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Adriann Hovey
University of Northern Iowa

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PRELIMINARY DEVELOPMENT OF QUANTITATIVE IMMUNOASSAYS FOR MITOCHONDRIAL PROTEINS

A Thesis

Submitted

in Partial Fulfillment

of the Requirements for the Designation

University Honors with Distinction

Adriann Hovey

University of Northern Iowa

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Entitled: Preliminary Development of Quantitative Immunoassays for Mitochondrial Proteins

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INTRODUCTION

Mitochondria are medium-sized organelles in which most cellular metabolic reactions are carried out. In postmitotic cells that are aged or diseased, such as myocardial, skeletal, and brain cells, abnormally large mitochondria ("giant mitochondria") are often observed. Mitochondrial size and shape are controlled by mitochondrial fusion and fission and disruption of this process may lead to their abnormally large size (Nauratil, Terman, Arriaga, 2008). These giant mitochondria exhibit variations from normal metabolism and altered membrane potentials (Nauratil *et al.*, 2008). An increased number of giant mitochondria may therefore disrupt cell function (Nauratil *et al.*, 2008). This accumulation may result either from the failure of macroautophagy to remove damaged organelles or from impaired fusion with normal mitochondria, which would allow repair by exchange of matrix contents. Since proteins such as mitofusin-1, mitofusin-2, and OPA1 participate in fusion, their under- or overexpression could lead to the development of giant mitochondria (Nauratil *et al.*, 2008). Measurement of the levels of these proteins in normal and giant mitochondria could provide insight into the development of giant mitochondria and their inability to fuse. This information could also be useful in understanding the aging of cells, in which giant mitochondria could play a prominent role.

Immunoassays have been used widely for detection of specific analytes in samples. These methods rely on the fact that an antibody binds to its antigen with high specificity to form an immune-complex (Manz, Pamme, Iossifidis, 2004). The equilibrium constant for the formation of the immuno-complex defines the affinity of an antibody for its antigen (Manz *et al.*, 2004). The equilibrium constants for antibodies used in immunoassays are typically greater than 10^8 L mol^{-1} (Manz *et al.*, 2004). This strong affinity of an antibody for its antigen is important to obtain accurate quantitation of the analyte. Most often monoclonal antibodies, which can be produced in cell cultures, are used in immunoassays, because they only bind to one unique epitope on the antigen (Manz *et al.*, 2004). In contrast, polyclonal antibodies can bind to many epitopes on an antigen, some of which may not be unique to that antigen. Monoclonal antibodies therefore increase the specificity of an immunoassay (Manz *et al.*, 2004). In order to use an immunoassay quantitatively, the antigen or antibody must be labeled (Manz *et al.*, 2004). In this research, a fluorescently labeled antibody was used. A primary antibody, which binds directly to

the antigen, can be labeled with a fluorescent tag; alternatively, a fluorescently tagged secondary antibody can be used to bind to some site on the primary antibody. This quantitative approach with immunoassays can give high specificity and low detection limits.

Capillary electrophoresis (CE) is a relatively new and powerful technique. Electrophoresis is the separation of analytes by the differences in their mobilities in the presence of an electric field (Manz *et al.*, 2004). The mobility depends on a charge to mass ratio of the analyte (Manz *et al.*, 2004). This technique therefore separates molecules based primarily on size difference. When CE is coupled with an immunoassay (IA), separation can be obtained with high specificity. This technique allows a separation of unbound fluorescently labeled antibody from an antibody-antigen complex, which has a molecular weight greater than that of the antibody alone. The overall charge of the complex may also be different, but the change in molecular weight is more significant. This change in mobility creates a new peak in the electropherogram corresponding to the fluorescently labeled immuno-complex. Total mobility is the mobility of the analyte with the electro-osmotic flow (EOF). Net mobility is only the mobility of the analyte corrected for the mobility of the EOF. Unless otherwise noted, calculated mobilities in this project are total mobilities. Known concentrations of the analyte and antibody can be analyzed by CE and used to quantitate the amount of analyte in a sample. CE also allows for quick separation time with high resolution (H. Zhang & Jin, 2006). It requires only a very small sample size and can be used to analyze a single cell (H. Zhang & Jin, 2006, Xiao, Li, Zou, L. Yang, Y. Yang, Y. Wang, *et al.* 2006). Laser-induced fluorescence (LIF) detection can be used to analyze fluorescently labeled samples with high sensitivity and low detection limits (Zhao, Xing, X. Wang, Q. Wang, & Z. Wang, 2007). Therefore, CEIA-LIF has the capability of being a very specific and sensitive separation technique.

Even though CEIA-LIF can be a powerful technique, one problem that can arise is the interaction of proteins with the capillary walls. Capillaries are made of fused silica that contains many negatively charged silanol groups on the walls of the capillary. Proteins are large biomolecules that contain many different charges. The positive charges on proteins can interact with the negative charges on the capillary wall, which causes broad peaks and irreproducible results. Capillary coatings can be used to minimize this effect. They work by reducing the electro-osmotic flow (EOF) and changing the physical chemistry of the capillary walls. Their

main purpose is to decrease interactions of the sample with the capillary walls to obtain better separation and more reproducible results. The use of capillary coatings helps to maintain the desirable high resolution of capillary electrophoresis.

Some previous experiments have been done using an immunoassay coupled with capillary electrophoresis separation techniques. H. Zhang and Jin determined the amount of human interferon-gamma (IFN-γ) on natural killer cells (2006). They used anti-IFN-γ monoclonal antibody labeled with fluorescein isothiocyanate (FITC) and introduced it into natural killer cells via electroporation (H. Zhang & Jin, 2006). Using this antibody and capillary electrophoresis with laser-induced fluorescence detection, Zhang and Jin quantitated two different forms of IFN-γ in a single cell, lysed on the capillary, with a limit of detection of a zeptomole (2006). Xiao *et al.* successfully used mouse-raised JSB-1 antibody as a primary antibody to bind to P-glycoprotein, and goat anti-mouse IgG-FITC (GAMIF) as a secondary antibody to fluorescently label the complex (2006). Xiao *et al.* obtained a detection limit for the GAMIF and JSB-1 complex of 40 zmol (2006). They were then able to quantitate the amount of P-glycoprotein on a single intact cell using CEIA-LIF (Xiao *et al.*, 2006). Su, X Zhang and Chang used a competitive immunoassay in which a known concentration of fluorescently labeled antigen competed with an unknown concentration of estrone (E_1) for anti-E_1 monoclonal antibody (2003). Through CEIA-LIF, they determined the concentration of estrone in women's serum at concentrations as low as 19.6 pg/ml (Su *et al.,* 2003). The experiments described show the ability of CEIA-LIF to separate and quantitate analytes at very low concentrations with high specificity.

This project studied Anti-OxPhos Complex IV Subunit 1, mouse IgG_{2A}, monoclonal 1D6, Alexa Fluor 488 conjugate ("anti-OxPhos"), an antibody against cytochrome oxidase subunit 1 (COX1). COX1 is a mitochondrial inner membrane protein involved in the oxidative phosphorylation pathway and is also known as OxPhos for this reason. This protein was used to develop a standard method for quantitation of mitochondrial proteins using CEIA-LIF. Mitochondria were isolated from rat liver and lysed by sonication in a buffer containing surfactants. The anti-OxPhos required no secondary antibody, because it was already fluorescently labeled with Alexa Fluor 488. The anti-OxPhos was incubated at room temperature with the mitochondrial protein sample and analyzed by CEIA-LIF for a change in

peak profile. The controls for the CEIA-LIF technique were: mitochondrial protein sample alone, anti-OxPhos alone, and anti-OxPhos spiked with fluorescein. Semi-permanent capillary coatings were also tested for their capacity to improve the peak profile of anti-OxPhos, but were unsuccessful. Analysis of incubation mixtures by CEIA-LIF suggested the presence of COX1 in the mitochondrial protein sample.

EXPERIMENTAL

Reagents

Anti-OxPhos Complex IV Subunit I, mouse IgG_{2a}, monoclonal 1D6, Alexa Fluor 488 conjugate (1 mg/ml) and Alexa Fluor 488 rabbit anti-goat IgG (H + L) (2 mg/ml) were purchased from Molecular Probes, Inc (Eugene, OR). Tris, NaCl, and $CaCl_2$ were purchased from Fisher Scientific (Fair Lawn, NJ). Triton X-100, boric acid, SDS, Fluorescent Molecular Weight Marker (M.W. 20,100-205,000), Bradford reagent, and HEPES were purchased from Sigma (St. Louis, MO). Anhydrous methyl alcohol, hydrochloric acid, and potassium hydroxide were purchased from Mallinckrodt Chemicals (Phillipsburg, NJ). D-mannitol was purchased from Sigma Aldrich (Steinheim, Germany). Ultrapure sucrose was purchased from MP Biomedicals (Aurora, OH). EDTA was purchased from Acros Organics (Morris Plains, NJ). Bovine serum albumin, Fraction V was purchased from Calbiochem (La Jolla, CA). UltraTrol™ LN was purchased from Target Discovery (Palo Alto, CA). DSPC (1,2-Distearoyl-sn-glycero-3-phosphocholine dihydrate) was purchased from Fluka brand of Sigma (St. Louis, MO).

Apparatus

A Beckman Coulter P/ACE™ MDQ capillary electrophoresis instrument with a LIF detector was used to analyze samples. Laser excitation was at 488 nm and fluorescence was

detected at 635 nm and 520 nm. The capillary and samples were kept at 25°C during separation. Mitochondria were separated in the eppendorf 5720 R and 5415 R centrifuges.

Capillary Preparation

A 365 μm outer diameter and 50 μm inner diameter capillary that was approximately 30 cm in length was used for all experiments. The capillary was conditioned for first time use, if the capillary had dried out, or if the sample had strongly adhered to the capillary walls. The method for conditioning the capillary for use with no capillary coating can be seen in Table 1. Table 2 shows the method for capillary conditioning for UltraTrol™ use, obtained from Target Discovery (2004). The method for conditioning the capillary before 1,2-Distearoyl-sn-glycero-phosphocholine (DSPC) coating can be seen in Table 3 (Gulcev & Lucy, 2008). All running buffers used for capillary electrophoresis were filtered with a 0.2 μm pore size filter.

Solution	**Conditions**	**Time**
0.1 M HCl	Rinse, 20 psi	10 min
H_20	Rinse, 20 psi	10 min
0.1 M KOH	Rinse, 20 psi	10 min
Buffer	Rinse, 20 psi	15 min
Buffer	Separate, +12 kV	1 min

Table 1 – Conditioning for uncoated capillary: This method was used for conditioning the capillary when no coating would be used. Conditioning was carried out on new capillaries, dried out capillaries, or capillaries with sample strongly adhered to the walls. Running buffer was either 10 mM Tris, 10 mM Boric Acid, pH 7.4 or 10 mM Tris-HCl pH 7.4.

Solution	**Conditions**	**Time**
Methanol	Rinse, 20 psi	5 min
H_20	Rinse, 20 psi	2 min
1 M HCl	Rinse, 20 psi	5 min
H_20	Rinse, 20 psi	2 min
1 M KOH	Rinse, 20 psi	10 min
H_20	Rinse, 20 psi	2 min

Table 2 – Conditioning for UltraTrol™ LN: The UltraTrol™ LN method for conditioning the capillary before coating was obtained from Target Discovery. Conditioning was carried out on new capillaries, dried out capillaries, or capillaries with sample strongly adhered to the walls. Running buffer was either 10 mM Tris, 10 mM Boric Acid, pH 7.4 or 10 mM Tris-HCl pH 7.4.

Solution	Conditions	Time
0.1 M KOH	Rinse, 20 psi	10 min
H_20	Rinse, 20 psi	5 min

Table 3 – Conditioning for DSPC: This method was used for conditioning the capillary before coating with DSPC. New capillaries were conditioned following this method if a DSPC coating would be used. Running buffer was 20 mM Tris-HCl pH 7.4.

The limit of detection (LOD) is the lowest amount of sample that can be distinguished from the background noise of the detector. Fluorescein samples were prepared from a 1 mM solution of fluorescein in methanol. Sample concentrations were 1 x 10^{-9} M, 5 x 10^{-10} M, and 5 x 10^{-11} M diluted in 10 mM Tris, 10 mM Boric Acid, pH 7.4 (TB buffer). A 100 µl volume was used for CE analysis by the Beckman Coulter P/ACE™ MDQ Instrument. The capillary was conditioned according to the procedure for conditioning an uncoated capillary (Table 1). The samples were injected at 0.5 psi for 3 seconds (sec) and separated at +12 kV for 10 minutes (min). A calibration curve of peak height versus fluorescein concentration was created and a linear series fit to the data points. This linear equation was used to find the LOD using three times the standard deviation of the background noise. Some error is associated with finding the LOD. This error can result from the background noise level varying from day to day. Also, different buffer systems and coatings can cause different patterns and levels in background noise. There is also some inherent error in fitting a linear regression to data points. The standard error for the linear regression calculated here was 0.015494.

UltraTrol™ Coating

This procedure and the UltraTrol™ LN capillary coating were obtained from Target Discovery (2004). The capillary was conditioned according to Table 2 in Capillary Preparation. Generally the capillary was recoated with UltraTrol™ LN between runs. However, when many sample injections of the same sample were being performed, the capillary was not recoated or rinsed between injections. For these experiments, separation time needed to be long enough to

expel the sample from the capillary. The general procedure for capillary coating and sample separation is in Table 4 (Target Discovery, 2004). The capillary coating time was extended from 2 min suggested by Target Discovery to 6 min to ensure a stable coating was achieved. If the capillary would not be used promptly or would be kept overnight, end rinses of 1 M KOH for 2 min at 20 psi and then water for 2 min at 20 psi were carried out. The buffer used for separation and sample dilutions was 10 mM Tris, 10 mM Boric Acid, pH 7.4 or 10 mM Tris-HCl pH 7.4.

Solution	Conditions	Time
1 M KOH	Rinse, 20 psi	2 min
H_20	Rinse, 20 psi	2 min
UltraTrol™	Rinse, 20 psi	6 min (time extended)
Buffer	Rinse, 20 psi	2 min
Sample	Inject, 0.3 psi	3 sec
Buffer Inlet and Outlet	Separate, -12 kV	10-25 min (variable)

Table 4 - UltaTrol™ LN coating and separation: This general procedure was used for coating the capillary after conditioning, and for typical CE runs. Consecutive sample injections of the same sample did not include a buffer rinse or recoating of the capillary. Running buffer was either 10 mM Tris, 10 mM Boric Acid, pH 7.4 or 10 mM Tris-HCl pH 7.4.

DSPC Coating

The DSPC coating (0.1 mM DSPC, 20 mM $CaCl_2$, 20 mM Tris-HCl pH 7.4) was prepared by adding $CaCl_2$ to the running buffer (20 mM Tris-HCl pH 7.4) and then adding DSPC (Cunliffe, Baryla, & Lucy, 2002). The solution was sonicated for 10 min and then stirred for 10 min a total of three times to create small unilamellar vesicles (Cunliffe *et al.*, 2002). The coating was stored at 4°C for no longer than 6 days as suggested by Cunliffe *et al.* (2002). The DSPC coating procedure is provided in Table 5 (Gulcev & Lucy, 2008). This procedure is for a newly conditioned capillary according to Table 3. This procedure was also repeated for every injection, meaning the capillary was recoated between runs. EOF direction and magnitude were determined for the DSPC coating by spiking the buffer outlet to a final concentration of 1×10^{-8} M fluorescein and using both polarities for separation with no sample injection. EOF was

calculated from the equation $\mu_e = v/E$, where μ_e is the EOF mobility, v is velocity of the EOF and E is the electric field applied.

Solution	Conditions	Time
DSPC coating	Rinse, 20 psi	5 min
Buffer	Rinse, 20 psi	1 min
Sample	Inject, 0.3 psi	3 sec
Buffer Inlet and Outlet	Separate, -12 kV	10-30 min (variable)

Table 5 – DSPC coating and separation: This procedure was used for coating and recoating the capillary before every injection of sample. Running buffer was 20 mM Tris-HCl, pH 7.4.

Mitochondria Isolation

A frozen rat liver sample from an aged rat was thawed in a 37°C water bath and 1.5 g of the thawed tissue was transferred to a glass pestle for homogenization. Further steps were all carried out at room temperature. A total of 5 ml Buffer M (210 mM mannitol, 70 mM sucrose, 5 mM HEPES, 5 mM EDTA, pH 7.4) was added to the tissue during homogenization. The tissue was homogenized until a smooth consistency was obtained. Then the homogenate was centrifuged at 600 x g for 10 min. The supernatant was separated into 1 ml portions and each aliquot was centrifuged at 10,000 x g for 10 min. The supernatants were removed and each pellet was suspended in 500 µl Buffer M. The suspensions were centrifuged again at 10,000 x g for 10 min and the supernatants removed. Each pellet was then resuspended in 500 µl Buffer M and stored at 4 °C.

Mitochondrial Protein Extraction

The mitochondrial suspension was centrifuged at 10,000 x g and room temp for 10 min. The supernatant was removed and 500 µl of RIPA buffer void of sodium deoxycholate (150 mM NaCl, 1.0% Triton X-100, 0.1% SDS, 50 mM Tris, pH 8.0) was used to resuspend the pellet and lyse the mitochondria (Abcam Inc., 1998-2008). The suspension was then disrupted through sonication at room temp for three 30 sec intervals spaced 15 sec apart at room temp. Total

protein was quantitated via the Bradford Assay and found to be 5130 μg/ml (Bradford, 1976). Protein solutions were stored at 4°C.

Anti-OxPhos Incubation and CE Analysis

Anti-OxPhos fluorescent antibody binds to cytochrome oxidase subunit 1 (COX1). Assuming 5% of the total protein from mitochondrial protein isolation is COX1, samples were diluted to approximately 2.5 μg COX1/ml and 5 μg COX1/ml. Anti-OxPhos was diluted to 5 μg/ml and was mixed with an equal volume of 2.5 μg/ml or 5 μg/ml COX1 estimated concentrations. Anti-OxPhos diluted to 2.5 μg/ml was also mixed with an equal volume of estimated 5 μg/ml COX1. Incubation time varied from 30 min to 2 hours. Then the incubation sample was spiked to a final concentration of 5×10^{-9} M fluorescein. Samples of anti-OxPhos concentrations used in incubations were spiked with fluorescein to a final concentration of 5×10^{-9} M and analyzed by CEIA-LIF as controls. Then separate samples of the diluted COX1 concentrations were each analyzed by CEIA-LIF as controls. The incubation mixtures spiked with fluorescein were then analyzed by CEIA-LIF. The capillary was conditioned according to Table 1. No capillary coating was used and only a 5 min buffer rinse was done before samples were injected at 0.3 psi for 3 sec. Separation was done at +12 kV for 15 min. For CEIA-LIF of the 1.25 μg/ml anti-OxPhos and ~2.5 μg/ml COX1 incubation and controls, a 3 min hydroxide rinse was done at the beginning of each run followed by a 6 min buffer rinse before injection to ensure proteins bound to the capillary walls were removed. Injection and separation conditions remained the same. The buffer used to dilute samples and as a running buffer for CE was 10 mM Tris-HCl pH 7.4.

RESULTS

No Capillary Coating

The limit of detection of the Beckman Coulter P/ACE® MDQ Instrument on fluorescein was found to be 151 zmol from the calibration curve of peak height vs. fluorescein concentration using 3σ (Figure 1). Then CEIA-LIF of anti-OxPhos alone (1 μg/ml) with no capillary coating was tested (Figure 2). This antibody peak had $w_{1/2}$ = 16.125 sec and some tailing. There was also the presence of a low intensity peak after the antibody peak which was observed in multiple electropherograms of anti-OxPhos. The mobility of anti-OxPhos was 4.87 x 10^{-4} $cm^2/V \cdot s$ and only 227 theoretical plates were obtained.

UltraTrol™ Coating

UltraTrol™ coating was chosen to improve peak width and reduce tailing. The electropherogram of fluorescein (1 x 10^{-9} M) with UltraTrol™ had $w_{1/2}$ = 1.5 sec and 77,100 theoretical plates (Figure 3). Mobility of fluorescein was -2.82 x 10^{-4} $cm^2/V \cdot s$. Migration time and peak intensity of fluorescein were fairly reproducible from day to day. UltraTrol™ coating was also tested on Anti-OxPhos (1 μg/ml) through CE-LIF (Figure 4). The anti-OxPhos peak had $w_{1/2}$ = 280.75 s, an electrophoretic mobility of -1.16 x 10^{-4} $cm^2/V \cdot s$, and only 13 theoretical plates. While using the same capillary, peak profile, intensity and migration time were not very reproducible and varied greatly from day to day.

Fluorescein LOD Experiment

0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0

Peak Height (A.U.)

0.00E+00 2.00E-10 4.00E-10 6.00E-10 8.00E-10 1.00E-09 1.20E-09

Fluorescein Concentration (mol/L)

Series1
Linear (Series1)
y = 32,550,922.50922x + 0.00108
R^2 = 0.99678
3σ = 0.000828

Figure 1 – LOD of fluorescein: Fluorescein concentrations analyzed by CE-LIF were 1 x 10^{-9} M, 5 x 10^{-10} M, and 5 x 10^{-11} M. Samples were injected at 0.3 psi for 3 sec and separated at +12 kV for 10 min with 10 mM Tris, 10 mM Boric Acid, pH 7.4 running buffer. A linear regression was fit to the data points and 3σ used to calculate the LOD from the linear equation.

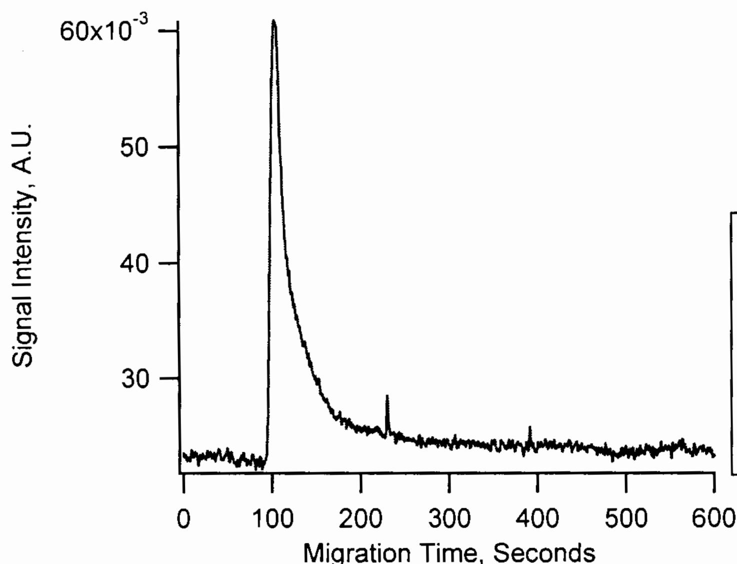

Figure 2 – Anti-OxPhos with no capillary coating: Anti-OxPhos (1 µg/ml) was injected at 0.3 psi for 3 sec and separated at +12 kV for 10 min with 10 mM Tris, 10 mM Boric Acid, pH 7.4 running buffer. No capillary coating was used. $w_{1/2}$ = 16.5 sec, μ_e = 4.87 x 10^{-4} $cm^2/V{\cdot}s$ and N = 215. (File AH061708-01)

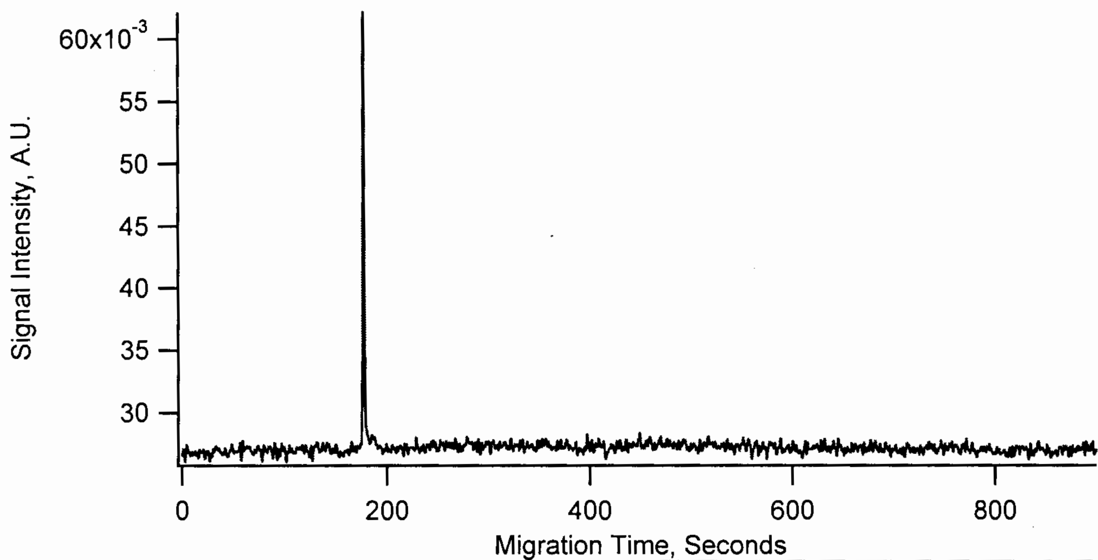

Figure 3 – Fluorescein with UltraTrol™ LN: Fluorescein (1 x 10^{-9} M) was injected at 0.3 psi for 3 sec and separated at -12 kV for 15 min with 10 mM Tris, 10 mM Boric Acid, pH 7.4 running buffer in an UltraTrol™ LN coated capillary. $w_{1/2}$ = 1.5 sec, N = 77,100, and μ_e = -2.82 x 10^{-4} $cm^2/V{\cdot}s$. (File AH063008-02)

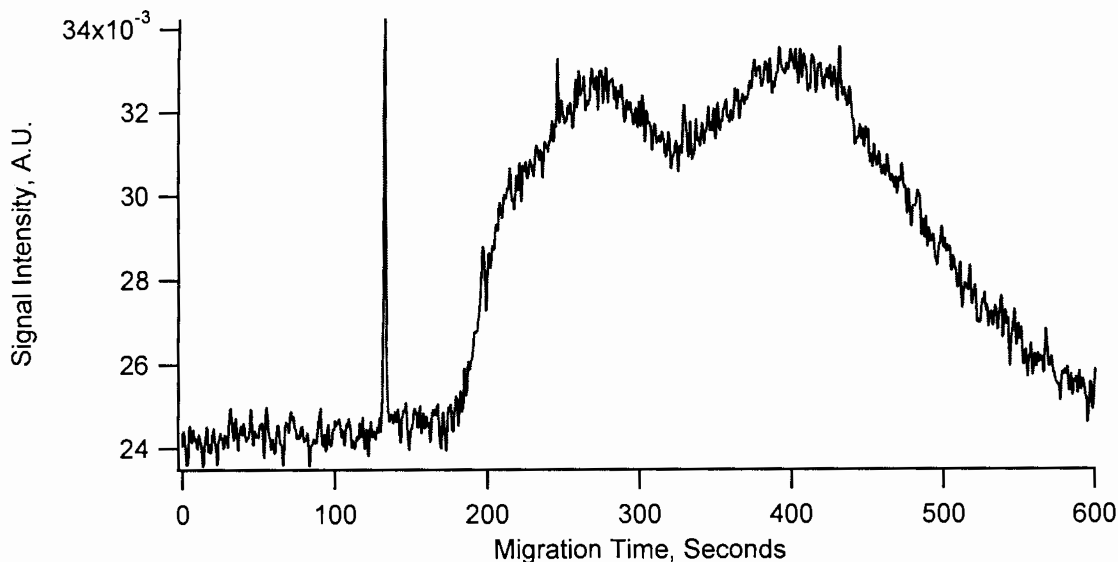

Figure 4 – Anti-OxPhos with UltraTrol™ LN and TB running buffer: Anti-OxPhos (1 µg/ml) was injected at 0.3 psi for 3 sec and separated at -12 kV for 10 min with 10 mM Tris, 10 mM Boric Acid, pH 7.4 running buffer in an UltraTrol™ LN coated capillary. Migration time was considered the time at which peak intensity was the greatest for the anti-OxPhos peak (not considering first, sharp peak). $w_{1/2}$ = 280.75 sec, N = 13, μ_e = -1.16 x 10^{-4} $cm^2/V{\cdot}s$. (File AH063008-05)

For the previously described separations, 10 mM Tris, 10 mM Boric Acid, pH 7.4 was used to dilute samples and as a running buffer. However, borates could bind to the sugars on anti-OxPhos and cause changes in migration time and peak broadening due to differences in molecular weight. UltraTrol™ LN coating was tested on anti-OxPhos (5 μg/ml) with a running buffer of 10 mM Tris-HCl pH 7.4 (T10 buffer) (Figure 5). Peak profile and width did not improve with $w_{1/2}$ = 361.25 sec and only 10 theoretical plates were achieved. Electrophoretic mobility of anti-OxPhos was -1.06 x 10^{-4} $cm^2/V \cdot s$.

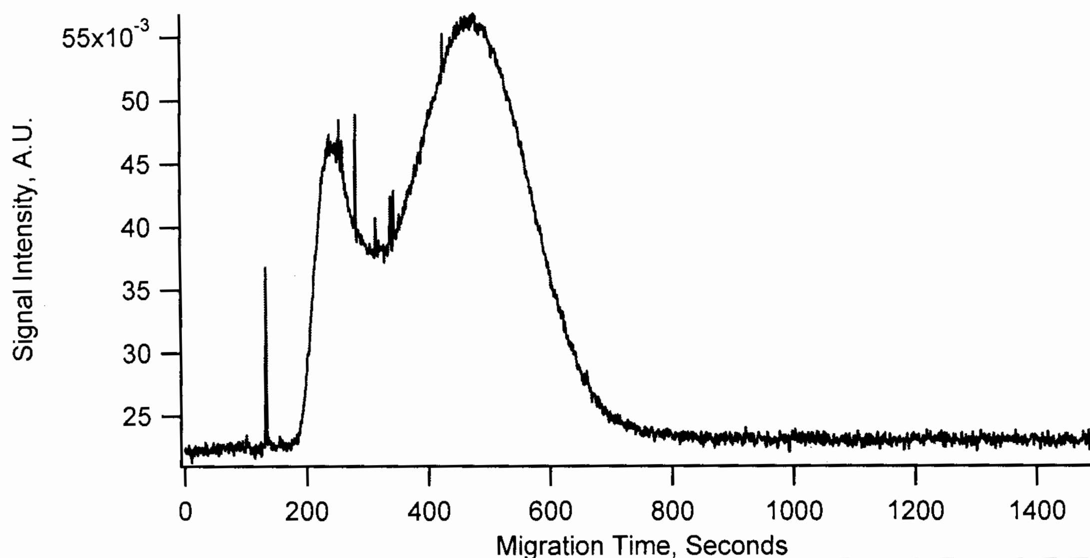

Figure 5 – Anti-OxPhos with UltraTrol™ LN and T10 running buffer: Anti-OxPhos (5 μg/ml) was injected at 0.3 psi for 3 sec. Separation was at -12 kV and 25 min with 10 mM Tris-HCl pH 7.4 running buffer in an UltraTrol™ LN coated capillary. $w_{1/2}$ = 361.25 s sec, N = 10, μ_e = -1.06 x 10^{-4} $cm^2/V \cdot s$. Migration time was the time at which the highest peak intensity was achieved. (File AH070908-05)

Another difficulty with the UltraTrol™ LN coating was the spike in current at the beginning of most runs. The current pattern was not always consistent from day to day. A series of tests were done to see if current could remain constant throughout a run (Figure 6). Run AH063008-02 was a standard run with the UltraTrol™ LN coating procedure on 1 x 10^{-9} M fluorescein sample. In run -03, a separation at -12 kV for 2 min was added just before sample injection of fluorescein (1 x 10^{-9} M). In run -04, the buffer rinse time was increased from 2 min to 6 min and 1 x 10^{-9} M fluorescein was injected. Runs -05 and -06 were the standard UltraTrol™ LN procedure done on a 1 μg/ml anti-OxPhos sample. Lastly, run AH063008-test

had the standard UltraTrol™ LN procedure, but no sample was injected. Current did not remain constant during any run, but the pattern was somewhat consistent for all runs.

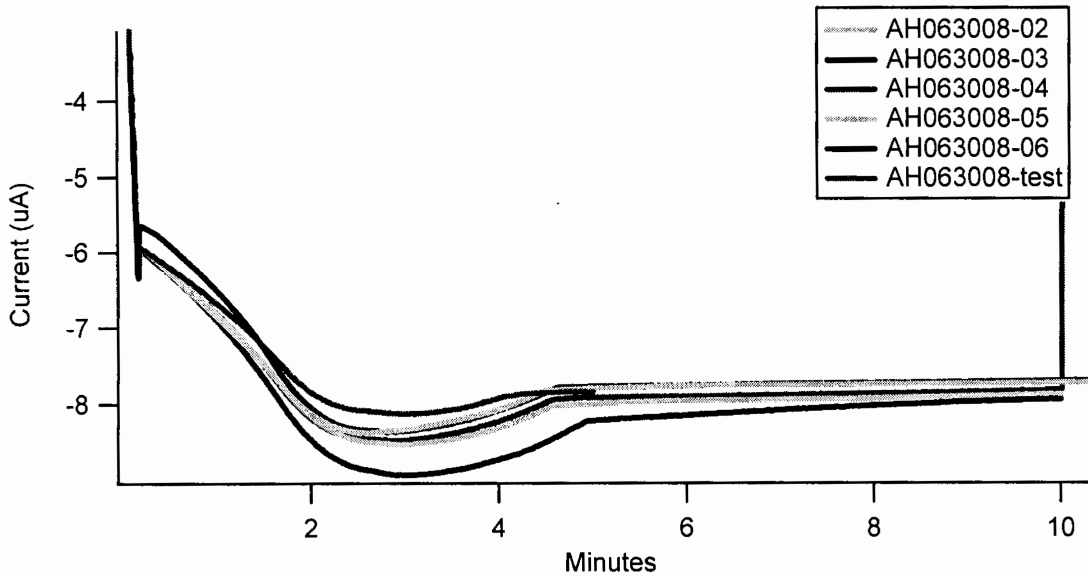

Figure 6 – Current tests with UltraTrol™ LN: UltraTrol™ LN coating time remained at 6 min for all runs. Samples were all injected at 0.3 psi for 3 sec. Runs 02 through 04 were on 1 x 10^{-9} M fluorescein while runs 05 and 06 were on 1 μg/ml anti-OxPhos. 02, 05 and 06 followed the standard procedure for UltraTrol™ LN runs; a separation at -12 kV for 2 min was added before sample injection in 03; buffer rinse was increased to 6 min in 04; and the test run was the standard procedure with no sample injection. (File AH063008-current)

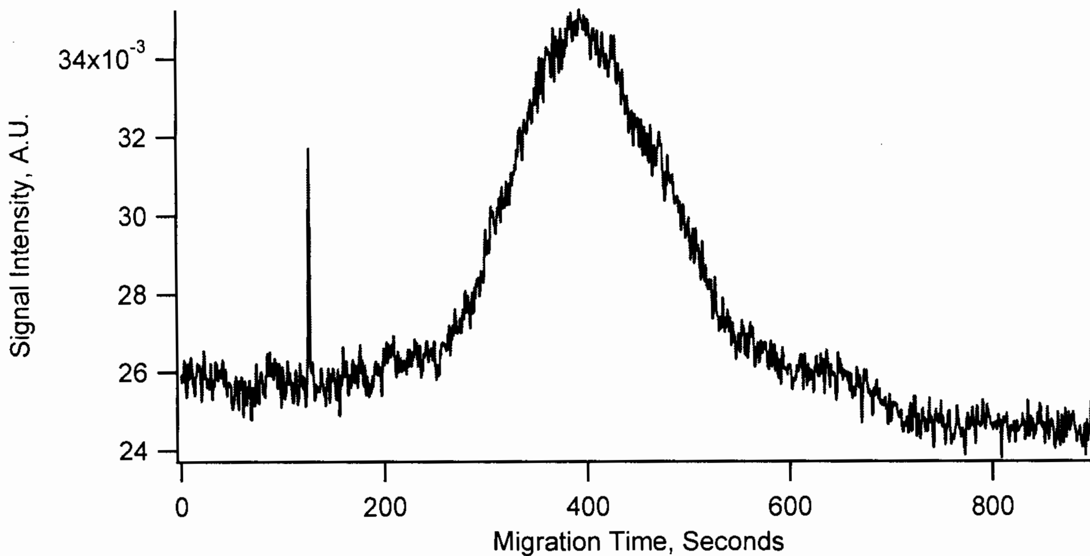

Figure 7 – Consecutive run of anti-OxPhos with UltraTrol™ LN: Anti-OxPhos (1 μg/ml) was injected at 0.3 psi for 3 sec and separated at -12 kV for 15 min with 10 mM Tris, 10 mM Boric Acid, pH 7.4 running buffer in an UltraTrol™ LN coated capillary. $w_{1/2}$ = 165.5 sec, N = 31, μ_e = -1.27 x 10^{-4} $cm^2/V \cdot s$. (File AH070208-02)

The stability of the UltraTrol™ LN coating was tested by doing a series of eight sample injections of 1 μg/ml anti-OxPhos with no rinses or recoating between runs. One electropherogram from this series can be seen in Figure 7. Peak width had decreased some with $w_{1/2}$ = 165.5 sec, but was still much broader than the anti-OxPhos peak with no capillary coating. Electrophoretic mobility was -1.27 x 10^{-4} cm^2/V·s and 31 theoretical plates were obtained. The peak profile, migration time, and intensity were fairly reproducible, but background noise level was not always steady. Electrophoretic mobilities of anti-OxPhos ranged from -1.27 x 10^{-4} to -1.14 x 10^{-4} cm^2/V·s for the eight runs.

To test the ability of UltraTrol™ LN coating to separate proteins, a fluorescent molecular weight marker (M.W. 20,100-205,000) was tested with CE-LIF. The proteins in the sample, their molecular weights, and their electrophoretic mobility ranges can be found in Table 6 below. The M.W. marker was tested with no recoating or rinses between runs, but results were very irreproducible and are not shown here or included in electrophoretic mobility ranges. This was most likely due to significant absorption of proteins to the capillary wall and coating. Figure 8 shows one electropherogram of the M.W. marker using the standard UltraTrol™ LN procedure. Electrophoretic mobilities decreased gradually as time elapsed and as more runs were completed. Intensity and peak profile were not reproducible. The UltraTrol™ LN coating was also tested on another antibody, Alexa Fluor 488 rabbit anti-goat IgG, to determine the coatings interaction with other antibodies (Figure 9). A high intensity peak was detected with a low peak width of $w_{1/2}$ = 2 sec, giving 23,900 theoretical plates. However, a lower intensity broad peak was also observed. The electrophoretic mobility of the antibody was -3.81 x 10^{-4} cm^2/V·s in Figure 9, but varied from -3.96 x 10-4 to 3.23 x 10-4 cm^2/V·s with the UltraTrol™ coating.

Protein	**Approximate M.W. (Daltons)**	**μ_e Range (cm^2/V·s)**
Trypsin inhibitor, soybean	20,100	-3.68 x 10^{-4} to -3.58 x 10^{-4}
Carbonic Anhydrase, bovine erythrocyte	29,000	-2.61 x 10^{-4} to -2.48 x 10^{-4}
Alcohol Dehydrogenase, equine liver	39,000	-2.54 x 10^{-4} to -2.41 x 10^{-4}
Albumin, bovine serum	66,000	-2.43 x 10^{-4} to -2.13 x 10^{-4}
B-galactosidase, *E. coli*	116,000	-2.24 x 10^{-4} to -2.09 x 10^{-4}
Myosin, rabbit muscle	205,000	-2.02 x 10^{-4} to -1.89 x 10^{-4}

Table 6 – Fluorescent M.W. marker: Fluorescent molecular weight marker from Sigma (St. Louis, MO). A 10 μl portion of the protein solution in 62 mM Tris-HCl,pH 8.0, 1 mM EDTA, 3% sucrose, 0.5% dithiothreitol, 2% SDS, and 0.005% bromophenol blue was diluted to 200 μl in 10 mM Tris-HCl pH 7.4 buffer. The fluorescent M.W. marker was injected at 0.3 psi for 3 sec and separated at -12 kV for 15 min with 10 mM Tris-HCl pH 7.4 running buffer in an UltraTrol™ LN coated capillary.

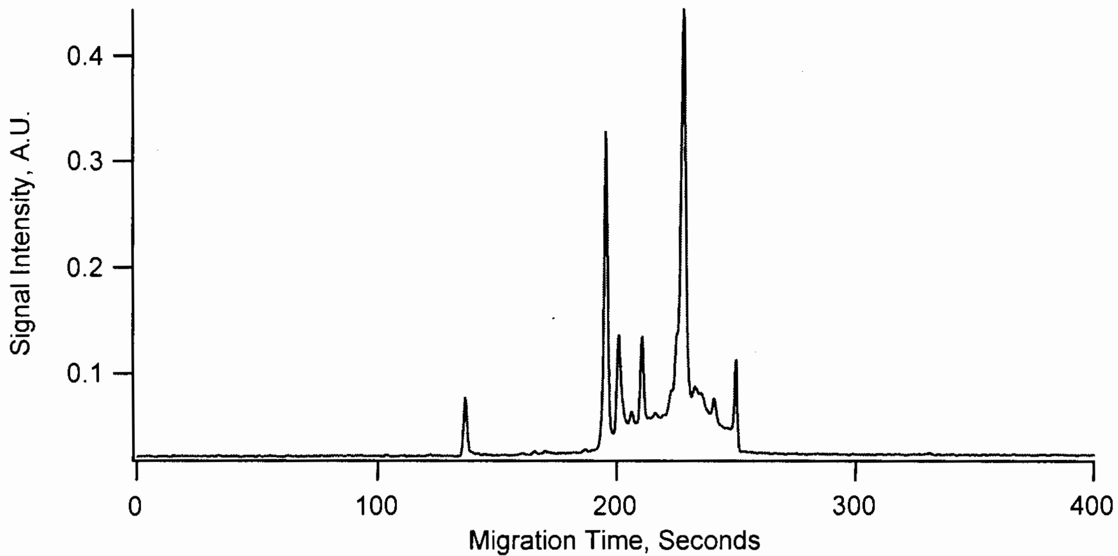

Figure 8 – Sigma fluorescent M.W. marker with UltraTrol™ LN: Sigma molecular weight marker (M.W. 20,100-205,000) was injected at 0.3 psi for 3 sec using an UltraTrol™ LN coated capillary. Separation was at -12 kV for 15 min with 10 mM Tris-HCl pH 7.4 running buffer. From left to right: μ_e = -3.66×10^{-4}, -2.56×10^{-4}, -2.49×10^{-4}, -2.38×10^{-4}, -2.20×10^{-4}, and -2.00×10^{-4} $cm^2/V{\cdot}s$, $w_{1/2}$ = 1.625, 1.625, 1.75, 1.5, 2.25, and 1.25 sec, N = 39,100, 80,000, 72,900, 109,000, 56,800, and 221,000. (File AH071008-06)

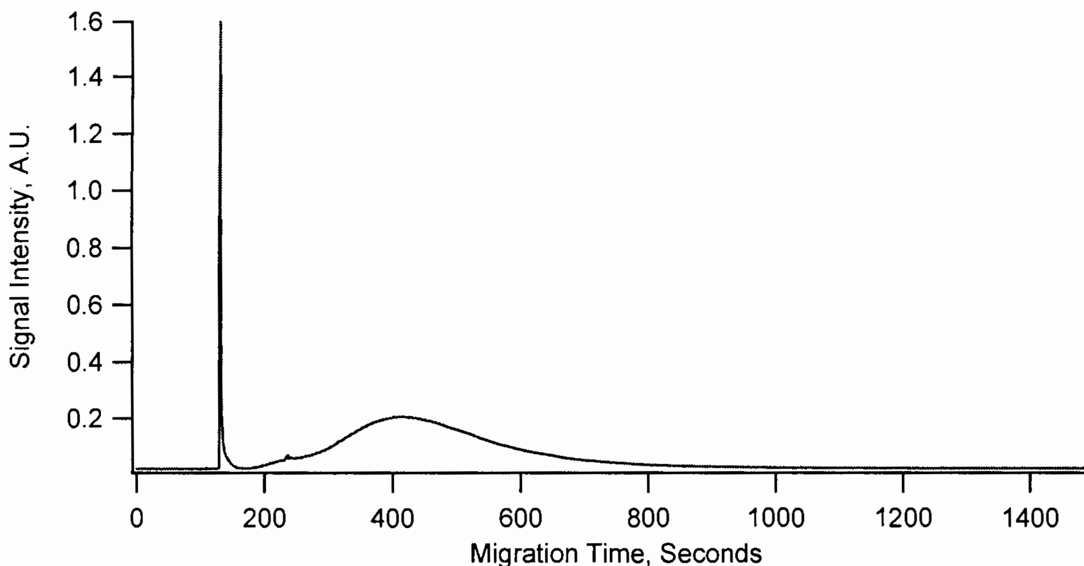

Figure 9 – Alexa Fluor 488 rabbit anti-goat IgG with UltraTrol™ LN: Alexa Fluor 488 rabbit anti-goat IgG (20 µg/ml) was injected at 0.3 psi for 3 sec and separated at -12 kV for 20 min with 10 mM Tris-HCl pH 7.4 in an UltraTrol™ LN coated capillary. First peak $w_{1/2}$ = 2 sec, N = 23,900, μ_e = -3.81×10^{-4} $cm^2/V{\cdot}s$. (File AH070908-06)

The calculated EOF with the DSPC coating was -1.73 x 10^{-4} $cm^2/V\cdot s$. The EOF with no capillary coating on the same capillary was 2.58 x 10^{-4} $cm^2/V\cdot s$. The DSPC coating was first tested on 1 x 10^{-8} M fluorescein sample (Figure 10). The fluorescein peak had an electrophoretic mobility of -2.37 x 10-4 $cm^2/V\cdot s$, $w_{1/2}$ = 2.5 sec, and 39,500 theoretical plates. The coating was then tested on a 5 μg/ml anti-OxPhos sample (Figure 11). The background noise had still not returned to the beginning level and was unsteady at the end of the run. The electrophoretic mobility of anti-OxPhos was -7.53 x 10^{-5} $cm^2/V\cdot s$. The electrophoretic mobility of anti-OxPhos corrected for EOF was 9.77 x 10^{-4} $cm^2/V\cdot s$.

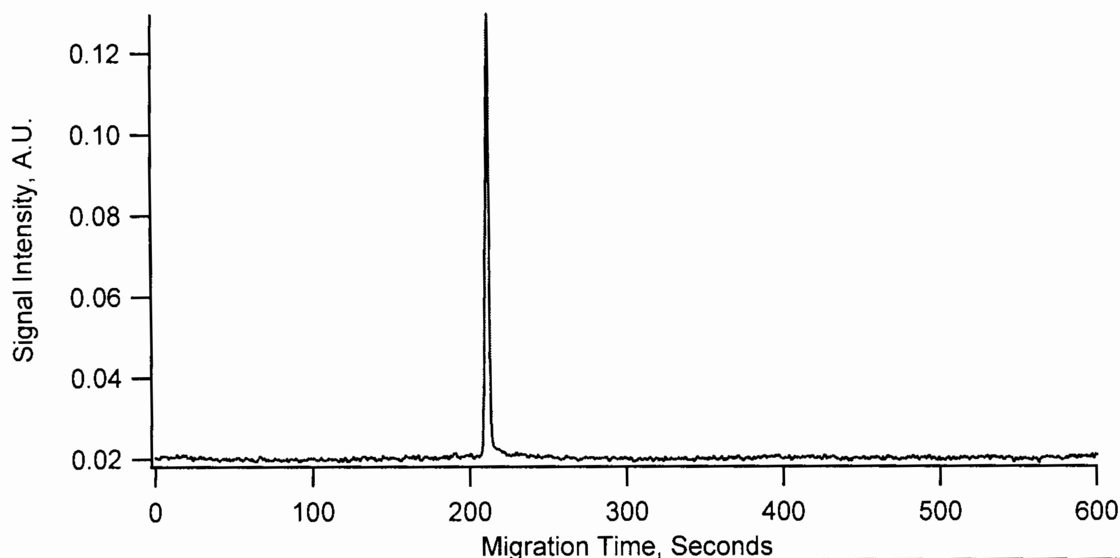

Figure 10 – Fluorescein with DSPC: Fluorescein (1 x 10^{-8} M) was injected at 0.3 psi for 3 sec and separated at -12 kV for 10 min with 20 mM Tris-HCl pH 7.4 in a DSPC coated capillary. $w_{1/2}$ = 2.5 sec, N = 39,500, μ_e = 2.37 x 10^{-4} $cm^2/V\cdot s$. (File AH071608-04)

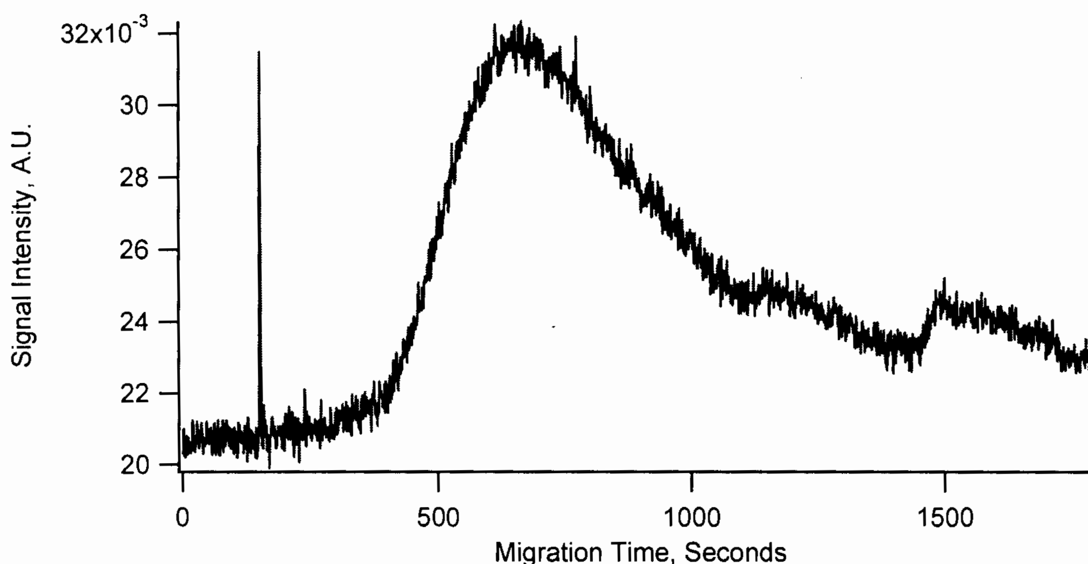

Figure 11 – Anti-OxPhos with DSPC: Anti-OxPhos (5 μg/ml) was injected at 0.3 psi for 3 sec and separated at -12 kV for 30 min with 20 mM Tris-HCl pH 7.4 running buffer in a DSPC coated capillary. Peak width and theoretical plates could not be obtained due to background noise level not returning to the beginning level. Migration time was determined by the highest intensity reached, which gave $\mu_e = 7.53 \times 10^{-5}$ cm^2/V·s. (File AH071608-07)

Incubation 2:1 Anti-OxPhos to COX1

Controls for this experiment were CEIA-LIF of samples of 2.5 μg/ml anti-OxPhos (Figure 12), 2.5 μg/ml anti-OxPhos spiked with 5×10^{-9} M fluorescein (Figure 13), and the mitochondrial protein sample with a concentration of approximately 1.25 μg/ml COX1 (Figure 14). Figure 15 is an electropherogram of the incubation mixture containing 2.5 μg/ml anti-OxPhos, 5×10^{-9} M fluorescein, and approximately 1.25 μg/ml COX1. The antibody peak electrophoretic mobility ranged from 3.80×10^{-4} to 4.18×10^{-4} cm^2/V·s. Fluorescein electrophoretic mobility ranged from 2.30×10^{-4} to 2.56×10^{-4} cm^2/V·s. The incubation electropherogram shows an increase in antibody peak width at half maximum ($w_{1/2}$) by about 8 sec compared to before incubation (Figure 15).

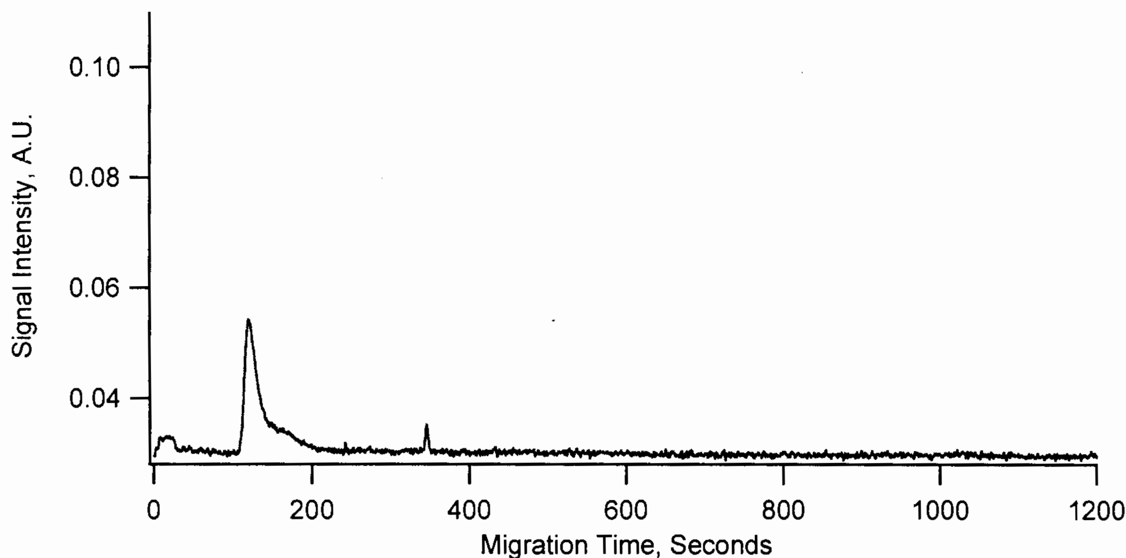

Figure 12 – Anti-OxPhos control 1: Anti-OxPhos (2.5 μg/ml) was injected at 0.3 psi for 3 sec and separated at +12 kV for 20 min with 10 mM Tris-HCl pH 7.4 running buffer in an uncoated capillary. $w_{1/2}$ = 17.5 sec, N = 259, $\mu_e = 4.18 \times 10^{-4}$ $cm^2/V \cdot s$. (File AH071708-02)

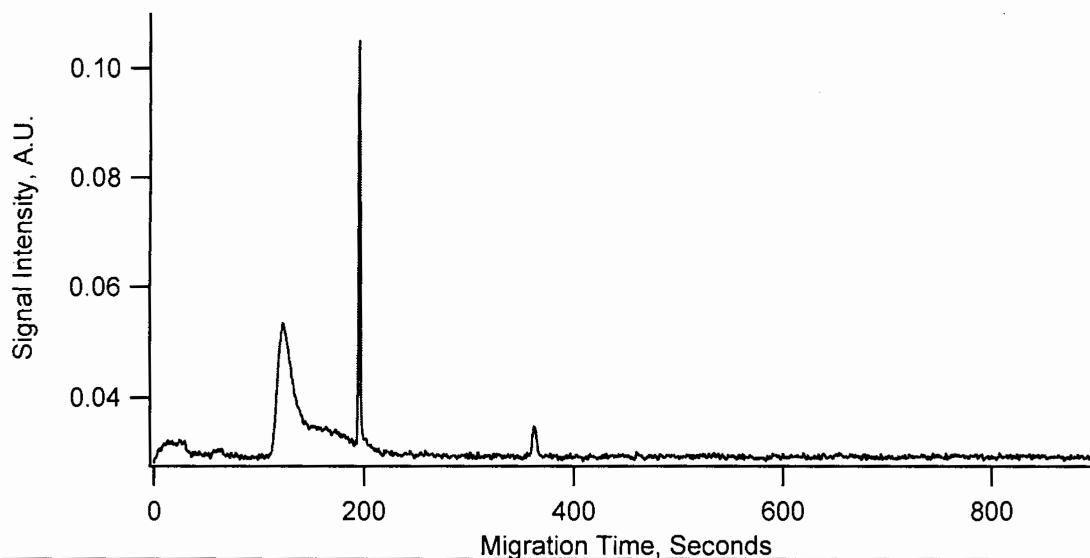

Figure 13 – Anti-OxPhos spiked w/ fluorescein control 1: Anti-OxPhos (2.5 μg/ml) spiked with fluorescein (5×10^{-9} M) was injected at 0.3 psi for 3 sec and separated at +12 kV for 15 min with 10 mM Tris-HCl pH 7.4 in an uncoated capillary. The first peak (anti-OxPhos): $w_{1/2}$ = 16.25 s, N = 308, $\mu_e = 4.07 \times 10^{-4}$ $cm^2/V \cdot s$. The second peak (fluorescein): $w_{1/2}$ = 2 sec, N = 52,800, $\mu_e = 2.56 \times 10^{-4}$ $cm^2/V \cdot s$.(File

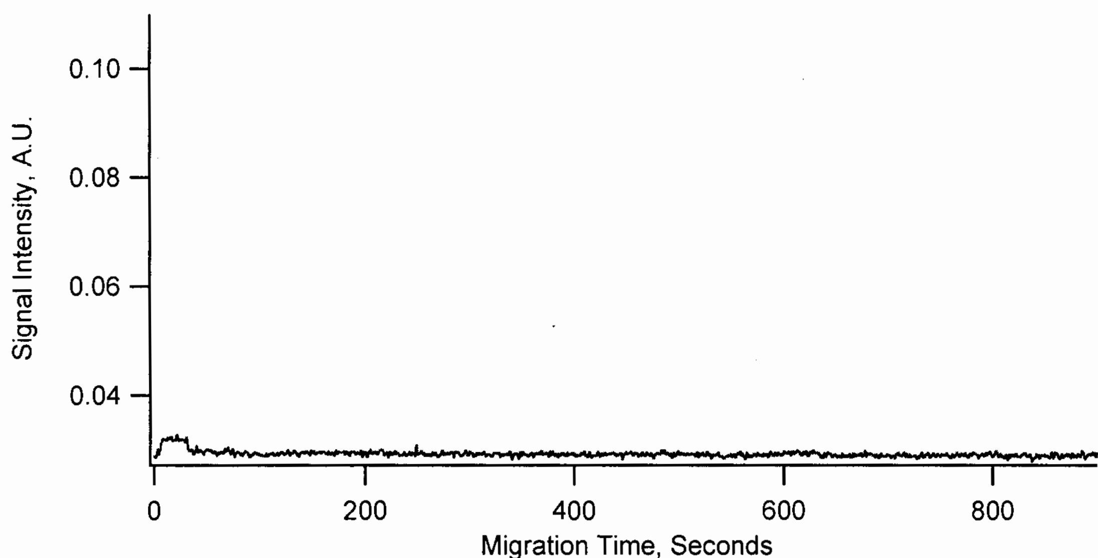

Figure 14 –Mitochondrial protein sample control 1: The mitochondrial protein sample (~1.25 μg/ml COX1) was injected at 0.3 psi for 3 sec and separated at +12 kV for 15 min with 10 mM Tris-HCl pH 7.4 in an uncoated capillary. (File AH071708-04)

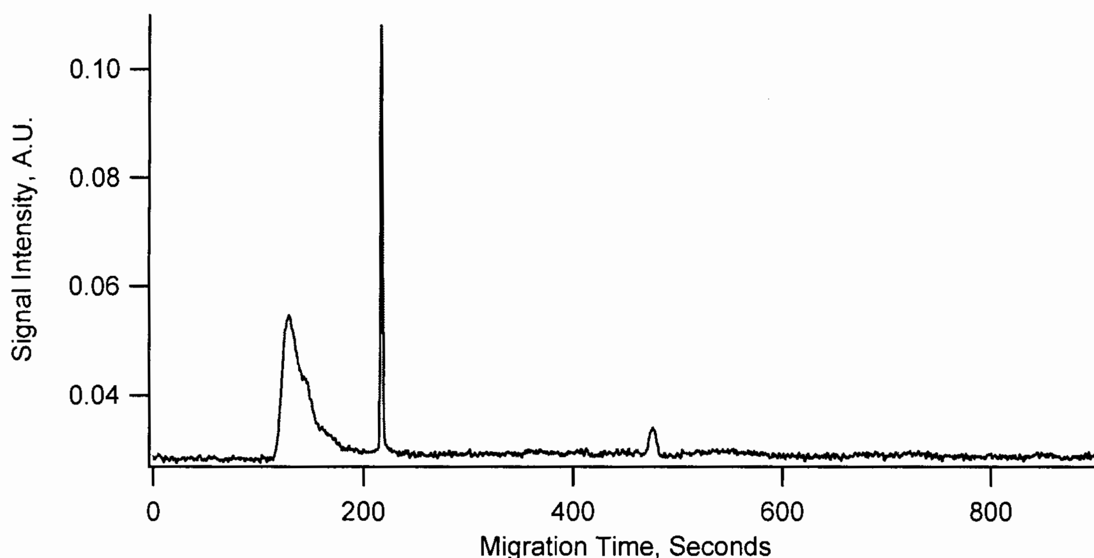

Figure 15 – 2:1 Incubation: Anti-OxPhos (2.5 μg/ml) was incubated with the mitochondrial protein sample (~1.25 μg/ml COX1) for 1 hr before CE analysis. Then the mixture was spiked with fluorescein (5 x 10^{-9} M). This electropherogram was taken approximately 2 hours after incubation began. This incubation mixture was injected at 0.3 psi for 3 sec and separated at +12 kV for 15 min with 10 mM Tris-HCl running buffer in an uncoated capillary. The first peak (anti-OxPhos): $w_{1/2}$ = 25.125 sec, N = 145, μ_e = 3.89 x 10^{-4} $cm^2/V{\cdot}s$. The second peak (fluorescein): $w_{1/2}$ = 2.375 sec, N = 46,250, μ_e = 2.30 x 10^{-4} $cm^2/V{\cdot}s$. (File AH071708-07).

Incubation 1:1 Anti-OxPhos to COX1

Controls for this experiment were CE-LIF of 2.5 μg/ml anti-OxPhos, (Figure 16), 2.5 μg/ml anti-OxPhos spiked with 5 x 10^{-9} M fluorescein (Figure 17), and the mitochondrial protein sample diluted to approximately 2.5 μg/ml COX1 (Figure 18). Incubation of the anti-OxPhos with the mitochondrial protein sample lasted 2 hrs before CEIA-LIF analysis. CEIA-LIF of the incubation mixture was taken at 120 min (Figure 19), 160 min (Figure 20), and 200 min (Figure 21) after the incubation began. The anti-OxPhos electrophoretic mobility ranged from 4.14 x 10^{-4} to 4.43 x 10^{-4} $cm^2/V{\cdot}s$. The fluorescein electrophoretic mobility ranged from 2.69 x 10^{-4} to 2.94 x 10^{-4} $cm^2/V{\cdot}s$. The second peak in the incubation electropherograms was not present before incubation and could possibly represent the immuno-complex. This peak's electrophoretic mobility ranged from 3.30 x 10^{-4} to 3.50 x 10^{-4} $cm^2/V{\cdot}s$. The fluorescein peak intensity was not consistent. However, the extra peak observed after incubation appeared to be decreasing in intensity over time relative to the fluorescein and anti-OxPhos peaks.

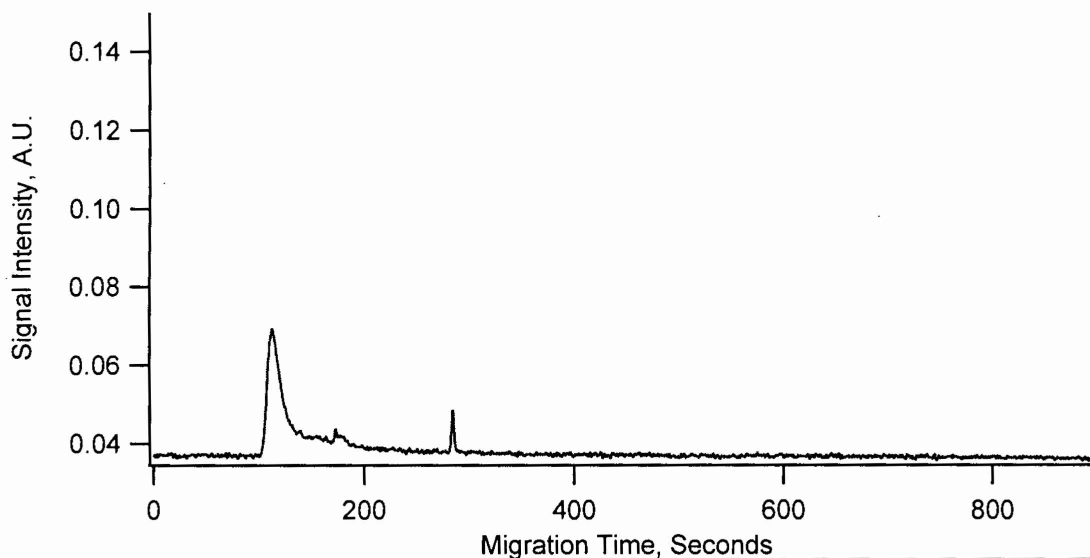

Figure 16 – Anti-OxPhos control 2: Anti-OxPhos (2.5 μg/ml) was injected at 0.3 psi for 3 sec and separated at +12 kV for 15 min with 10 mM Tris-HCl pH 7.4 running buffer in an uncoated capillary. $w_{1/2}$ = 14.625 sec, N = 329, μ_e = 4.43 x 10^{-4} $cm^2/V{\cdot}s$. (File AH072808-02)

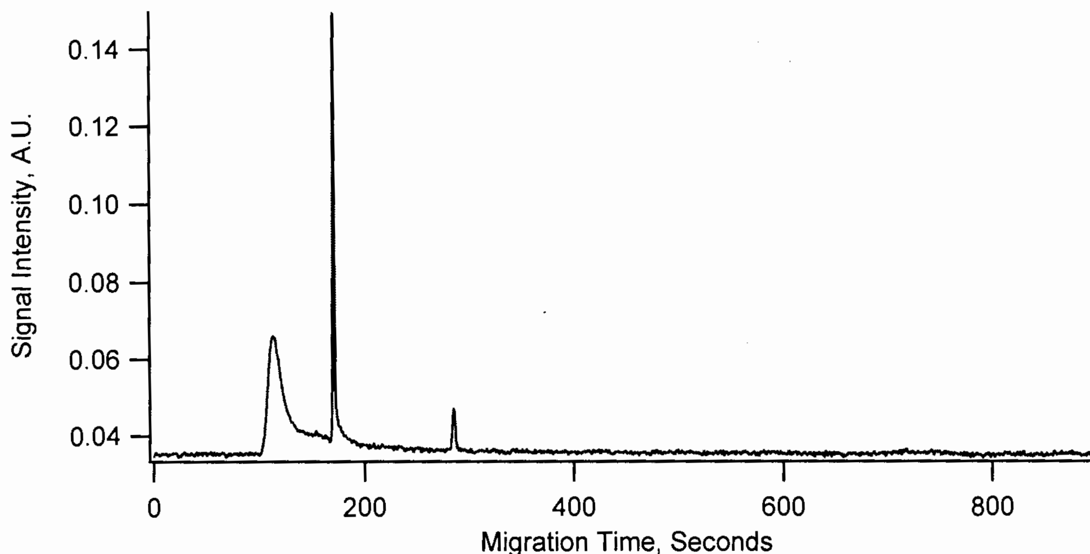

Figure 17 – Anti-OxPhos spiked w/ fluorescein control 2: Anti-OxPhos (2.5 μg/ml) spiked with fluorescein (5 x 10^{-9} M) was injected at 0.3 psi for 3 sec and separated at +12 kV for 15 min with 10 mM Tris-HCl pH 7.4 running buffer in an uncoated capillary. First peak (anti-OxPhos): $w_{1/2}$ = 14 sec, N = 359, μ_e = 4.43 x 10^{-4} cm^2/V·s. Second peak (fluorescein): $w_{1/2}$ = 1.75 sec, N = 52, 400, μ_e = 2.94 x 10^{-4} cm^2/V·s. (File AH072808-03)

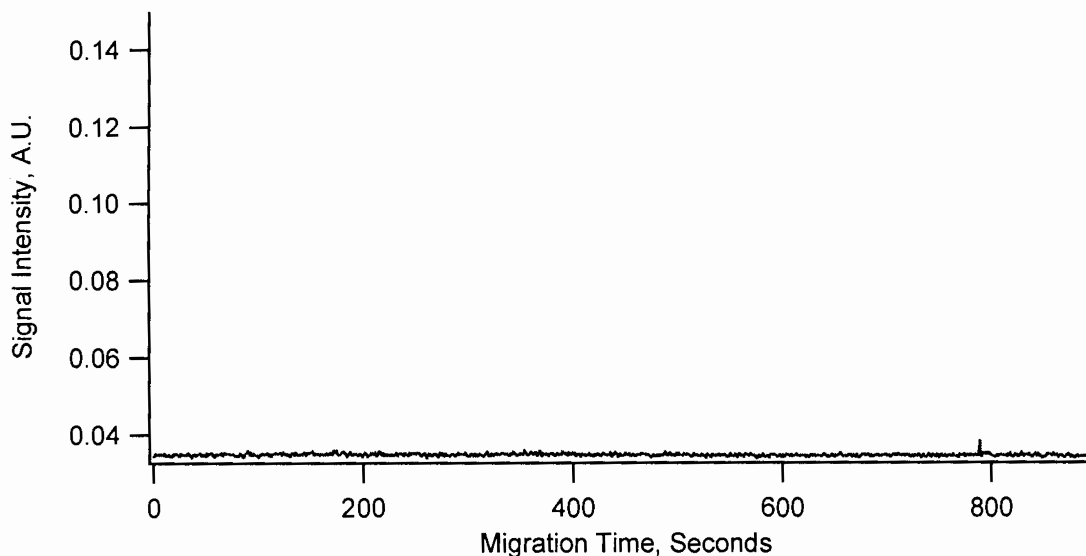

Figure 18 – Mitochondrial Protein Sample Control 2: The mitochondrial control sample (~ 5 μg/ml COX1) was injected at 0.3 psi for 3 sec and separated at +12 kV for 15 min with 10 mM Tris-HCl pH 7.4 running buffer in an uncoated capillary. (File AH072808-06)

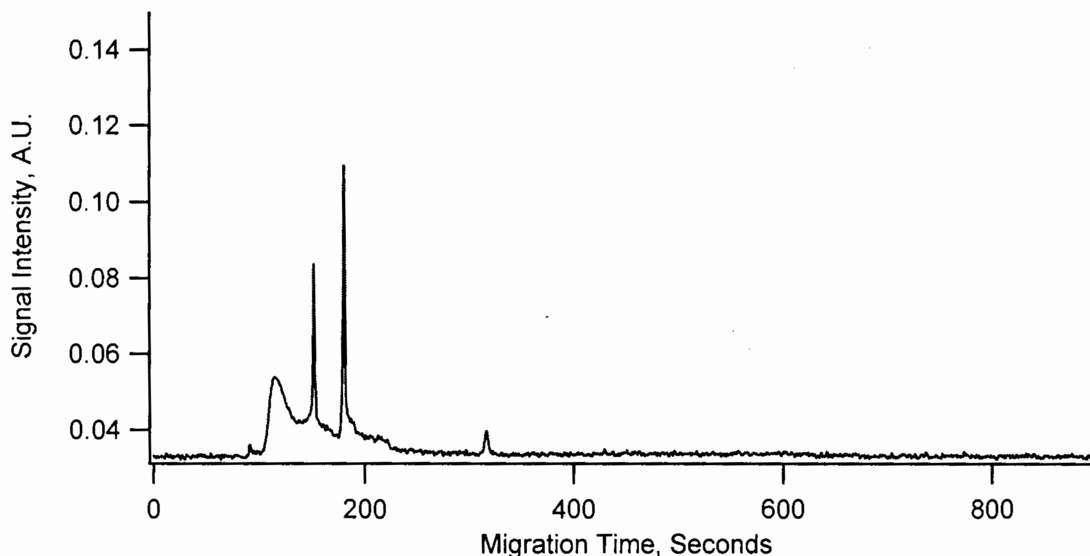

Figure 19 – Incubation 1:1, 120 min: Anti-OxPhos (2.5 µg/ml) was incubated with the mitochondrial protein sample (~2.5 µg/ml COX1) for 2 hours before CE analysis. Then the mixture was spiked with 5 x 10^{-9} M fluorescein. This electropherogram was taken about 120 min after incubation began. The incubation mixture was injected at 0.3 psi for 3 sec and separated at +12 kV for 15 min with 10 mM Tris-HCl pH 7.4 running buffer in an uncoated capillary. First peak (anti-OxPhos): $w_{1/2}$ = 17.125 sec, N = 250, μ_e = 4.35 x 10^{-4} $cm^2/V{\cdot}s$. Second peak (possible immuno-complex): $w_{1/2}$ = 1.625 sec, N = 48,100, μ_e = 3.30 x 10^{-4} $cm^2/V{\cdot}s$. Third peak (fluorescein): $w_{1/2}$ = 2 sec, N = 45,000, μ_e = 2.77 x 10^{-4} $cm^2/V{\cdot}s$. (File AH072808-08)

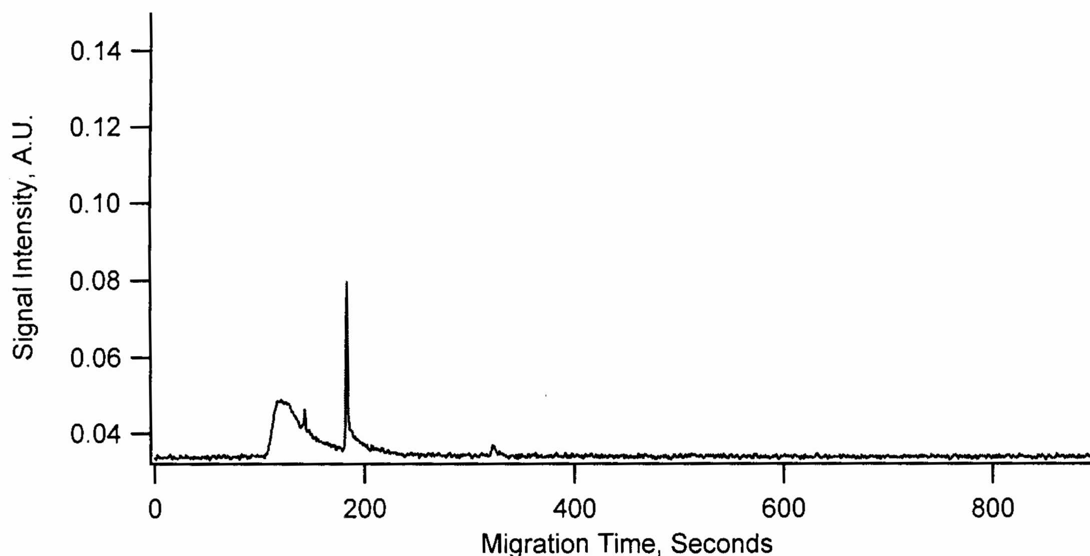

Figure 20 – Incubation 1:1, 160 min: Anti-OxPhos (2.5 µg/ml) was incubated with the mitochondrial protein sample (~2.5 µg/ml COX1) for 2 hours before CE analysis. Then the mixture was spiked with 5 x 10^{-9} M fluorescein. This electropherogram was taken about 160 min after incubation began. The incubation mixture was injected at 0.3 psi for 3 sec and separated at +12 kV for 15 min with 10 mM Tris-HCl pH 7.4 running buffer in an uncoated capillary. First peak (anti-OxPhos): $w_{1/2}$ = 25.5 sec, N = 124, μ_e = 4.14 x 10^{-4} $cm^2/V{\cdot}s$. Second peak (possible immuno-complex): $w_{1/2}$ = 1.5 sec, N = 50,400, μ_e = 3.50 x 10^{-4} $cm^2/V{\cdot}s$. Third peak (fluorescein): $w_{1/2}$ = 2 sec, N = 46,400, μ_e = 2.73 x 10^{-4} $cm^2/V{\cdot}s$ (File AH0720808-10)

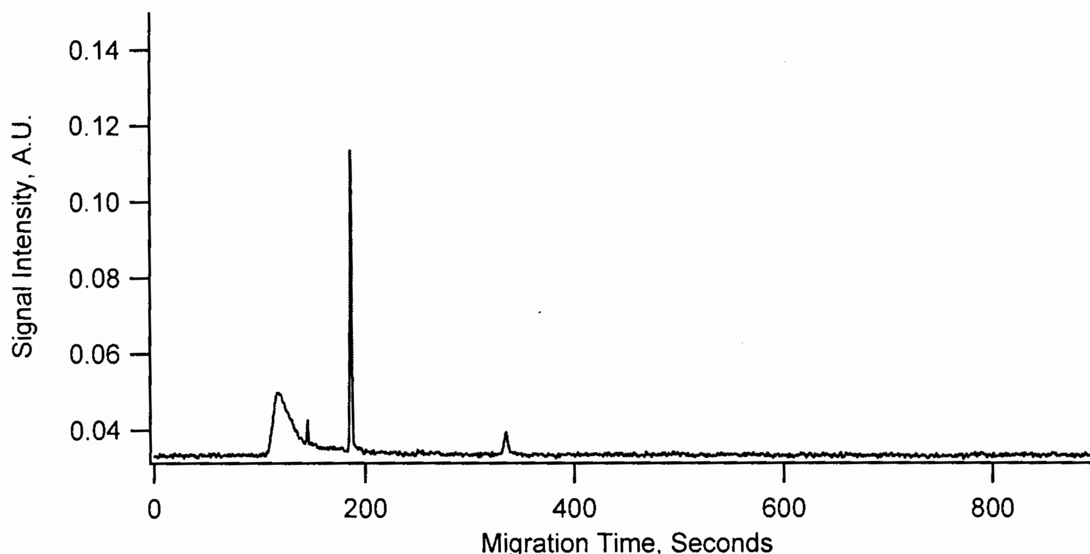

Figure 21 – Incubation 1:1, 200 min: Anti-OxPhos (2.5 μg/ml) was incubated with the mitochondrial protein sample (~2.5 μg/ml COX1) for 2 hours before CE analysis. Then the mixture was spiked with 5 x 10^{-9} M fluorescein. This electropherogram was taken about 200 min after incubation began. The incubation mixture was injected at 0.3 psi for 3 sec and separated at +12 kV for 15 min with 10 mM Tris-HCl pH 7.4 running buffer in an uncoated capillary. First peak (anti-OxPhos): $w_{1/2}$ = 18.5 sec, N = 221, μ_e = 4.28 x 10^{-4} $cm^2/V{\cdot}s$. Second peak (possible immuno-complex): $w_{1/2}$ = 1.25 sec, N = 74,800, μ_e = 3.44 x 10^{-4} $cm^2/V{\cdot}s$. Third peak (fluorescein): $w_{1/2}$ = 1.825 sec, N = 57,600, μ_e = 2.69 x 10^{-4} $cm^2/V{\cdot}s$ (File AH072808-12)

Incubation 1:2 Anti-OxPhos to COX1

Controls for this experiment were CE-LIF of 1.25 μg/ml anti-OxPhos (Figure 22), 1.25 μg/ml anti-OxPhos spiked with 5 x 10^{-9} M fluorescein (Figure 23), and the mitochondrial protein sample diluted to approximately 2.5 μg/ml COX1 (Figure 18). The 1.25 μg/ml anti-OxPhos with approximately ~2.5 μg COX1 mixture was incubated for 30 min before CEIA-LIF analysis. Figure 24 is 40 min after the incubation began and Figure 25 is 90 min after incubation began. Anti-OxPhos electrophoretic mobility ranged from 4.94 x 10^{-4} to 5.06 x 10^{-4} $cm^2/V{\cdot}s$. Fluorescein electrophoretic mobility ranged from 3.45 x 10^{-4} to 3.68 x 10^{-4} $cm^2/V{\cdot}s$. The antibody peak increased in $w_{1/2}$ by about 7 sec in Figure 24 and by about 10 s in Figure 25 after incubation.

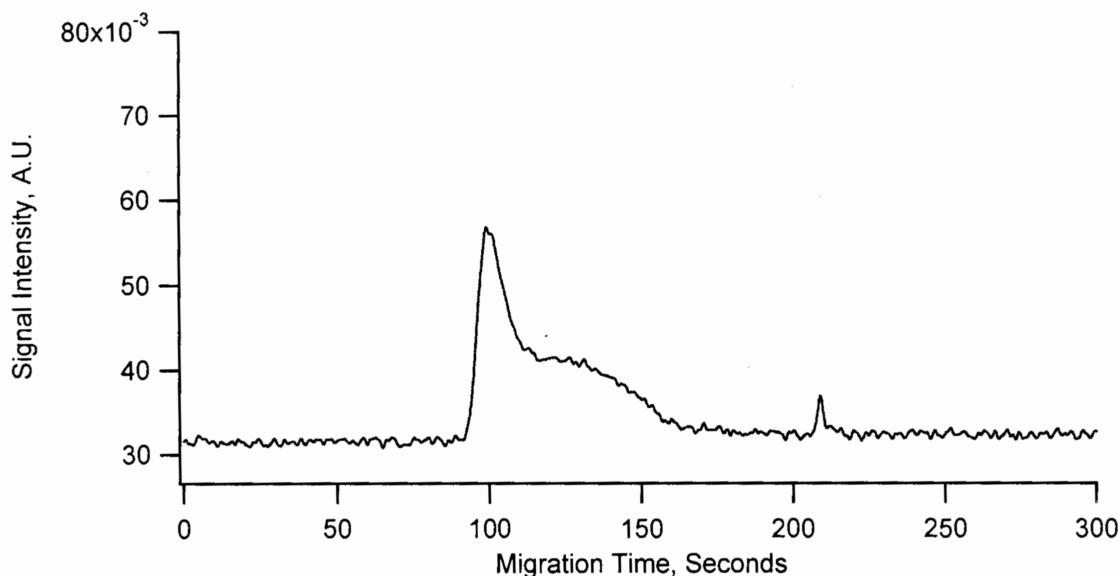

Figure 22 – Anti-OxPhos Control 3: Anti-OxPhos (1.25 μg/ml) was injected at 0.3 psi for 3 sec and separated at +12 kV for 15 min with 10 mM Tris-HCl pH 7.4 running buffer in an uncoated capillary. $w_{1/2}$ = 12.75 sec, N = 334, $\mu_e = 5.05 \times 10^{-4}$ $cm^2/V{\cdot}s$. Electropherogram is a close up of initial graph. (File AH073008-13)

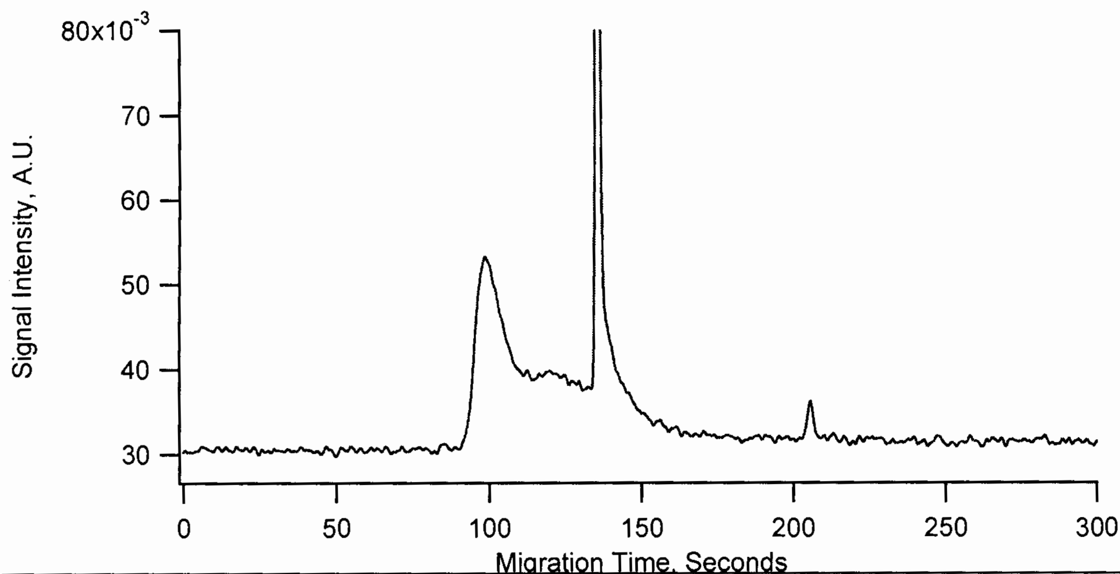

Figure 23 – Anti-OxPhos Spiked w/ Fluorescein Control 3: Anti-OxPhos (1.25 μg/ml) spiked with fluorescein (5×10^{-9} M) was injected at 0.3 psi for 3 sec and separated at +12 kV for 15 min with 10 mM Tris-HCl running buffer in an uncoated capillary. First peak (anti-OxPhos): $w_{1/2}$ = 10 sec, N = 540, $\mu_e = 5.06 \times 10^{-4}$ $cm^2/V{\cdot}s$. Second peak (fluorescein): $w_{1/2}$ = 1.5 sec, N = 45,400, $\mu_e = 3.68 \times 10^{-4}$ $cm^2/V{\cdot}s$. Electropherogram is a close up of initial graph. (File AH073008-14)

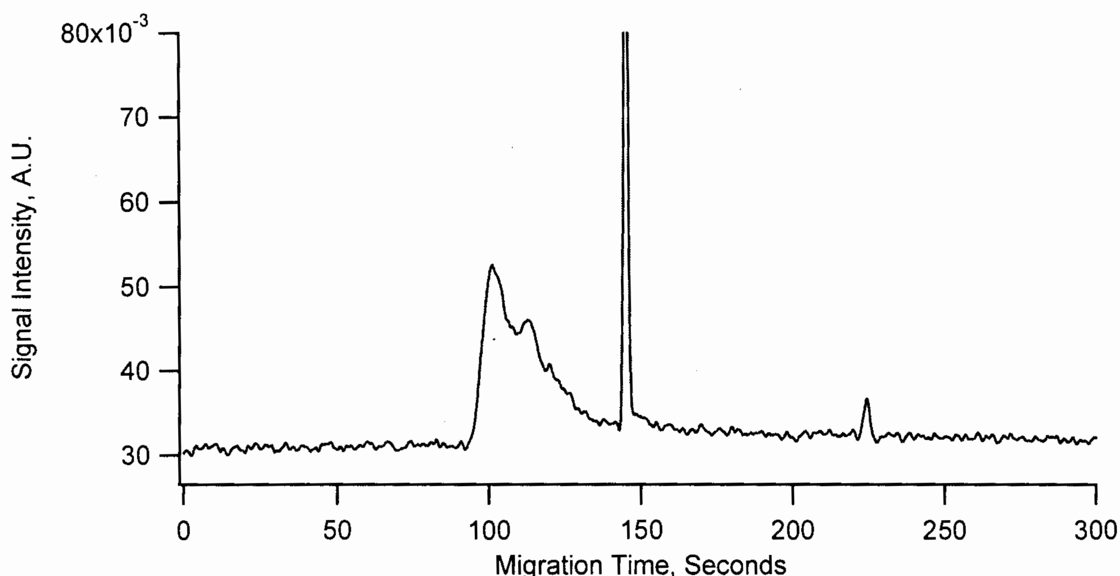

Figure 24 – Incubation 1:2, 40 min: Anti-OxPhos (1.25 μg/ml) was incubated with the mitochondrial protein sample (~ 2.5 μg/ml COX1) for 30 min before CEIA-LIF analysis. Then the mixture was spiked with 5 x 10^{-9} M fluorescein. This electropherogram was taken about 40 min after incubation began. The incubation mixture was injected at 0.3 psi for 3 sec and separated at +12 kV for 15 min with 10 mM Tris-HCl pH 7.4 running buffer in an uncoated capillary. First peak (anti-OxPhos): $w_{1/2}$ = 18.75 sec, N = 161, μ_e = 4.95 x 10^{-4} cm^2/V·s. Second peak (fluorescein): $w_{1/2}$ = 1.5 sec, N =51, 800, μ_e = 3.45 x 10^{-4} cm^2/V·s. Electropherogram is a close up of the initial graph. (File AH073008-10)

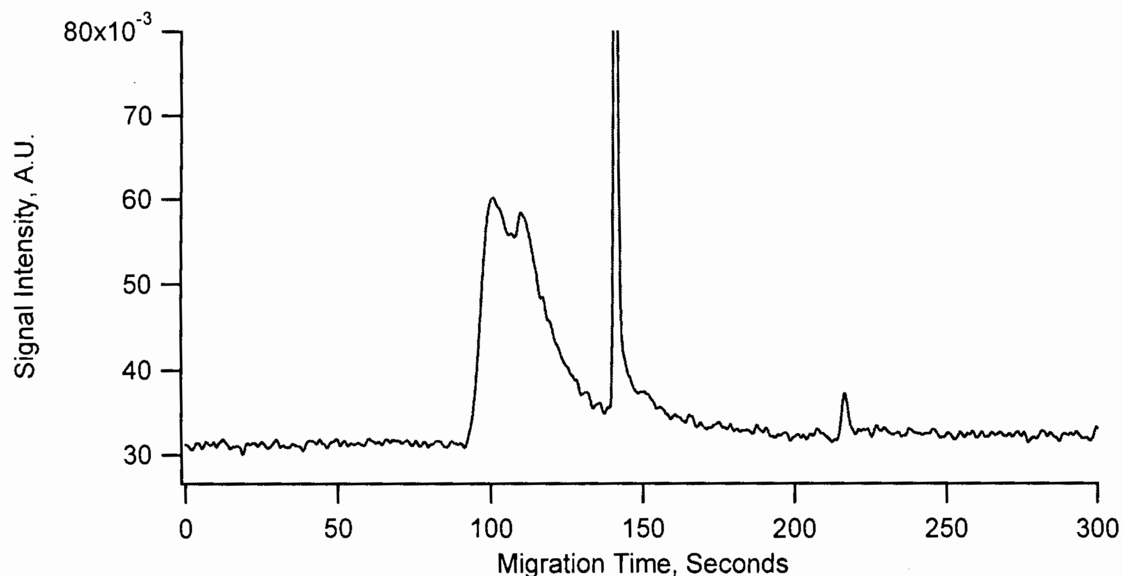

Figure 25 – Incubation 1:2, 90 min: Anti-OxPhos (1.25 μg/ml) was incubated with the mitochondrial protein sample (~ 2.5 μg/ml COX1) for 30 min before CEIA-LIF analysis. Then the mixture was spiked with 5 x 10^{-9} M fluorescein. This electropherogram was taken about 90 min after incubation began. The incubation mixture was injected at 0.3 psi for 3 sec and separated at +12 kV for 15 min with 10 mM Tris-HCl pH 7.4 running buffer in an uncoated capillary. First peak (anti-OxPhos): $w_{1/2}$ = 21.5 sec, N = 122, μ_e = 4.95 x 10^{-4} cm^2/V·s. Second peak (fluorescein): $w_{1/2}$ = 1.5 sec, N = 49,100, μ_e = 3.54 x 10^{-4} cm^2/V·s. Electropherogram is a close up of the initial graph. (File AH073008-012)

DISCUSSION

The LOD of fluorescein on the Beckman Coulter P/ACE™ MDQ Instrument was 151 zmol, showing the high resolution and sensitivity of detection. The observed anti-OxPhos peaks were broad, which can be attributed to (i) anti-OxPhos interacting strongly with both UltraTrol™ and DSPC coatings and/or (ii) molecular microheterogeneity of the antibody. The anti-OxPhos peaks with the smallest peak width and highest reproducibility were achieved with no capillary coating. These peaks were typically around 17 sec wide at half maximum. Also, the anti-OxPhos appeared to have an impurity in the stock solution, because a low intensity peak was repeatedly observed in electropherograms from different sample preparations. The mitochondrial protein sample appeared to be efficiently solubilized by the RIPA buffer (void of sodium deoxycholate), because after suspension in RIPA buffer the protein concentration was 5130 μg/ml and the solution turned from cloudy to clear. Incubations of anti-OxPhos with the mitochondrial protein sample suggest the presence of an immuno-complex between anti-OxPhos and COX1 with the appearance of a shoulder peak next to the anti-OxPhos peak.

The UltraTrol™ LN coating had significant interactions with anti-OxPhos. The anti-OxPhos peak width at half maximum was much wider with the UltraTrol™ LN coating than with no capillary coating. The peak width at half maximum for anti-OxPhos ranged from 165.5 sec to 361.25 sec, while the width at half maximum with no capillary coating was approximately 17 sec. The peak profile, intensity and electrophoretic mobility were not reproducible from day to day. The repeated sample injections without rinsing or recoating the capillary with UltarTrol™ LN did not help decrease peak width. However, it did suggest that the UltraTrol™ LN coating was stable, because the peak profile was fairly reproducible with no recoating. Diluting samples in and switching the running buffer to a buffer lacking borates (10 mM Tris-HCl pH 7.4) did not help decrease peak width. Interactions of borates with the sugars on the antibody were not significant enough to cause a visible change in mobility. Because anti-OxPhos peaks with UltraTrol™ LN coating were broader than with no capillary coating and were irreproducible, anti-OxPhos must have been interacting with the capillary coating.

Other tests with the UltraTrol™ LN coating indicated the possible microheterogeneity of anti-OxPhos. The Alexa Fluor 488 rabbit anti-goat IgG had a sharp, high intensity peak and a second broad, lower intensity peak. The high intensity peak had a width at half maximum of only 2 sec. However, the Alexa Fluor 488 rabbit anti-goat IgG still seemed to be interacting with the capillary, because the migration time of the first, high intensity peak varied by about 20 sec and the second broad peak profile varied greatly. The CE-LIF of the fluorescent molecular weight marker suggested that UltraTrol™ LN coating could be used to separate other protein mixtures. The six peaks were fairly well resolved and electrophoretic mobilities were reproducible. However, the peak intensity and profile were not always reproducible. A seventh and eighth peak began to appear over time. These extra peaks could be signs of degradation of proteins in the sample. The higher resolution of protein peaks compared to the peak width of anti-OxPhos suggested that anti-OxPhos may be microheterogeneous. The anti-OxPhos sample could be microheterogeneous due to uneven glycosylation and fluorescent labeling (Hoffstetter-Kuhn, Alt, & Kuhn, 1996, Michels, Brady, Guo, & Balland, 2007). When the EOF is reduced or reversed with capillary coatings, small differences in mobility can cause broader peaks. In general, capillary electrophoresis is a very sensitive technique with high resolution and has the ability to detect small differences in mobility that may not be detected by other analytical techniques.

The DSPC coating had significant interactions with anti-OxPhos. The broad peak of anti-OxPhos was not completely expelled from the capillary after a 30 min run. The background noise had not leveled out relative to the beginning level and was unsteady at the end of the run. This also suggests that anti-OxPhos may be microheterogeneous. The DSPC coating reduced and reversed the EOF, which could have increased the sensitivity of the instrument to small changes in mobility. The peak width was too great and resolution too low to continue on with incubations. These results could be due to anti-OxPhos interacting with the DSPC coating and/or microheterogeneity.

The best peak profile for anti-OxPhos was obtained with no capillary coating, which had $w_{1/2}$ = 17 sec and a higher level of reproducibility. Anti-OxPhos could have a better peak profile with no capillary coating because of the faster EOF. When the EOF is faster, small changes in mobility are more difficult to distinguish. Though peaks could not be resolved, a shoulder peak

was observed on the anti-OxPhos peak after incubation. The anti-OxPhos peak also had an increase in $w_{1/2}$ by about 7 to 10 sec after incubation. This shoulder peak suggests that COX1 was present in the mitochondrial protein sample, because the peak width increase was consistent and only occurred after incubation. The 1:1 incubation was the only experiment that resulted in an additional sharp peak after incubation. The gradual decrease in intensity of this extra peak relative to fluorescein and anti-OxPhos suggested that the immuno-complex was unstable and degrading over time. This incubation had a higher concentration of surfactants from the RIPA buffer and a longer incubation time than did the 2:1 incubation. It is possible that this caused the immuno-complex to be less stable and degrade. Overall, the presence of a shoulder peak in the 1:2 incubations suggested that COX1 was present in the mitochondrial sample and that an immuno-complex had possibly formed.

The immuno-complex between anti-OxPhos and COX1 appeared to be unstable and in low yield. The anti-OxPhos peak profile did not change greatly after incubation, suggesting that not much immuno-complex formed. This could be due to an overestimation of COX1 in the mitochondrial sample or instability of the immuno-complex. A different buffer could be used to lyse the mitochondria containing less surfactant. Because anti-OxPhos is a membrane protein, surfactants are necessary in the buffer. However, the RIPA buffer contained both SDS and Triton X-100. A buffer with only one surfactant or a lower concentration of surfactants could be tested. The immuno-complex could be more stable at different conditions for incubation. A higher temperature and longer incubation time could be tested. To obtain more significant results, a greater change in the anti-OxPhos peak profile is needed.

Some adjustments can be made to improve results obtained from CEIA-LIF of the incubations. To obtain quantitative results, the anti-OxPhos needs to be tested on known concentrations of COX1. However, this could be inconclusive due to the low resolution of peaks. Some different capillary coatings could be tested, such as a permanent capillary coating or a capillary coating that reduces the EOF more. If anti-OxPhos is microheterogeneous, a different antibody could be used to develop a general method for CEIA-LIF quantitation of mitochondrial proteins. The Alexa Fluor 488 rabbit anti-goat IgG could be tested with its primary antibody, mfn2 goat polyclonal IgG. The first peak for Alexa Fluor 488 rabbit anti-goat IgG had a peak width at half maximum of only 2 sec. However, the lower intensity, broad peak

could cause irreproducible results. This broad peak suggests that some of the antibody is interacting with and blocking the capillary. In general, a higher resolution of peaks is needed to obtain quantitative results.

The main goal of this research was to develop a specific and sensitive method to quantitate mitochondrial proteins through capillary electrophoresis-based immunoassays. The overall goal was not achieved, but certain conclusions will help in developing a standard method for the quantitation of mitochondrial proteins. Mitochondria were isolated and lysed successfully. The presence of the shoulder peak on anti-OxPhos suggested that this procedure was successful in freeing COX1 from the mitochondrial inner membrane. This shoulder peak also suggested that the incubation conditions had been sufficient for the immuno-complex to form. To obtain quantitative results, a protein standard for COX1 needs to be tested. The major challenge in this research was obtaining a high resolution of peaks. The suboptimal resolution could be due to microheterogenity of the antibody or interactions of the antibody with the capillary walls and coatings. Future work needs to focus on testing new capillary coatings to obtain a higher resolution of peaks or testing a new antibody to develop a standard method.

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