1. Characteristics of prepared hydrogels containing acid or base solution

Hydrogels containing acid or base solution were successfully prepared as shown in Fig. 1. They showed sufficient mechanical strength, facilitating treatment as compared with the use of solutions alone, as no additional tools were required such as a pipette and many vials. When pH test paper strips were in contact with the hydrogels, their colors changed, which indicated that the acid or base solutions were successfully contained in the hydrogels.

Fig. 2 shows the fluorescence images obtained 10 s after applying a height difference to both ends of the capillaries filled with fluorescein solution. Leakage of the sample solution from the capillary was clearly observed as soon as the height difference was applied to the reservoirs filled with background solutions (Fig. 2a), whereas no leakage was observed from the capillaries when RRGs were used instead of the solutions (Fig. 2b). Furthermore, no leakage was observed even when the capillary contacting the hydrogels was tilted 90°. These results indicate that hydrogels can effectively prevent the flowing out of the sample solution from the capillary caused by hydrodynamic flow. Thus, it was expected that use of the hydrogel as an ion supplier could improve the reproducibility in conventional CIEF.

Fig. 1. Prepared hydrogels containing an acid or a base solution. (a) pH test of the prepared hydrogel using test paper strips; (b), (c) change in the color of pH test paper strips upon contact with the acidic (orange, b) or basic (blue, c) hydrogel.

Fig. 2. Suppression of leakage of the sample solution due to hydrodynamic flow. A height difference for generating hydrodynamic flow was applied by controlling the added amount of the background solution in the reservoirs (a), or tilting the capillary connected to the hydrogel (b).

Fig. 3. Variation of the fluorescence intensity in the capillary by electrokinetic introduction of the fluorescent dye. Blue and green lines indicate the change in fluorescence intensity over time of electrokinetically injected sulforhodamine B (negative charge) using solutions and hydrogels, respectively. Red dotted lines indicate fluorescence intensity upon the direct injection of different concentration of the dye solution into the capillary.

3.2. Confirmation of introduction of ionic species and formation of pH gradient

To evaluate the applicability of the prepared hydrogels as a supplier of ionic species, a hydrogel and solution containing ionic fluorescent dye were prepared for visualizing the electrokinetic introduction of the ionic molecule. The fluorescence intensity close to the anodic end of the capillary was measured when voltage was applied to the capillary *via* solutions or hydrogels containing an anionic fluorescent dye, sulforhodamine B. After applying voltage, the dye was electrokinetically introduced from the cathodic reservoir filled with the solutions or hydrogels into the capillary. Under the experimental conditions, the dye reached the detection area following an approximately 50-s application of the voltage, as shown in Fig. 3. The red dotted lines in Fig. 3 represent the fluorescence intensity obtained by the direct introduction of each concentration of the standard solutions of the dyes, which provides an estimation of the concentration of introduced ions from the solution or hydrogel. Although the estimated concentration of the introduced dye from the hydrogel was relatively lower than that from the solutions, this result confirmed that ionic species could be supplied and their concentrations were on the order of 10^{-5} M when the prepared RRG containing 10^{-4} M sulforhodamine B was employed. Therefore, it was suggested that the order of 10^{-1} M acid or base solution in hydrogels are more appropriate to supply a sufficient amount of ions since the order of 10^{-2} M acid and base solutions are generally used for typical IEF experiments.

To confirm the migration of the a sufficient amount of acid and base ions from the RRGs into the capillary, IEF

Fig. 4. Evaluation of the formed pH gradients. Magenta and blue squares show the focused points of the *pI* markers by employing 20 mM acid and base solutions or hydrogels containing 100 mM acid and base solutions, respectively.

analyses of the *pI* markers were conducted to measure the pH gradient obtained when employing 20 mM acid and base solutions or RRGs containing 100 mM acid and base solutions as ion suppliers. As shown in Fig. 4, the observed profile of the pH gradient using RRGs was similar to that observed using solutions, which indicated the formation of a nearly identical pH gradient using the prepared RRGs.

In conventional IEF using the liquid reservoirs, it was sometimes observed that the focused bands moved by the hydrodynamic flow generated by the height difference in the liquid surface of the reservoirs. Therefore, it was necessary to careful handling of the acidic/basic solutions for the conventional IEF experiment using liquid reservoirs. In the proposed IEF using the RRGs, on the other hand, the hydrogels suppressed the hydrodynamic flow in the capillary as described above, which stabilized the positions of IEF markers after focusing. Therefore, it is confirmed that a simple handling only inserting a capillary to RRGs provides a simple and stable IEF analysis as compared to conventional IEF analysis using acidic/basic solutions.

3.3. Evaluation of storage stability of RRGs containing acid or base solution

The profiles of the formed pH gradients in CIEF were measured using RRGs stored for 1–5 weeks at room temperature to evaluate the stability of the RRGs. As a result, there was no significant variation upon increasing the period of the storage, and approximately the same pH gradient profiles were obtained as found for the fresh RRGs. The relative standard deviation of the formed pH gradient ($\Delta pI / \Delta \text{distance}$) was calculated to be 3.8% ($n = 5$), indicating the sufficiently high stability of the stored RRGs.

It is known that hydrolysis of the amide group in

poly(acrylamide) to a carboxyl group occurs under a basic condition [18]. Thus, we were concerned that this hydrolysis might progress in the prepared RRGs, especially in those containing the basic solution, during storage. However, the high reproducibility of the formed pH gradient and the absence of change in their appearance or mechanical strength over five weeks indicated that storage did not significantly affect the ability of the RRGs to supply the ions. As compared to gel electrophoresis, the proposed RRGs act not as a separation medium to achieve a sieving effect, but rather as a supplier of ions, and ensure the suppression of hydrodynamic flow in the capillary. Therefore, it was concluded that the slight hydrolysis during storage at room temperature would have a negligible effect on the proposed CIEF with RRGs.

4. Conclusion

In this work, hydrogels containing an acid or a base solution were developed as an ion supplier for CIEF. The prepared RRGs efficiently suppress the hydrodynamic flow in the capillary, and they remained stable for at least five weeks at room temperature while maintaining their ability to supply ions. In CIEF with RRGs, the profile of the formed pH gradient was almost the same as that obtained using the acid and base solutions. These results show that the proposed RRGs are applicable to bioassays using CIEF with RRGs [25], which will realize a rapid, simple and easy-to-use bioassay based on RRG-IEF.

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