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Cytokine Analysis by Immunoaffinity Capillary Electrophoresis

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Abstract

Immunoaffinity capillary electrophoresis (ICE) is a powerful tool used to detect and quantify target proteins of interest in complex biological fluids. The target analyte is captured and bound to antibodies immobilized onto the wall of a capillary, labeled in situ with a fluorescent dye, eluted and detected online using laser-induced fluorescence following electrophoretic separation. Here, we illustrate how to construct an immunoaffinity capillary and utilize it to run ICE in order to capture and quantify target cytokines and chemokines from a clinical sample.

Keywords

Immunoaffinity capillary electrophoresis; Analyte concentrator; Cytokines; Proteins

1. Introduction

Capillary electrophoresis (CE) is an extremely versatile technique used for the rapid separation of proteins. CE-coupled with laser-induced fluorescence (LIF) detection has allowed for detection and quantitation of cytokines, chemokines, neuropeptides, and other biomarkers found in clinical samples (1–3). Immunoaffinity capillary electrophoresis (ICE) is a customized technique that tailors the capillary to capture the analytes of interest. The use of an antibody to capture its target analyte directly on the capillary enhances the signal to noise ratio by eliminating analytes of non-interest and concentrating the target analytes prior to detection. ICE has been used to quantify biomarkers in different clinical samples such as urine, blood serum, and CSF (4–6), quantify phosphorylation of STAT proteins (7), and quantify erythropoietin glycoforms biological fluids (8). ICE is particularly useful in the detection and quantitation of cytokines released in an immunological response (9), and has been used to quantify cytokine release in head trauma (10), after triggering myofacial points (11), after neuropeptide stimulation (12), and to detect a decrease in IL-1 β with the consumption of salt water (13).

Like other immunoaffinity techniques, ICE immobilizes analyte-specific antibodies so they may capture its targeted analyte. Once captured, the analyte is isolated by eliminating the remaining sample, incubating the analyte with a fluorescent dye, eluting with an acidic buffer and detecting through LIF. Combining ICE with LIF allows for the quantitation of one or multiple cytokines using an extremely small sample volume (less than 1 μ L) with increased sensitivity. In addition, the small reagent quantity and short time provide for an efficient, cost-effective means to quantify biomarkers. Since ICE performs analyte pre-

concentration and cleanup, it is particularly useful when working with biological samples where one or a few cytokines are selected from a clinical sample with multiple proteins. When compared to ELISA, ICE offers several advantages including less reagent and sample use, greater resolving power and less false positive results due to poly-reactivity of antibodies (14). This chapter outlines the steps necessary to build an immunoaffinity capillary and run an effective CE protocol to capture, label, detect, and quantify cytokines.

2. Materials

2.1. Immunoaffinity Capillary

1. Flexible fused silica capillary: Fused silica capillary with an internal diameter of 100 μm . Use a tile to cut the capillary at a length of 70 cm (see Note 1).
2. Ten % v/v 3-aminopropyltriethoxysilane: Transfer 1 mL 3-aminopropyltriethoxysilane to 9 mL H_2O and mix thoroughly. Store at 4°C.
3. 50 mM $\text{Na}_2\text{B}_4\text{O}_7$: Dilute 1 mL 0.5 M $\text{Na}_2\text{B}_4\text{O}_7$ with 9 mL deionized H_2O .
4. Sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC): Sulfo-SMCC is moisture sensitive and should be stored at -20°C. To prepare, weigh 1 mg Sulfo-SMCC and transfer to 1 mL 50 mM $\text{Na}_2\text{B}_4\text{O}_7$, pH 7.6.
5. Antitumor necrosis factor- α antibodies (anti-TNF- α): Reconstitute to a concentration of 20 $\mu\text{g}/\text{mL}$. After reconstitution, solution is stable for up to 12 months at -20°C. Avoid repeated freeze-thaw cycles. Although this protocol was designed using anti-TNF- α antibodies, any antibodies that are specific to a target antigen may be substituted to quantify any cytokine in an unknown sample.
6. 25 mM sodium acetate, pH 4.5: 1.501 g of acetic acid (MW 60.05) in 900 mL deionized H_2O . Titrate with 1 N HCl to a pH of 4.5.
7. Immobilized pepsin: Stored at 4°C.
8. 10 mM EDTA: Prepare by diluting 1 mL of 0.5 M EDTA pH 8.0 in PBS to a final volume of 5 mL.
9. 2-Mercaptoethylamine HCl (2-MEA): 1 vial contains 6 mg 2-MEA. Store at 4°C.
10. 100 mM sodium phosphate buffer, pH 7.4: Use commercially available 100 mM sodium phosphate buffer for HPLC, pH 7.0. Adjust the pH to 7.4 using 1 M NaOH.
11. 1 mL syringe and attached capillary flush system: Constructed using a micro-tight PEEK tube, part F-185 in micro-tight PEEK sleeve kit, CE flushing unit P-662, and female tube slip unit P-686. All parts are available from Upchurch Scientific (Oak Harbor, WA, USA) The female tube slip is attached to the micro-tight PEEK tube and placed inside the CE flushing unit. Use the smallest size PEEK tube that can securely fit over the capillary so no leakage occurs during the flushing procedures.

¹The length of the capillary may be increased. This allows for greater separation of cytokines once a voltage is applied, and may be useful if multiple cytokines similar in size are being measured. Use safety goggles when cutting the capillary.

Attach a 1 mL syringe to the CE flushing unit. The construction of the capillary flush is illustrated in Fig. 1.

12. MagnaBind™ protein A magnetic beads: Store at 4°C.
13. 0.22 µm Millex®-GS syringe riven filters.
14. Magnetic separation rack.
15. Scotch tape.

2.2. CE

1. Running buffer: 100 mM sodium phosphate buffer, pH 7.4. To prepare, use commercially available 100 mM sodium phosphate buffer for HPLC, pH 7.0. Buffer is supplemented with 0.01% v/v Brij 35 (see Note 2) and filtered through a 0.22 µm syringe filter.
2. Elution Buffer: 100 mM sodium phosphate buffer, pH 1.5. To prepare, use commercially available 100 mM sodium phosphate buffer for HPLC, pH 2.5. Adjust the pH by titrating 1 N HCl to a pH of 1.5. Elution buffer is supplemented with 0.01% v/v Brij 35 (see Note 2) and filtered through a 0.22 µm syringe filter.
3. 5 ng/mL AlexaFluor 633 laser dye: Dissolve 5 mg of AlexaFluor 633 in 50% formamide in 0.1 M sodium bicarbonate, pH 9.0. Store at -20°C (see Note 3).
4. Window maker (see Note 4).
5. Recombinant tumor necrosis factor-α (TNF-α): This will be used to create a standard dilution curve. It is important to use the recombinant cytokine that is targeted by the specific antibody tethered in the capillary. Reconstitute and store according to the manufactured instructions. Note the final concentration of the TNF-α.

2.3. Instrumentation

Instrumentation used to run ICE is illustrated in Fig. 2.

1. Capillary electrophoresis (CE) system: The CE system used in this chapter is an Agilent Technologies 7100 Capillary Electrophoresis. CE procedures were written and run through a computer running 3D-CE ChemStation software. This chapter outlines how to perform ICE on a generic CE instrument.
2. Laser-induced fluorescence (LIF) detector. The LIF detector used in our procedures incorporates a 12 mW red-light emitting, 633 nm helium-neon laser. Signals from the detector were filtered through a 650 nm interference filter before being transferred to a computer running 3D-CE ChemStation software.

²To decrease friction with the walls of the capillary, higher concentrations of Brij-35 may be used, up to a 1% v/v. Other non-ionic surfactants like Igepal CA-630 (Sigma-Aldrich, St. Louis, MO, USA) may be substituted for Brij-35.

³The AlexaFluor laser dye used is dependent on the LIF detector coupled to the CE. Our procedure incorporates a 12 mW red-light, 633 nm helium-neon emitting laser; thus, AlexaFluor 633 is used to label our cytokine samples for detection.

⁴If you do not have access to a Window maker, it is possible to create a window with a butane lighter. This procedure will be discussed when the window is installed on the capillary.

3. Methods

To build a capillary suitable to run ICE, antibodies must be processed so they are able to attach to a thiol-derivatized capillary. The antibodies are digested with pepsin to yield F(Ab')₂ fragments (Fig. 3a), which are further reduced to 2 FAb fragments (Fig. 3b) and attached to the capillary (Fig. 3c).

The following ICE protocol was designed to use anti-TNF- α to capture TNF- α . This protocol may be extended to introduce one or many antibodies. In order to properly capture and quantify a target antigen, an antibody that is specific to the target antigen must be added to the capillary. Figure 4 illustrates how ICE operates to capture and elute a targeted cytokine from a clinical sample. Carry out all procedures at room temperature unless otherwise specified.

3.1. Thiol-Derivatization of a Capillary

This method has been modified from a previously published technique (15).

1. Pipette 500 nL of prepared 10% v/v 3-aminopropyltriethox-ysilane solution onto a parafilm sheet. Allow the labeled capillary end to take up the solution through capillary action (see Notes 5 and ⁶).
2. Use scotch tape to tape both ends of the capillary and place the capillary in a glass Petri-dish. Incubate for 1 h at 100°C.
3. Using the 1.0 mL syringe with the attached capillary flush, purge the capillary with air three times.
4. Repeat steps 1–3 four more times (see Note 7).
5. Add 500 nL of prepared Sulfo-SMCC solution to the silanized end of the capillary (see Note 8).
6. Use scotch tape to tape both ends of the capillary. Place the capillary in a glass Petri-dish. Incubate for 1 h at 30°C.
7. Using the 1.0 mL syringe with the attached capillary flush, flush the capillary three times with 0.2 mL of 50 mM Na₂B₄O₇, pH 7.6 from the non-silanized end (see Note 9).
8. Using the 1.0 mL syringe with the attached capillary flush, flush the capillary with air until all the 50 mM Na₂B₄O₇ is removed (see Note 10).

⁵Placing 500 nL on a parafilm sheet is often difficult and it is acceptable to add 600–700 nL to ensure the capillary takes up the 10% v/v 3-aminopropyltriethoxysilane.

⁶Only one end of the capillary will be anchored with antibodies. It is important to distinguish what end is silanized with 10% v/v 3-aminopropyltriethoxysilane. Mark this end with a permanent marker to distinguish the two ends.

⁷The first time 10% v/v 3-aminopropyltriethoxysilane is added, capillary action should occur in a few seconds. Each time after that, the addition of 10% v/v 3-aminopropyltriethoxysilane will take progressively longer for capillary action to occur. The final time may take up to 15 min for capillary action to occur.

⁸500 nL is only the minimum volume to be added and it is acceptable to place 600–700 nL on the parafilm sheet, to ensure the capillary takes up the solution.

⁹The capillary is being properly flushed if liquid comes out of the other end. If this does not happen, first check to see if there is a leak when using the syringe with the flush system. If there is no excessive leakage, the antibody-immobilized end might be blocked. If this happens, cut 0.5 cm off the end of the antibody-immobilized end of the capillary and try to flush again.

9. Seal ends using scotch tape, labeling the silanized end. Place the capillary in a Petri-dish or any other container, covering it with aluminum foil to protect from light, and store at room temperature.

3.2. Preparation of F(Ab')₂ Antibody Fragments

This method has been modified from a previously published technique (16).

1. Dissolve 50 µg of anti-TNF-α in 1 mL of 25 mM sodium acetate, pH 4.5. Warm the solution to 37°C, mixing at 400 rpm for 30 min (see Note 11).
2. Dissolve 100 µg of pepsin in 10 mL of 25 mM sodium Acetate, pH 4.5. Warm to 37°C.
3. Mix the anti-TNF-α and pepsin solutions, protecting the solution from light by mixing in black tubes. Incubate the mixture at 37°C with 400 rpm shaking for 24 h.
4. Spin tubes at 12,000 × g for 5 min.
5. Remove the supernatant from the pepsin pellet and place in a tube with 50 µL protein A magnetic beads and 200 µL of PBS.
6. Place on an overhead mixer for 30 min.
7. Place tubes on a magnetic holder to sediment the beads from the solution. Once the beads separate from the supernatant, remove the supernatant and filter through a 0.22 µm filter. Store solution at 4°C until antibody fragments are ready to be reduced.

3.3. Reduction of F(Ab')₂ Antibody Fragments to FAb Fragments

1. Mix 1 mL of the anti-TNF-α F(Ab')₂ fragments with 500 µL of prepared 10 mM EDTA in one vial containing 6 mg of 2-MEA.
2. Mix the solution and incubate the reaction mixture for 90 min at 37°C (see Note 12).

3.4. Addition of Reduced FAb Antibody Fragments Onto a Thiol-Derivatized Capillary

This method has been modified from a previously published technique (17).

1. Using the 1.0 mL syringe with the attached capillary flush, flush the capillary three times with 0.3 mL of 100 mM sodium phosphate, pH 7.0 through the non-labeled end.
2. Using the 1.0 mL syringe with the attached capillary flush, flush the capillary with air until all the 100 mM sodium phosphate is removed.

¹⁰When all the Na₂B₄O₇ is flushed out, air bubbles will be observed from the antibody-immobilized end of the capillary.

¹¹If more than one antibody is being used, the total antibody amount should be 50 µg (i.e., if two antibodies are used, 25 µg of the first antibody and 25 µg of the second antibody should be added to 1 mL of 25 mM sodium acetate, pH 4.5).

¹²The manufactured instructions indicate that 2-MEA and the reduced F(Ab')₂ fragments are to be separated using a desalting column. Experience has shown that the use of a desalting column leads to significant loss of the reduced antibody, and that the presence of salts does not hinder the formation of the sulfur-sulfur bond. Any excess salt will be washed away with sodium phosphate in an upcoming step.

3. Place 500 nL of reduced anti-TNF- α FAb fragment solution in 2-MEA on a parafilm sheet and allow uptake of liquid through capillary action (see Note 13).
4. Place the capillary in a Petri-dish, leaving the ends of the capillary uncovered, at 4°C for 24 h. To protect from light, wrap the glass Petri-dish in aluminum foil.
5. Using the 1.0 mL syringe with the attached capillary flush, flush the capillary three times with 0.3 mL of 100 mM sodium phosphate, pH 7.0 through non-labeled end.
6. Seal the ends of the capillary with scotch tape and store in a Petri-dish covered with aluminum foil to protect from light. Store at 4°C until use.

3.5. Immunoaffinity Capture and Separation of Cytokines

This method has been modified from a previously published technique (18)

1. Using the Window maker, burn a window into the antibody-coated capillary. The window length should be 45 cm from the inlet (see Note 14).
2. Mount the capillary into a CE instrument. Make sure the window is aligned with the detector port (see Notes 15 and ¹⁶). The antibody-immobilized end should be placed at the CE inlet and the other end should be placed at the outlet (see Note 17).
3. If the CE instrument has the capability of changing the capillary temperature, set this at 4°C.
4. Flush the capillary with the prepared run buffer at a pressure of 1,000 mbar for 5 min.
5. Inject labeled sample into the antibody-coated side of the capillary at 50 mbar for 5 s.
6. Wait 10 min without applying any pressure or current to allow analyte capture.
7. Flush the capillary with the prepared run buffer at a pressure of 1,000 mbar for 5 min to wash out any unbound protein.
8. Inject prepared AlexaFluor 633 solution at 50 mbar for 5 s.
9. Wait for 10 min without applying any pressure or current to allow labeling.
10. Flush the capillary with the prepared run buffer at a pressure of 1,000 mbar for 5 min to wash any excess dye.

¹³500 nL is only the minimum volume to be added. It is acceptable to add 600–700 nL to ensure the capillary takes up the solution.

¹⁴The length from the beginning of the capillary to the window may be increased to allow for a greater separation of analytes. This may be helpful if multiple cytokines are being analyzed that are close in size.

¹⁵If you do not have access to the Window Maker, it is possible to create one with a butane lighter. With a permanent marker, mark 1.0 cm at the desired window location on the capillary. Burn the marked location with a lighter for 5 s. Use methanol to remove the charred coating to reveal transparent silica.

¹⁶Once the window is created, it is extremely fragile and is prone to breaking if any stress is placed on the capillary. Avoid putting any stress on this location. Cover the capillary with PEEK tubing when storing the capillary to decrease any tension on the capillary.

¹⁷The first time the capillary is used, it may be necessary to flush the capillary with the run buffer at 1,000 mbar for 20 min to set the baseline. After every ten samples are run and before the capillary is being stored, a buffer flush should be performed by flushing using the running buffer at 1,000 mbar for 20 min. Perform this same procedure prior to storing the capillary.

11. Run 100- μ A constant current using the prepared elution buffer for 30–40 min at room temperature or until the single peak is detected on an electropherogram (see Notes 18 and ¹⁹). A sample electropherogram from an unknown sample of TNF- α is presented in Fig. 5 (see Note 20).
12. Once all samples runs are complete, store the capillary at 4°C in a glass Petri-dish. Tape the ends with scotch tape, marking the labeled end. Cover dish in aluminum foil to protect from light.

3.6. Quantitation of Cytokine Concentration in an Unknown Sample Using a Standard Curve

In order to properly quantify the concentration of a targeted cytokine in an unknown sample, a standard curve must be constructed. This section illustrates how to create a standard curve and use it to quantify the cytokine concentration in an unknown sample.

1. Create a dilution series of the reconstituted recombinant TNF- α in PBS. Note the concentration of the recombinant TNF- α in each dilution. Dilutions of TNF- α can range in concentrations from 1 pg/mL to 10 μ g/mL. A minimum of five dilutions should be made to create an effective standard curve.
2. Run the ICE protocol as indicated in Subheading 3.5, steps 4–11 (see Note 21). Perform the ICE protocol to collect electropherograms for the prepared dilutions of TNF- α .
3. Determine the area under the electropherogram peak for each of the TNF- α standards.
4. Create a plot of the area under an electropherogram peak vs. its corresponding TNF- α concentration in the standard TNF- α solution. An example of this plot may be observed in Fig. 6. Determine the slope of the standard curve using $y=mx+b$, where m is the slope of the curve, x is the concentration of the known sample, y is the corresponding area under the curve for the sample, and b is the y-intercept.
5. To calculate the concentration of TNF- α in the unknown sample, determine the area under the electropherogram peak obtained when running ICE on the unknown sample. Using the slope and y-intercept of the standard curve and the area under the TNF- α unknown sample electropherogram peak, calculate the concentration of TNF- α in the unknown sample. Make sure the peak area of the electropherogram for the cytokine captured in the unknown sample falls on the standard curve. Depending on the concentration of cytokine in the unknown sample, it may be necessary to adjust the dilution series of the standards.

¹⁸If the observed peaks range more than 3–5 min, an internal pressure of 5 mbar may be applied to create a tighter peak. This pressure is applied when the voltage is applied.

¹⁹Depending on the time the analyte signal peak comes off on the electropherogram, the time of the elution may be adjusted accordingly.

²⁰Figure 5 presents an electropherogram where only TNF- α was selected for using ICE. If multiple antibodies are used to capture multiple cytokines, each peak will reflect one cytokine. A sample electropherogram from a previously published paper performing ICE on multiple captured cytokines is presented in Fig. 7 (19).

²¹All conditions must be identical when running ICE on both standard and unknown samples.

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Fig. 1. Construction of the 1 mL syringe flush system to flush a derivatized capillary. **(a)** Individual components used to construct the capillary flush system from **left to right**: 1 mL syringe, CE flushing unit P-662 (Upchurch Scientific, Oak Harbor, WA, USA) part that connects to the syringe, female tube slip unit P-686 (Upchurch Scientific, Oak Harbor, WA, USA) attached to part F-185 in the Micro-tight PEEK Sleeve Kit (Upchurch Scientific) and the end of the CE flushing unit P-662. **(b)** One milliliter syringe with capillary flush attached. First, the syringe is filled with the desired flushing solution and attached to the flushing system. The capillary is then placed into the PEEK tubing.

Fig. 2. Instrumentation used for ICE. **(a)** Setup of the Agilent Technologies 7100 Capillary Electrophoresis (**left**) (Agilent Technologies, Santa Clara, CA, USA) coupled to the laser-induced fluorescence (LIF) detector (**right**) (Picometrics, Cambridge, MA, USA). **(b)** The interior of the CE shows the buffer and sample vial tray, vial lifts, and electrodes. Capillary ends are placed inside the electrodes (indicated with **arrows**) to allow for a single puncture into the inlet and outlet vials. **(c)** The LIF detector is attached to the CE through a detection cell (indicated with an **arrow**) attached to a cassette that holds the capillary, aligned with the window of the capillary.

Fig. 3.

Diagram of the preparation of an antibody-coated immunoaffinity capillary. **(a)** Pepsin digests an antibody to produce an $F(ab')_2$ fragment and an Fc' fragment. **(b)** $F(ab')_2$ fragments undergo reduction by 2-mercaptoethylamine HCl (2-MEA) in 10 mM EDTA to produce 2 Fab fragments. **(c)** Fab fragments bind to a thiol-derivatized capillary by forming S-S bonds.

Fig. 4.

Diagram of ICE. **(a)** An unknown sample with target cytokines is injected into an immunoaffinity capillary, followed by incubation time to allow for the Fab fragments to bind its targeted analyte. **(b)** Running buffer is used to flush the capillary, removing all other analytes found in the clinical sample that does not bind to the bound Fab fragments. **(c)** A fluorescent marker, Alexa Fluor 633, is injected into the immunoaffinity capillary and is allowed to bind to the captured analytes. **(d)** An elution buffer is injected and a voltage is applied to elute the analytes from the Fab fragments. The analytes elute as concentrated sample plugs and are detected by the LIF detector.

Fig. 5.

An ICE electropherogram for m-TNF- α from an unknown sample. The peak area between the **dotted lines** indicates the area that is integrated to find the area under the curve. This information is used to plot against a standard curve to determine the TNF- α concentration. Peak 1 = TNF- α ; peak 2 = free Alexa Fluor 633 dye.

Fig. 6. Typical ICE electropherogram of recombinant cytokines recovered by microdialysis from cell-free micro-droplets. Peak 1 = IL-2; peak 2 = IL-4; peak 3 = IL-10; peak 4 = IL-12; and peak 5 = IL-5 (reproduced from (19) with permission from Elsevier).

Fig. 7.

A sample standard curve used to determine TNF- α concentrations in unknown samples.

(Filled square) Known concentrations of TNF α were used to construct the standard curve.

(Filled circle) Unknown TNF α concentration measured in two unknown samples and plotted on the standard curve.