Direct coupling of immobilized metal ion affinity chromatography and capillary isoelectric focusing in a single capillary

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SUMMARY

A procedure for the direct coupling of affinity chromatography and capillary isoelectric focusing in a single capillary is described. A unified capillary device was made to implement this procedure. The inner surface of a fused-silica capillary was tandemly coated with a chelating polymer, polyiminodiacetate, at the inlet side and with a neutral polymer, polydimethylacrylamide, at the outlet side. After loading a fluorescence-labeled recombinant Fab with a hexahistidine tag, a model sample, the device was rinsed with a high-salt buffer and then with a carrier ampholyte solution to fill the device. The bound Fab was eluted by filling the nickel-chelate column with an anode solution, 100 mM phosphoric acid. A positive voltage was applied at the chelate column side with a pressure at the same side, so as to slightly overwhelm the anodic electroosmosis produced in the acidified chelate column and gradually move the focused protein bands through a stationary fluorescence detector. The mixture of the labeled recombinant Fab at variable concentration and labeled bovine serum albumin at 50 nM was analyzed with the capillary device. A linear relationship between the peak area and the concentration was demonstrated for the Fab at 3.2 pM to 10 nM. The coefficients of variation for the peak area and detection time at 10 nM were 4.3% and 4.4%, respectively. The coupled procedure described here allows total transfer of the specifically adsorbed proteins from the affinity column to the capillary for isoelectric focusing without any compromise in separation efficiency. Removal of excess salts and concentration of dilute samples are also attractive features of the coupled procedure that can provide a new option for the analysis of charge variants of a protein in biological samples.

Key words: affinity chromatography, isoelectric focusing, capillary, IMAC, CIEF

INTRODUCTION

Capillary isoelectric focusing (CIEF) is a high resolution separation method that can resolve proteins and peptides based on the differences of isoelectric points as small as 0.02 pH unit at a micro-scale. It is a method of primary importance in the analysis of charge variants produced by post-translational modifications. However, when minor components in biological samples are analyzed, the complexity of the sample matrix could readily overwhelm the resolution of CIEF and hide them from detection. To address this problem, two methods have been developed: one is immunochemical staining of photo-chemically immobilized proteins on the inner surface of a capillary after CIEF separation, and the other is specific non-covalent labeling of target proteins with a fluorescence-labeled antibody fragment prior to CIEF separation, i.e., affinity probe capillary electrophoresis (APCE). Another possibility is the on-line coupling of CIEF with affinity chromatography (AC). The issue in the coupling of CIEF with AC is the setup of solutions in the necessary arrangement, i.e., an anode solution (acid), a sample and a carrier ampholyte solution, and a cathode solution (base). One of the authors of the current study has previously investigated the integration of AC and CIEF in a microfluidic glass chip. In the glass chip, the channel for CIEF was flanked with two manifolds, each with three branch channels. An immunoaffinity chromatographic column was placed in one of the anodic branch channels. The sample adsorbed on the column was eluted with a urea solution containing carrier ampholytes and transferred into the CIEF channel. Both ends of the CIEF channel were connected to electrode vessels with a branch channel filled with an electrode solution. Three problems hindered the practicality of this chip. First,
the eluted zone of the adsorbed protein from the chromatographic column tended to broaden, and only a part of the eluted sample could be analyzed by CIEF, which degraded the performance of quantification of a target protein. Second, the leak of micro valves that had been used for the control of fluids in the channels compromised the precision of the fluid control required for the coupling of AC and CIEF. Third, the stability of the neutral-polymer coating in the channel filled with cathode solution was not enough for repeated analytical cycles required for practical use.

The coupling of AC and capillary zone electrophoresis (CZE) in a directly connected AC column and CZE capillary has already been developed\(^{13}\), and its utility has been demonstrated especially with immunoaffinity mode\(^{12–17}\). Here, “the direct connection” means a simple connection without any branching at the junction. With our experience of the microfluidic chip, we started investigating the possibility of a simple tandem connection between an AC column and a capillary for IEF. To the best of our knowledge, no attempts have so far been reported about the direct connection of AC and CIEF. As a model of AC, we chose an open-tubular capillary AC, in which nickel chelate was immobilized on the inner surface of the capillary, and a hexahistidine-tagged and fluorescence-labeled recombinant Fab fragment (rFab) was used as a model sample. The air-pressure pumping system of the commercial capillary electrophoresis instrument was used for the control of solutions in the capillary. The key to success of this coupling is the control of electroosmotic flow (EOF) produced in the AC column during focusing. The developed coupling method allows the complete transfer of adsorbed analytes on the AC column to CIEF that can be carried out under the most favorable conditions, thereby producing a highly efficient separation.

MATERIALS AND METHODS

Chemicals and Materials

The following were obtained from commercial sources: fused-silica capillaries (Polymicro Technologies, Phoenix, AZ); Pharmalyte 3–10, HiTrap Chelating HP column (GE Healthcare Japan, Tokyo); 3-methacryloyloxypropyltrimethoxysilane (Shin-Etsu Chemical, Tokyo); tris(hydroxymethyl)aminomethane (Tris), \(N,N\)-dimethylacrylamide, glycidyl methacrylate, iminodiacetic acid, \(N,N,N',N''\)-tetramethylethlenediamine (TEMED), \(\mathrm{NiCl}_2\cdot6\mathrm{H}_2\mathrm{O}\) (Wako Pure Chemical Industries, Osaka); tetramethylrhodamine-5-iodoacetamide dihydroiodide (5-TMRIA, T6006), 5-carboxymethylrhodamine, succinimidyl ester (5-TAMRA, SE, C2211) (Life Technologies Japan, Tokyo); bovine serum albumin (BSA) (Cohn V fraction, A4503, Sigma-Aldrich Japan, Tokyo). Hexahistidine peptide was obtained from a commercial peptide synthesis service. Fluorescence-labeled peptide pI markers were prepared as described previously\(^{16}\).

Hexahistidine-tagged rFab against insulin

The anti-insulin rFab with an E tag at the C-terminal of the light chain was prepared and used as previously described\(^{16}\). The vector, pAK400E, bearing an E tag sequence was changed to pAK400 bearing a hexahistidine (6His)-tag sequence\(^{19}\). The rFab was purified from the periplasmic extract using a HiTrap Chelating HP column loaded with nickel ions. After labeling with 5-TMRIA at the cysteine residue located at the C-terminal of the Fd chain, which is a polypeptide chain of Fab and derived from a heavy chain, a single pI variant of pI 7.70 was purified on a slab gel IEF, as described previously\(^{30}\), and used as a model sample, 6His-rFab.

Fluorescence labeling of BSA

BSA, 5.0 mg, dissolved in 0.1 mL of 0.1 M sodium borate buffer (pH 9.0) was reacted with 9.5 \(\mu\)L of 41 mM 5-TAMRA, SE in dimethylformamide for 1 h at room temperature. The reaction mixture was passed through a 2 mL column of Sephadex G50 equilibrated with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM NaH2PO4, 2 mM KH2PO4, pH 7.4). The pass-through fraction was collected, and the labeling ratio was determined spectrophotometrically to be 2.3 using the absorbance at 280 nm and 554 nm. The following values were used for calculation: \(\varepsilon_{280,\text{BSA}} = 4.3 \times 10^4 \text{M}^{-1} \text{cm}^{-1}; \varepsilon_{564,\text{dye}} = 8.7 \times 10^4 \text{M}^{-1} \text{cm}^{-1}; \varepsilon_{580,\text{dye}} = 2.3 \times 10^4 \text{M}^{-1} \text{cm}^{-1}; \) molar mass of BSA = 64000.

Preparation of unified capillaries

For the coupling of AC and CIEF, we prepared a unified capillary, in which the inlet side of the inner wall was coated with polyiminodiacetate (PIDA) and the outlet side with a neutral polymer, polydimethylacrylamide (PDMA). The PIDA part held nickel ion and acted as an affinity adsorbent for 6His-tagged recombinant proteins. On the other hand, the PDMA part, in which EOF and non-specific protein adsorption being suppressed, was suitable for IEF.

A fused-silica capillary (50 \(\mu\)m i.d., 375 \(\mu\)m o.d., 70 cm long) was treated with 3-methacryloyloxypropyltrimethoxysilane, rinsed with acetone, and dried by pulling air through the capillary. The capillary was marked at 30 cm from the inlet with a felt-tip pen to indicate the border of the two types of coating. The silanization and PDMA coating was made according to the published procedure\(^{21}\). A solution of \(N,N\)-dimethylacrylamide (water 9.8 mL, \(N,N\)-dimethylacrylamide 0.2 mL) was deaerated at 25°C for 20 min under a pressure of about 20 mmHg. Aliquots of TEMED 5 \(\mu\)L, isopropanol 50 \(\mu\)L, and 10% (w/v) ammonium persulfate 50 \(\mu\)L were added to the monomer solution, and the mixture was then introduced into the capillary from the outlet by applying reduced pressure with a syringe at the inlet. The meniscus was observed under a stereo microscope. When the meniscus approached close to the mark, the syringe was detached and the solution was allowed to enter the capillary by capillary action. When it reached the mark, the outlet end
was lifted from the solution to stop the move of the meniscus. The outlet end was capped by placing it into a rubber septum for gas chromatography. Care must be taken to avoid an air bubble at the end before piercing it into the septum, because the air bubble would expand and could dislocate the meniscus when the capillary is heated for polymerization. When a bubble was observed, a 2–3 mm segment containing the bubble was cut off at the end. Then, the inlet was also capped with a rubber septum. Polymerization was carried out at 50°C for 2 h. The capillary was then rinsed with injections of 50 μL of each of water and acetone, successively, from the inlet side before being air-dried.

A mixture of 1,4-dioxane (8 mL), water (2 mL), and glycidyl methacrylate (0.3 mL) was deaerated as described above. A 50-μL portion of isopropanol, 5 μL of TEMED, and 50 μL of 10% ammonium sulfate were added to the mixture to form a polymerizing solution. It was introduced into the capillary from the inlet until the meniscus reached the mark. Both ends were then capped as described above. Polymerization was carried out overnight at 50°C. The capillary was then rinsed with 50 μL of each of water and acetone successively, and air-dried. The part of the capillary coated with poly(glycidyl methacrylate) was filled with a 0.5 M iminodiacetate solution titrated to pH 9.5 with 5 M NaOH and reacted at 50°C for 1.6 h. It was successively rinsed with 50 μL of each of water and acetone, then air-dried. The capillary was labeled a PIDA-PDMA unified capillary, and stored in a refrigerator until use.

**Open-tubular capillary column chromatography**

Open-tubular capillary column chromatography was performed using an automated capillary electrophoresis instrument (Beckman-Coulter P/ACE MDQ, Brea CA) with fluorescence detection through a band pass filter (FF01-593/40, Semrock Inc., Rochester NY) using a 532 nm laser (Model CL532-010, CrystaLaser LC, Reno NV) for excitation. PIDA or PDMA coated capillaries (50 μm i.d., 30.5 cm in length) were installed in the capillary cartridge, and detection was carried out at 20.5 cm from the inlet. Buffers and solutions were pumped through by air applied at the inlet vial at 2.0 psi, which corresponds to a linear flow rate of 24 cm/min calculated using the Hagen-Poiseuille equation and a volumetric flow rate of 0.48 μL/min. Sample loading was carried out at a lower pressure, typically at 0.5 psi (6.0 cm/min) for better capture of rFab on the column. The temperature of the cartridge was set at 25°C.

**Standard CIEF conditions with PDMA-coated capillaries**

A PDMA-coated fused-silica capillary (50 μm i.d., 365 μm o.d., 30.5 cm or 48.5 cm in length) was installed in the capillary cassette of the P/ACE MDQ instrument with a detection window 10 cm from the outlet end. The polyimide outer coating on the detection window was removed with a razor blade. The capillary was filled with a sample and/or PI markers dissolved in 1xC A solution (2.5% [v/v] Pharmalyte 3–10, 0.1% acetic acid, 0.6% TEMED, 0.1% Tween 20). A voltage of 15 kV for the 30.5 cm capillary or 25 kV for the 48.5 cm capillary was applied with a current limit of 20 μA using 100 mM phosphoric acid as the anode solution and 100 mM sodium hydroxide as the cathode solution. For the detection of focused labeled proteins and/or PI markers, the pH gradient was mobilized by applying a pressure of 0.1 psi at the anode after 5 min focusing on the 30.5 cm capillary, or after 7 min focusing on the 48.5 cm capillary. Fluorescence detection at 590 nm was carried out with excitation by the 532 nm laser. The temperature of the capillary cassette was set at 25°C.

**Zebra experiment**

To visualize the bulk flow inside the capillary, a zebra pattern of a neutral molecule, acrylamide, was made at three different concentrations in the capillary. Acrylamide at 40, 20, and 10 mg/mL in 1xC A solution was consecutively injected from the inlet (anodic side) at 0.5 psi for 10 s, between each of which 1xC A solution alone was injected at 0.5 psi for 20 s. In total, 12 sets of the triplet were injected to fill the 48.5 cm capillary with the zebra pattern of acrylamide. The zebra pattern was then detected with ultra-violet absorption at 254 nm to observe bulk fluid flow inside the capillary, of which inner wall was coated with PIDA and PDMA, under different voltage and pressure settings.

**On-line coupling of AC and CIEF**

The operation of the unified capillary was carried out using the P/ACE MDQ instrument with the same fluorescence detection system as with chromatography. The unified capillary was installed in the capillary cartridge of the instrument with 18.5 cm PIDA part at the inlet side and 30 cm PDMA part at the outlet side. When the coolant tube of the capillary cartridge for a nominal total capillary length of 50 cm was used, the actual length between the detection point and the inlet end was 38.5 cm, a little shorter than the nominal length of 40 cm. This made the length of the PIDA-coated part shorter than the planned 20 cm. The detection window was set at 10 cm from the outlet. The capillary was equilibrated by injecting 20 mM Tris-HCl buffer (pH 7.4) containing 0.5 M NaCl (binding buffer) at 3.2 psi (24 cm/min) for 1 min, and Ni ion was loaded with 0.1 M NiCl2 in water at 3.2 psi for 1 min. The capillary was rinsed to remove excess Ni ions with 20 mM Tris- HCl buffer (pH 7.4) containing 0.5 M NaCl, 10 mM imidazole, and 0.1% Tween 20 (rinse buffer) at a pressure of 3.2 psi for 1 min. A sample solution containing 6His-rFab in 0.1 M Tris-HCl buffer (pH 8.0) containing 0.1% Tween 20 was injected into the capillary at 0.8 psi (6.0 cm/min) for 8 min, which corresponds to the sample plug length of 48 cm and to the sample volume of 0.94 μL. The capillary was rinsed with rinse buffer at 3.2 psi
for 3 min, and further rinsed with 20 mM Tris-HCl (pH 7.4) containing 0.1% (w/v) Tween 20 at 3.2 psi for 1 min to remove excess salts. A 1xCA solution containing 2.5 nM each of the fluorescence-labeled peptide pl markers was injected at 3.2 psi for 1.6 min, corresponding to a plug length of 38 cm. The anode solution (100 mM H₂PO₄) was injected at 2.0 psi for 1.5 min, which corresponds to a plug length of 22.5 cm, to acidify the PIDA part and elute bound proteins. Focusing was started at a voltage of 25 kV with a pressure of 0.2 psi at the anode. Two minutes later, the pressure was reduced to 0.1 psi, and focusing and mobilization were continued for 30 min. Finally, the capillary was rinsed with 0.1 M HCl and water successively at 50 psi for 2 min each.

RESULTS AND DISCUSSION

Affinity chromatography on nickel-chelate open-tubular capillary column

The pressure vs. flow properties of open-tubular PIDA-coated capillary columns were examined using the automated capillary electrophoresis instrument. The column was irrigated with buffers by pressurizing the inlet vial with air. The time required to bring an injected plug of acrylamide solution to the detection point was measured for the 50 μm i.d. open-tubular column with a length of 30.5 cm and 48.5 cm. The experimental values fitted well with theoretical ones calculated using the Hagen-Poiseuille equation over a range of 0.1–2.0 psi (Fig. 1). No significant difference was observed among PIDA coating, PDMA coating, and the loading states with nickel ions.

The PIDA-coated capillary (50 μm i.d., 30.5 cm long) was loaded with nickel ions, and its chromatographic characters were examined using 6His-rFab as a model sample. During the loading of 20 nM of the sample at 2.0 psi for 2 min (0.96 μL, 19 fmol of rFab), a fraction of the sample overflowed from the column (Fig. 2, solid line). The capillary was rinsed with rinse buffer, which contained 0.5 M NaCl and 10 mM imidazole. The high salt concentration should be effective to suppress ionic interaction between proteins and the adsorbent, and 10 mM imidazole should exclude proteins interacting weakly with the metal. A bound fraction was eluted from the column as a sharp peak with a neutral buffer containing 0.5 M imidazole, which is a standard eluting agent for 6His-tagged proteins from an immobilized nickel ion affinity column. On the other hand, any retention of the sample was observed on the PIDA-coated capillary without nickel loading, and on the PDMA-coated capillary with nickel loading. The results indicate that the nickel-loaded open-tubular PIDA-coated capillary column shares principal characteristics with immobilized metal ion AC columns with a packed adsorbent, although the capacity of the open-tubular col-

![Fig. 1. The pressure vs. water flow relationships of Beckman P/ACE MDQ instrument with a fused silica capillary. A plug of acrylamide solution was injected into a fused-silica capillary (50 μm i.d., 30.5 or 48.5 cm in length) filled with water, and the measurement was started by injecting water by pressure. The linear flow rate was calculated from the time taken to the rear end of the plug with UV absorption at 254 nm. Filled circles for 30.5 cm capillary and open circles for 48.5 cm capillary. The lines were drawn for the calculated values with the Hagen-Poiseuille equation. The temperature of the capillary cartridge was set at 25°C.](image1)

![Fig. 2. Affinity chromatography of 6His-rFab on the PIDA- or PDMA-coated open-tubular capillary column. All buffers and the sample were pumped into the capillaries (50 μm i.d., 30.5 cm in length) with a pressure of 2.0 psi at the inlet vial (linear flow rate of 24 cm/min). The coated open-tubular columns were equilibrated with binding buffer for 0.5 min, and nickel ion (Ni) was loaded with 0.1 M NiCl₂ for 0.5 min. The columns were re-equilibrated with rinse buffer (R) for 1 min, and 20 nM 6His-rFab (rFab) in 0.1 M Tris-HCl buffer (pH 8.0) containing 0.1% Tween 20 was applied for 2 min, which corresponds 0.96 μL of the sample and 19 fmol of rFab. After rinsing the columns with rinse buffer for 1 min, bound rFab was eluted with 20 mM Tris-HCl buffer (pH 7.4) containing 0.5 M NaCl and 0.5 M imidazole. The nickel ion-loaded PIDA-coated column, solid line; the nickel ion-unloaded PIDA-coated column, which was irrigated with binding buffer instead of the NiCl₂ solution, dotted line; and the PDMA-coated column with a nickel-loading procedure, broken line. The temperature of the capillary cartridge was set at 25°C. Detection was carried out with fluorescence at 590 nm. RFU represents relative fluorescence unit.](image2)
umn is limited. When chromatography on the nickel ion-loaded PIDA-coated capillary column was repeated six times, the coefficient of variation of the peak area of the eluted fraction with 0.5 M imidazole was 2.9%.

Since a capillary bore of 50 μm might be large enough to impair rapid approach to the binding equilibrium between the mobile phase and the immobile phase, the effect of the flow rate at sample loading on the amount of retained rFab on the column was examined. The pumping pressure and loading time were changed to keep the sampling volume constant at 0.96 μL. When these chromatograms were examined, it was noted that the pass-through fraction was reduced as the flow rate decreased (Fig. 3a). It is noteworthy that the area of the fluorescence signal is reciprocally proportional to the flow rate of the solutions in the capillary when the signal is plotted against time, and thus the comparison of the area of the pass-through fraction is difficult. Instead, the comparison of eluted peaks with 0.5 M imidazole is straightforward, since all elution was performed at 2.0 psi. As the sample-loading pressure decreases and the loading flow rate became smaller, it is evident that a larger amount of rFab was retained (Fig. 3b). This indicates that the optimum flow rate for the maximum capacity of the open-tubular column with the macromolecular analyte is very small, which is understandable when the large bore of the open-tubular column and slow diffusion of macromolecules are considered. As a compromise for loading time and effective capacity, sample loading at 0.5 psi for 30.5 cm capillary, which corresponded to a linear flow rate of 6.0 cm/min, were used in the later analysis. For 48.5 cm capillary, 0.8 psi was used corresponding to a linear flow rate of 5.8 cm/min.

Imidazole was a very efficient eluting agent in the immobilized metal AC and, in the early part of this investigation on the coupling of the nickel chelate chromatography and CIEF, we attempted the focusing of rFab eluted with imidazole in the presence of carrier ampholytes. However, in simple CIEF experiments, we observed that the presence of imidazole at 500 mM severely impaired focusing when using carrier ampholytes. The focusing time elongated and the pH gradient deformed to have a shallower slope in the weakly alkaline region. We tested other conditions for eluting rFab from the nickel chelate column. Hydrochloric acid at 10 mM and phosphoric acid at 100 mM were both very efficient eluents, which was comparable to imidazole at 0.5 M in binding buffer (Fig. 4). Surprisingly, efficient elution was not observed with EDTA, which should be a far stronger chelating agent compared to the immobilized iminodiacetate. The results may relate to the flexible nature of the polymeric chelate immobilized on the wall. High efficacy in the competitive elution was expected for hexahistidine peptide. However, it was less effective than 0.2 M imidazole even at 20 mM. It was found that acid was the only eluent that was comparable to 0.5 M imidazole.

Fig. 3. The effect of the flow rate of sample loading on the affinity chromatography of 6His-rFab on the nickel ion-loaded PIDA-coated open-tubular capillary column (50 μm i.d., 30.5 cm in length). (a) The pressure and loading time were changed so as to keep the product of the two values constant in order to maintain a constant sampling volume of 0.96 μL: grey broken line, 8.0 psi (97 cm/min) for 0.5 min; grey solid line, 4.0 psi (49 cm/min) for 1.0 min; dotted line, 2.0 psi (24 cm/min) for 2.0 min; broken line, 1.0 psi (12 cm/min) for 4 min; and solid line, 0.5 psi (6 cm/min) for 8 min. Other conditions were the same as those in Fig. 2, and other buffers and the imidazole solution were pumped at a pressure of 2.0 psi. (b) The peak areas of the eluted 6His-rFab with 0.5 M imidazole were plotted against linear flow rate in the sample loading process.

Fig. 4. Elution of 6His-rFab from nickel ion-loaded PIDA-coated open-tubular capillary column (50 μm i.d., 30.5 cm in length) under several different conditions. The PIDA-coated capillary was loaded with nickel ion and 6His-rFab as described in the Fig. 2 legend, and after rinse with rinse buffer for 1 min, different eluents were pumped at a pressure of 2.0 psi, HCl, 10 mM; imidazole, 0.2 M (broken line) and 0.5 M (solid line) in binding buffer; EDTA, 0.05 M (broken line) and 0.5 M (solid line) in binding buffer; hexahistidine peptide, 10 mM (solid line) and 20 mM (broken line) in binding buffer; and H3PO4, 100 mM.
and one positive charge, i.e., one effective negative charge (Fig. 6a). When Ni²⁺ ion is loaded, the proton on the tertiary amino group is replaced with metal, and the effective charge should be zero (Fig. 6b). Under acidic conditions, the protonation of the carboxyl groups leads to the loss of its chelating function, leaving one positive charge in the tertiary amino group (Fig. 6c).

EOF was checked under different conditions of PIDA, as described in Fig. 6. At neutral pH, nickel-unloaded PIDA-coated capillary presented rapid EOF to the cathode, which agree with the effective negative charge of the PIDA structure. No EOF was expected for the structure of Fig. 6b, but substantial cathodic EOF was still observed after nickel-loading at neutral pH. This is likely due to the presence of free chelator moiety under equilibrium of nickel ion-PIDA complexation. Under acidic conditions, the EOF was towards the anode as anticipated from the positive charge on the amino group.

If CIEF separation is carried out in the total length of the unified capillary, the nickel-PIDA part experiences a change of pH and consequently its EOF should change in the focusing process. In the beginning of focusing with the inlet end at the anode, the EOF should be towards the cathode, because the initial pH of the carrier ampholyte solution is weakly alkaline due to the addition of TEMED. This EOF should result in partial loss of carrier ampholytes from the cathodic end of the capillary and influx of anode solution at the anodic end. As the formation of pH gradient proceeds, the nickel-PIDA part is acidified, and the PIDA part will gradually change to a positively charged structure losing nickel ions, as depicted in Fig. 6c, and the EOF should be reversed to the anode. Consequently, the alkaline cathode solution will enter the capillary from the cathodic end. The anodic mobilization of the pH gradient will gradually increase the pH of the PIDA-coated part and will finally cease the bulk flow in the capillary by the balance of the anodic EOF at the anodic side of the PIDA part and the cathodic EOF produced at its cathodic side. When the PIDA part is involved in IEF, the
change of EOF in the capillary should result in partial loss of carrier ampholytes and influx of the alkaline cathode solution that should be unfavorable for the PDMA coating of the capillary.

**Zebra experiments**

To observe bulk flow in the unified capillary during the IEF process, a repeating pattern of acrylamide at three different concentrations was made in a carrier ampholyte solution and the acrylamide zones were detected with a fixed ultra-violet detector under focusing conditions, which we call a zebra experiment (Fig. 7a). When the zebra pattern was made in a PDMA-coated capillary and focusing was started under standard CIEF conditions (without application of pressure at the anode), no acrylamide peak was observed, indicating the EOF of the fused-silica capillary was well suppressed with the coating. The zebra pattern was now observed when pressure was applied from the beginning of focusing under the same conditions. A typical observation of the pattern with application of 0.3 psi at the anode under focusing conditions is presented in Fig. 7b. The results show that about 7.5 sets of the three-acrylamide zones were present between the anodic end and the detection point at the start of focusing and pressure application. The rise of the valleys of the first to the fifth set is due to UV absorption of the focused carrier ampholyte. Without application of voltage, the valleys aligned flat. For the sake of convenience of explanation of the observed zebra pattern, each set of zones is labeled with a number. H (high), M (medium), and L (low) were used to distinguish zones with different concentrations. Using this numbering system, the UV absorption trace in Fig. 7b can be said to start with Zone 0M and the following peak was Zone 0L.

When the same experiment was performed in the unified capillary, a different pattern was observed (Fig. 7c). The pattern started at the valley of the acrylamide peak between an H and an M zone. The difference in the start point of the UV traces for one peak is often observed due to an accumulation of small errors of the repeated injection process. The initial movement of the fluid should be towards the cathode, since both the pressure and the expected EOF were in the same direction. The first peak should be Zone 0M. A rough mound followed at 2–4 min. The roughness of the trace, which is not apparent in Fig. 6b, is ascribed to the passing of focusing carrier ampholytes at the detection window from the cathodic side of the capillary. If this mound had been ascribed to the passing of Zone 0L and the cathodic flow had continued, the next expected peak would have been Zone 1H, but was actually detected as an M zone (Fig. 7c, 6–9 min). This result indicates that the direction of the bulk flow in the capillary had reversed at some point. We suspect that this occurred at the cathodic slope of Zone 0L. The reversed flow, which is against the pressure applied at the anode, continued, and an H zone was detected (Fig. 7c, 10–15 min), i.e., Zone 0H.

We expected an L zone to appear next if the flow continued, but this was not the case. An M zone was detected, and the order of H, M, and L did not change thereafter. This means that reversed flow of the fluid had occurred again, most probably when the center of Zone 0H reached close to the detection point.

The observation of bulk flow in the unified capillary can be explained with the pH-dependent ionization of its PIDA part discussed in the earlier section. In the presence of pressure at the anode, the pH gradient is gradually replaced with the anode solution. Of note is that the anode solution, 100 mM H₃PO₄, has a much higher electric conductivity than the pH gradient. The strength of the electric field in the region filled with the anode solution is low in comparison with that of the pH gradient. The anodic EOF of the PIDA part becomes very small as it is filled with the anode solution. The observed distance of peaks for zones three to seven was not much different from that observed in the PDMA capillary, indicating a similar bulk flow in the two capillaries. Furthermore, it is interesting to note the degradation in the resolution of the last three sets of zones. Every triplet of
zones five to seven was detected almost as a single peak. The loss of resolution can be explained by the local mixing caused by the opposite flow produced by electroosmosis and pressure in the PIDA-part of the unified capillary.

**Suppression of EOF by eluting proteins with anode solution**

Due to the strong impact of imidazole on the formation of a pH gradient, and the troublesome EOF produced in the PIDA-part of the unified capillary, elution with imidazole in carrier ampholyte solution and subsequent focusing was not successful. Considering the effectiveness of acid in eluting bound 6His-rFab and the effective suppression of EOF with the anode solution, we realized that elution of bound proteins by filling the PIDA part with an acidic anode solution could set up very favorable conditions for the start of IEF separation. If the pressure balances with the residual anodic EOF, it should be possible to avoid the influence of the anodic EOF produced in the PIDA part on the PDMA part where IEF occurs. Under these conditions, local circulating flow should occur by counteracting flow of electroosmosis and pressure in the PIDA part, as mentioned in the previous section. On the other hand, there should be no turbulence in the fluid in the PDMA part, and focusing should occur without any compromise (Fig. 8).

To find the necessary pressure to balance with EOF produced in the PIDA part filled with the anode solution, zebra experiments were carried out under different pressures applied to the anode. After forming the zebra pattern of acrylamide in 1xCA solution in the nickel-loaded unified capillary, the anode solution of 100 mM phosphoric acid was injected at 2.0 psi for 1.5 min, which was necessary to fill the 22.5 cm range of the anodic side of the capillary, including the PIDA part. The application of voltage (25 kV) and a pressure at 0.3 psi at the anode was started, and the UV absorbance was traced (Fig. 9a). In contrast to the case without filling the PIDA part with the anode solution (Fig. 7c), the fluid in the capillary was rapidly transferred to the cathodic side, and it is almost comparable to the case of PDMA-coated capillary (Fig. 7b). The number of zebra patterns observed was reduced due to the injection of the anode solution without acrylamide zones. This result indicates that the EOF in the unified capillary was mostly suppressed by filling the PIDA part with 100 mM phosphoric acid. This is due to the higher electric conductivity of the anode solution than that of the carrier ampholyte solution. Therefore, the

**Fig. 8.** The concept of the direct coupling of nickel-chelate affinity chromatography and CIEF in the unified capillary device. The specifically captured proteins are eluted from the nickel-PIDA coated part of the unified capillary with the anode solution, an acid, and subsequently focused in the PDMA-coated part with the anodic pressure that balances with the EOF produced in the acid-filled PIDA-coated part. Local circulating flow should be generated in the PIDA-coated part by the counteracting flow by pressure and electroosmosis; however, the bulk flow is suppressed. Focusing in the PDMA-coated capillary can be achieved without any influence of the EOF and the pressure. When a detector with a fixed detection point is used, as in the present report, mobilization of pH gradient by pressure or electrophoresis is necessary. The thick vertical lines in the pH gradient represent focused proteins.

**Fig. 9.** Selection of a balancing pressure setting by zebra experiments. The zebra patterns in 1xCA solution were made in the unified capillary (48.5 cm in length) as described in the Fig. 7 legend. Further, the anode solution, 100 mM phosphoric acid, was injected to fill a 22.5 cm range of the anodic side of the capillary, including the PIDA part. The application of voltage (25 kV) and a pressure at 0.3 psi at the anode was started, and the UV absorbance was traced (Fig. 9a). In contrast to the case without filling the PIDA part with the anode solution (Fig. 7c), the fluid in the capillary was rapidly transferred to the cathodic side, and it is almost comparable to the case of PDMA-coated capillary (Fig. 7b). The number of zebra patterns observed was reduced due to the injection of the anode solution without acrylamide zones. This result indicates that the EOF in the unified capillary was mostly suppressed by filling the PIDA part with 100 mM phosphoric acid. This is due to the higher electric conductivity of the anode solution than that of the carrier ampholyte solution. Therefore, the
voltage drop in the part filled with anode solution became small in comparison with the part filled with the 1xCA solution.

When the pressure was reduced to 0.2 psi, we observed that the first peak was broadened, i.e., the bulk flow decreased in the beginning of focusing (Fig. 9b). It became more evident with the flat valley observed at the start of the focusing at 0.1 psi (Fig. 9c). These results indicate that the anodic EOF was effectively suppressed with the anode solution, but the residual EOF was especially evident at the beginning of the focusing process. This observation is related to the change of conductivity of the carrier ampholyte solution in the course of focusing. As it is always observed during isoelectric focusing, the electric current is high at the beginning of focusing and decreases as a pH gradient is established when a constant voltage is applied between the electrodes. The conductivity of the part filled with the anode solution is constant during the focusing process, but the conductivity of the rest of the capillary, i.e., the part filled with carrier ampholyte, decreases as focusing proceeds. This change in the relative conductivity of the two parts results in the decrease of the voltage drop in the part filled with the anode solution, as well as the reduction of EOF in the course of the development of the pH gradient. The result obtained without application of pressure was difficult to interpret, but it seemed that the EOF was very small (Fig. 9d).

Application of pressure at the anode was also necessary to bring focused proteins to the detection point. Taking the observation of relatively high anodic EOF at the initial part of the focusing process into consideration, we chose a pressure program of 0.2 psi for the initial 2 min and 0.1 psi for the rest of focusing at 25 kV for the unified capillary. The result of the zebra experiment under this pressure program is presented in Fig. 9e. Lower pressure may be enough to balance the EOF after the initial part of focusing, but 0.1 psi is the smallest pressure setting of the instrument. Since we were anxious about damage to the PDMA coating at the cathodic side of the capillary by the influx of the cathode solution (100 mM NaOH), a pressure setting that ensured a continuous flow towards the cathode was selected.

**On-line coupling of AC and CIEF in the unified capillary**

As a model sample, a mixture of 10 nM 6His-rFab and 50 nM labeled BSA in a neutral buffer was applied to the unified capillary. The capillary was rinsed with rinse buffer containing 0.5 M NaCl, and then with a low salt buffer to remove excess salt in the capillary. Following that, a carrier ampholyte solution containing fluorescent pI markers was injected, corresponding to a plug length of about 40 cm. Injection of the carrier ampholyte solution was minimized, because it destabilizes the binding of 6His-rFab to the nickel-chelate column. TEMED was added to the carrier ampholyte solution at a relatively high concentration as a spacer between the cathodic end of the pH gradient and the cathode solution to cover the distance from the detection point to the cathode end of the capillary. Finally, anode solution (100 mM H3PO4) was injected to form a plug of 22.5 cm, which is enough to cover the PIDA-coated part of the unified capillary. Isoelectric focusing was started by applying 25 kV and pressure was applied according to the predetermined program, i.e., 0.2 psi for 2 min and 0.1 psi after 2 min.

The trace of fluorescence signal showed a small peak at around 12.5 min (Fig. 10a). Of note is that the peak area is reciprocally proportional to the speed of the labeled materials that pass through the detection point. The speed of the fluid inside the capillary was 5.8 cm/min at 0.8 psi, 23 cm/min at 3.2 psi and about 1 cm/min in the mobilization process of the pH gradient. Most of the pass-through fraction was detected at the front edge of rinse buffer delivered at 3.2 psi, and thus the peak area looked about 20 times smaller.

![Fig. 10. Specific adsorption of 6His-rFab and subsequent analysis by CIEF using the nickel ion-loaded unified capillary.](image)

A mixture of 6His-rFab and 50 nM labeled BSA was applied to the unified capillary at 0.8 psi for 8 min (3–11 min, 0.94 μL). The 6His-rFab was trapped on the nickel-chelate column in this process. The capillary was rinsed with rinse buffer at 3.2 psi for 3 min and with a low ionic strength buffer for 1 min. Most of BSA without 6His-tag was removed from the devise through this rinse process. A plug of 1xCA solution with two pI markers (2.5 nM each) was injected at 3.2 psi for 1.6 min, and the bound 6His-rFab was eluted with 100 mM phosphoric acid at 2.0 psi for 1.5 min until the front of the acid solution just passed the border between the PIDA and PDMA coating. Focusing was started at 25 kV with a pressure of 0.2 psi at the anode. After 2 min, the pressure was reduced to 0.1 psi, and focusing and mobilization of pH gradient was continued. The concentration of 6His-rFab was (a) 10 nM and (b) 3.2 pM, both with 50 nM BSA. Thin dotted lines, voltage; broken lines, current. Please note that the sensitivity of the fluorescence detection was about 20 times less in the rinse process due to the higher flow speed of solution inside the capillary than that of the mobilization process of a pH gradient.
in the pass-through fraction than that in the pH gradient. From the peaks of pI markers, pI 9.56 and 3.64, it can be seen that the pH gradient from pH 10 to 3 spanned from about 25 min to 45 min. A sharp peak corresponded to 6His-rFab of pI 7.70 appeared at about 31 min. The labeled BSA, which is expected to appear at the acidic side of the rFab, was scarcely observed in the pH gradient and is suspected to have already been separated from the rFab during the rinse process. The small peak at about 36 min should be the residual labeled BSA that remained after rinsing. The result clearly shows that the unified column specifically trapped 6His-rFab and allowed its analysis by IEF in a directly coupled manner without any loss of the trapped sample.

The strong point of this coupling process is that there is no compromise in separation by CIEF. The focusing occurs in an essentially EOF-free capillary and, if the EOF generated in the affinity column is effectively balanced with pressure, the conditions in the CIEF capillary are completely the same as those in a simple CIEF analysis, except for the initial position of a sample. In a simple CIEF analysis, a sample is usually included in the carrier ampholyte solution, but in this on-line coupling the sample is eluted with the anode solution in the unified capillary and resides close to the boundary with the carrier ampholyte solution in the CIEF capillary. The application of voltage initiates the formation of a pH gradient and the migration of positively charged eluted proteins into the pH gradient. The final pressure of 0.1 psi at the anode slowly mobilized the pH gradient and focused proteins, making them pass through the detection point. In addition to the uncompromised CIEF condition, the coupling allows removal of sample matrix including excess salt, which interferes with the focusing process. The concentration of a target protein from a dilute sample could also be expected for the affinity column coupled to the CIEF capillary.

The detection limit of this analysis was examined by serially reducing the concentration of 6His-rFab, while keeping the concentration of the labeled BSA constant at 50 nM in the sample solution. Even at 3.2 pM, the peak of 6His-rFab was readily discernible (Fig. 10b). The peak that originated from the residual labeled BSA not removed by rinsing was apparent at this magnification of the ordinate. The result represents the superiority of the combination of affinity adsorption and separation by CIEF over AC alone, with which it would be impossible to accurately estimate a small amount of the target in the presence of other protein species that cannot be removed completely by rinsing. The relationship of the peak area and the concentration of 6His-rFab in the sample was linear from 3.2 pM to 10 nM (Fig. 11). The precision of the analysis was evaluated using 10 nM 6His-rFab with or without 50 nM labeled BSA. The coefficients of variation for peak area and detection time were 4.3% and 4.4% for triplicate experiments with labeled BSA, and 4.6% and 1.2% for octuplicate experiments without labeled BSA, respectively. To test the effect of a real sample matrix, rFab in 50% serum was tested as a sample. It was found that the presence of serum weakened the affinity of 6His-rFab to the nickel-chelate column considerably, probably due to the competitive binding of serum proteins to the nickel-chelate. To solve this specificity problem, we are testing the use of immunoaffinity columns.

**CONCLUSION**

In striking contrast to zone electrophoresis, the resolution of IEF does not depend on the width of a sample zone at the start of focusing. This is the inherent advantage of IEF in coupling with solid-phase extraction, which allows not only for a high degree of sample concentration but also for removal of salts that compromise CIEF in the application to biological fluids. The unified capillary of the immobilized metal affinity column and IEF capillary should be useful to check the production of recombinant proteins with a 6His-tag in an expression experiment. In that case, however, ultra-violet absorption detection is preferable, but the capacity of the open-tubular column is not enough for ultra-violet detection, which is less sensitive than fluorescence detection. The use of a higher capacity column is under investigation. The problem of EOF in the direct coupling of AC and CIEF should be common to any AC column, since most affinity adsorbents change ionization state depending on pH, and contain bases that produce anodic EOF under acidic conditions. The principle of the direct combination of AC and CIEF should be applicable to any kind of AC column. The combination with an immunoaffinity column seems to be successful and will be reported before long.

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