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Capillary electrophoresis monitors changes in the electrophoretic behavior of mitochondrial preparations

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Abstract

The presence of electrical charges on the surface of an organelle is the source of the organelle's electrophoretic mobility. Recently, we reported that capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) can be used to determine the electrophoretic mobility of individual mitochondria. Here, we describe the use of CE-LIF to monitor changes in the electrophoretic mobility distributions of: (i) mitochondria isolated from cultured NS-1 mouse hybridoma cells disrupted by nitrogen cavitation or mechanical homogenization; (ii) mitochondria isolated from rat liver and purified by gradient centrifugation before and after being frozen in liquid nitrogen; and (iii) mitochondria chemically transformed into mitoplasts. These results indicate that the organelle electrophoretic mobility observed by researchers is affected by preparation procedures and that CE-LIF is a complementary technique for monitoring the quality of mitochondrial preparations.

Keywords: Electrophoretic mobility; Mitochondrial preparations

1. Introduction

Centrifugation techniques have been used extensively to fractionate organelles since the pioneering work of de Duve et al. [1,2]. Often, it is difficult to fractionate pure components using these techniques because different organelle types have overlapping densities and sedimentation rates. For example, lysosomes and mitochondria from human liver are difficult to purify by gradient density centrifugation because their densities are 1.15–1.20 and 1.20 g mL^{-1}, respectively [3].

Electrophoretic techniques that separate organelles based on their electrophoretic mobilities are an attractive alternative to centrifugation techniques for preparative purposes. Included among these techniques are free flow electrophoresis (FFE) [4–13] and density gradient electrophoresis (DGE) [14–19] that has been used to purify rat liver lysosomes from mitochondria [4] as well as to separate rat liver mitochondrial membranes [5,6]. The electrophoretic mobility of colloidal particles, including organelles, is thought to be a complex function of the electrical charge on the particle's surface, its morphology, and its size [5]. The contribution of each factor varies depending on the nature of the particle. For organelles at biological pH, the electrophoretic mobility is negative because a net negative electrical charge accumulates on the surface organelle as a result of the contributions of ionized phospholipids, proteins, and glycoproteins. Thus, organelles that are kept at biological pH tend to migrate towards the anode in the presence of an electric field when other factors such as electroosmotic flow are suppressed [20–24]. In contrast to the electrical surface charge, morphology and size do not seem to have a strong effect on the electrophoretic mobility of an organelle. Size-dependent electrophoretic separations may be accomplished if a sieving matrix is added to the separation media as has been done for non-biological particles [25–32].

In the case of mitochondria, reports have suggested that electrophoretic mobility is mainly a function of the electrical charge and not morphology because both intact mitochondria and isolated outer mitochondrial membranes showed identical average electrophoretic mobilities [5]; mitochodrial mobility also remained unaffected by mitochondrial swelling (i.e., change in size) [21].

It has also been previously reported that the average electrophoretic mobilities for mitochondria vary among those from different sources and those measured by different

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methods. The average mobilities of mitochondria from NS-1 cells (determined by CE-LIF [23]), from rat liver (determined by microscopy [21]), and from guinea pig liver (determined by paper electrophoresis [20]), were -1.9×10^{-4}, -1.06×10^{-4}, -2.97×10^{-4} cm^2 V^{-1} s^{-1}, respectively. In all of these cases, the mitochondria showed a wide electrophoretic mobility range, which is attributable to the natural variations in their surface composition.

Electrophoretic separations of mitochondria for analytical and characterization purposes, which is the main focus of this report, were reported as early as 1965 [21]. Using a chamber mounted on a microscope, the velocity of mitochondria under the presence of an electric field could be measured and used to calculate their electrophoretic mobilities. Rat liver and kidney mitochondrial average electrophoretic mobilities were -1.06×10^{-4} and -0.78×10^{-4} cm^2 V^{-1} s^{-1}, respectively, when the organelles were suspended in 125 mM KCl, 20 mM Tris, pH 7.4. In 1973, Valdivia et al. used paper electrophoresis to measure the electrophoretic mobilities of beef heart and guinea pig liver mitochondria [20]. The average electrophoretic mobilities of the beef heart and guinea pig liver mitochondria were -3.74×10^{-4} and -2.97×10^{-4} cm^2 V^{-1} s^{-1}, respectively, when the organelles were suspended in 210 mM mannitol, 60 mM sucrose, 0.2 mM EDTA, pH 7.4. A direct comparison between these electrophoretic mobility values is not possible because of the different experimental approaches, separation conditions, and mitochondrial source.

Recently, we reported the use of capillary electrophoresis to separate organelles including mitochondria [22–24], acidic organelles [33], and nuclei [34]. Using this technique, about 1 nl of organelle suspension is sampled electrokinetically or hydrodynamically into a separation capillary (30–40 cm long, 50 μm internal diameter). The application of an electric field (ca. -400 V cm^{-1}), that is relatively higher than those applied in other separation techniques results in organelle electrophoretic migration towards the highly sensitive post-column laser-induced fluorescence detector with single organelle detection capability. This detector has been used to measure individual organelle electrophoretic mobilities, count mitochondria, and estimate the cardiolipin content of individual organelles. Worthy noting in some of these reports [22–24] was that the distributions resulting from individual mitochondrial electrophoretic mobility measurements varied even when using the same experimental approach and separation conditions. Although some of these variations in electrophoretic mobility may be attributed to the variation in the mitochondrial source, we hypothesized that the isolation or preservation procedures for mitochondria may have an impact on the electrophoretic mobilities of these organelles.

In this report, we show that capillary electrophoresis with post-column laser-induced fluorescence (CE-LIF) is an adequate analytical resource for monitoring the quality of mitochondrial (or other organellar) preparations. We report that the electrophoretic properties of mitochondria vary with the nature of the disruption process used. In addition, we describe the effects that freezing and thawing have on the electrophoretic mobilities of highly pure mitochondria. Based on these results, we propose that CE-LIF analysis of individual mitochondria may be useful as a sensitive bioanalytical tool for monitoring the quality of isolated mitochondrial preparations. In addition, CE-LIF may help determine what conditions are adequate for preparing separations of organelles by FFE or DGE, techniques that promise to become important in subcellular proteomic studies.

2. Experimental

2.1. NS-1 cell culture maintenance and disruption

NS-1 mouse myeloma cells were grown at 37 °C and 5% CO_2. The cells (kindly donated by Dr. Sally Palm, Department of Laboratory Medicine and Pathology, University of Minnesota) were cultured in 90% Dulbecco's Modified Eagle's Medium—10% bovine calf serum (Sigma, St, Louis, MO, USA) and were maintained by the addition of new media every 2–3 days. Biosafety level I was observed for all the preparations.

A protocol based on procedures from Howell et al. [35] and Bogenhagen and Clayton [36] and described by Fuller et al. [22], was followed for disrupting NS-1 cells by nitrogen cavitation. Briefly, 3.2 to 8.6 $\times 10^6$ cells were suspended into 1 mL mitochondrial isolation buffer consisting of 210 mM D-mannitol, 70 mM sucrose, 5 mM HEPES and 5 mM EDTA, adjusted to pH 7.38 with potassium hydroxide (all buffers were made with milli-Q deionized water and filtered (0.2 μm) prior to use). A 100 mg mL^{-1} digitonin (Aldrich, Milwaukee, WI, USA) stock solution was prepared in DMSO, and diluted to 10 mg mL^{-1} in mitochondrial isolation buffer before being added to the cells. The cells were incubated on ice for 5 min and placed in the chamber of an ice cooled cell disruption bomb (Parr Instrument Co., Moline, IL, USA). The bomb was charged to approximately 650 psi with nitrogen and equilibrated for 20 min prior to pressure release. Varying the concentration of digitonin relative to cell density produced different degrees of breakage. To achieve 100% breakage, the cavitation procedure was repeated a second time. For NS-1 cells disrupted by homogenization, cells suspended in mitochondrial isolation buffer were homogenized on ice using a Potter–Elvehjem tissue homogenizer. The extent of cell breakage using both disruption techniques was assessed by light microscopy.

2.2. Labeling and isolation of NS-1 mitochondria

A 10^{-3} M 10-nonyl acridine orange (NAO, Molecular Probes, Eugene, OR, USA) stock solution was prepared in DMSO. Enough NAO solution was added to the disrupted cells to achieve a final concentration of 5–10 μM NAO. Then the preparation was incubated on ice for 5 min. Whole cells,

nuclei, and large cell debris were removed from the suspensions of disrupted cells by centrifugation at 1400 × *g* for 5 min in an Eppendorf 5415D centrifuge (Eppendorf, Westbury, NY, USA). The supernatants were removed and centrifuged again, a process that was repeated two more times. Mitochondria were pelleted from the final supernatant by centrifugation at 14,000 × *g* for 20 min and resuspended in CE buffer containing 250 mM sucrose and 10 mM HEPES that was adjusted to pH 7.47 with potassium hydroxide.

2.3. Isolation and labeling of fresh rat liver mitochondria

Intact, coupled, purified mitochondria were provided by Dr. Cecilia Giulivi (University of Minnesota, Duluth). Liver mitochondria were obtained from adult, male Wistar rats and prepared and characterized as described in [37]. Briefly, after separation by differential centrifugation, purification by Percoll gradient centrifugation, and two washes, respiration was evaluated in the absence (State 4) or in the presence (State 3) of 0.2 mM ADP using a Clark-type oxygen electrode in a medium containing 220 mM sucrose, 50 mM KCl, 10 mM KH_2P0_4, 5 mM $MgCl_2$, 1 mM EDTA, and 10 mM HEPES, at pH 7.4 with 10 mM succinate added as substrate. Under these conditions, only preparations that exhibited an RCR above 5.0 and a P/O ratio of 2.0 ± 0.5 were kept and used further.

Fresh mitochondria were shipped in ice overnight for CE-LIF analysis from Duluth, Minnesota to Minneapolis, Minnesota. Upon arrival in Minneapolis, the mitochondria were diluted and stained with 5 mM NAO for 20 min on ice, and pelleted at 6600 × *g* for 10 min, then resuspended in 500 mL of mitochondrial isolation buffer. The centrifugation and resuspension were repeated twice.

2.4. Frozen rat liver mitochondria and mitoplasts

Fresh rat liver mitochondria were stored at −78 °C in 10% DMSO, 220 mM D-mannitol, 70 mM sucrose, 0.5 mM EGTA, 2.0 mM HEPES, 0.1% fatty-acid free bovine serum albumin, at pH 7.4 with KOH. After 101 days of storage, an aliquot of frozen mitochondria was thawed out, washed twice in mitochondrial isolation buffer, and analyzed using the same procedures as for fresh mitochondria.

Mitoplasts, mitochondria with the outer membrane removed, were prepared from rat liver mitochondria that were frozen for 107 days according to the procedure of Technikova-Dobrova et al. [38]. Briefly, the mitochondria were stained with 5 mM NAO for 25 min on ice, pelleted at 6600 × *g* for 10 min, resuspended in 1 mL mitochondrial isolation buffer and pelleted. Stained mitochondria (9.7 mg protein) were suspended in 3.22 mL of buffer containing 4.8 mg digitonin, 80 mM sucrose, 10 mM Tris–HCl, 100 mM EGTA (pH 7.4) and 0.25 mM phenylmethyl sulfonyl fluoride (EM Science, Gibbstown, NJ, USA). Following incubation for 15 min at 0 °C, the mitoplasts were pelleted at 11,000 × *g* for 15 min, resuspended in 500 mL of buffer containing 80 mM sucrose, 10 mM Tris–HCl, 100 mM EGTA (pH 7.4), repelleted and resuspended in 1 mL mitochondrial isolation buffer. Prior to CE-LIF analysis, the mitoplast suspension was diluted five-fold in mitochondrial isolation buffer.

2.5. CE-LIF setup

The design and setup of the electrophoresis system with post-column LIF detection used for this study has been described previously [23,39]. The 488 nm line from an Argon-ion laser (Melles Griot, Irvine, CA, USA) was used for excitation. Fluorescence emission was monitored spectrally with an interference filter transmitting in the range of 517–552 nm (535DF35, Omega Optical, Brattleboro, VT, USA). In order to reduce the scattering at 488 nm that is caused by interactions between the laser beam and mitochondria or air bubbles, an additional rejection band filter (488-53D, OD4, Omega Optical) was placed in front of the interference filter.

2.6. CE-LIF analysis of mitochondria

Separations were carried out using poly-acryloylaminopropanol (poly-AAP) coated [40] fused silica capillaries (50 μm i.d. and 150 μm o.d.). The poly-AAP coating reduces the interactions between proteins associated with the mitochondrial surface and the capillary wall, and suppresses the electroosmotic flow. Stock solutions of 10^{-3} M fluorescein (Molecular Probes) were made in ethanol and diluted immediately prior to use. The detector was aligned by the continuous electrokinetic introduction of a 10^{-9} M solution of fluorescein in CE buffer at $-200\ V\ cm^{-1}$. Detector optimization was completed by observing the reproducibility of the fluorescence intensity produced by individual 1 μm diameter fluorescently-labeled latex beads (Polysciences Inc., Warrington, PA, USA). The relative standard deviation of their fluorescence intensities was found to be approximately 20% for approximately 300 events.

To reduce sample carryover between consecutive runs, the capillary was flushed sequentially with water (1 min), methanol (2 min), water (1 min), and CE buffer (1 min) using a syringe adaptor (Valco, Houston, TX, USA) and gentle manual pressure. It is our experience that the condition of a capillary changes over the course of several experiments, possibly due to degradation of the capillary coating or to modification of the capillary walls by the biological components in the sample. This change results in electroosmotic flow variations that introduce systematic changes in the observed electrophoretic mobility. To correct for electroosmotic flow changes, we used the migration time of fluorescein as a reference which resulted from electrokinetically injecting a 10^{-9} M solution of fluorescein in CE buffer at $-200\ V\ cm^{-1}$. Migration times for individually detected mitochondria, referred as detected events, were corrected using the migration time of fluorescein as a reference. This correction was needed then for carrying

out comparisons of mitochondrial separations performed on different days or different capillaries.

2.7. Data analysis

The output from the photomultiplier tube was electronically filtered (RC = 0.01 s) and then digitized using a PCI-MIO-16E-50 I/O board driven by LabVIEW software (National Instruments, Austin, TX, USA). The data were acquired at 50 or 100 Hz, stored as binary files, and then analyzed using Igor Pro software (Wavemetrics, Lake Oswego, OR, USA). The fluorescence intensities and migration times for individual events were tabulated using PickPeaks, an in-house written Igor Procedure that has been previously described [39]. The program selects those events with signal intensities higher than three times the standard deviation of the background. The PickPeaks output was divided into a pre-migration window, defined as the time frame prior to the detection of mitochondria, and a migration window (the time frame in which the mitochondria were detected). The fluorescence intensities of the events that occurred in the two windows were sorted and compared to facilitate the selection of a new threshold. The new threshold was applied to the PickPeaks output and the events in the migration time window were used to calculate the individual electrophoretic mobilities. Since some experiments were preformed on different days, all the electrophoretic mobilities had to be corrected based on the electrophoretic mobility of fluorescein.

3. Results and discussion

3.1. Effects of cell disruption techniques on electrophoretic mobility

Previously, we reported that isolated mitochondria selectively labeled with NAO can be detected individually by CE-LIF [23,24]. The resulting electropherograms show a collection of narrow events (ca. 70 ms wide) that were caused by individual mitochondria traveling through the tightly focused argon-ion laser beam of the LIF detector. As seen in Fig. 1(i), these narrow events span a wide migration time range, indicative that there is a wide electrophoretic mobility range for the sampled organelles. Based on the capillary length (L), the electric field (E), and the measured migration time (t) for each event, the apparent electrophoretic mobility (μ) is calculated as $\mu = LE^{-1}t^{-1}$. The set of electrophoretic mobility values is represented as a distribution (Fig. 1(ii)).

As suggested by the error bars located on each of the bins of the electrophoretic mobility distributions (Fig. 1(ii)), there is analytical variation among the CE-LIF replicate analyses. These variations may be caused by changes in the surface of the capillary that have led to changes in the electroosmotic flow. As indicated in Section 2, we used the migration time of fluorescein injected in a separate run to correct the migration times of the detected events so that we could compare

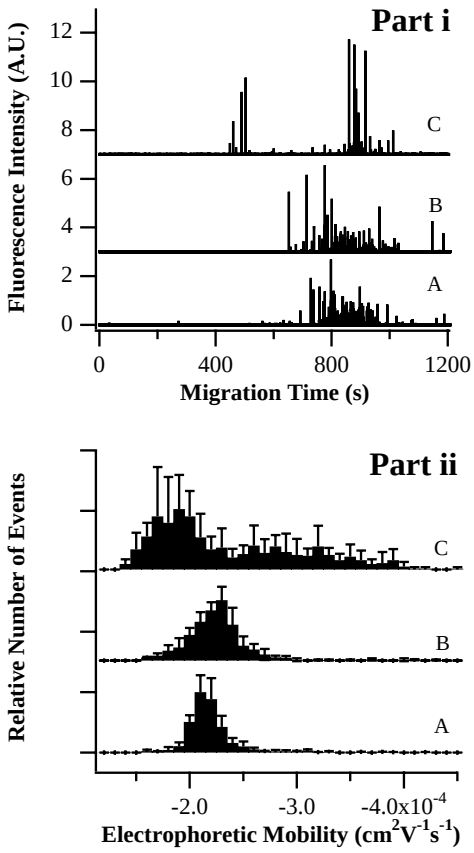

Fig. 1. (i) Electropherograms and (ii) electrophoretic mobility distributions of mitochondria from NS-1 cells. (A) NS-1 cells were disrupted by mechanical homogenization resulting in 75% breakage. (B) NS-1 cells were disrupted by two rounds of nitrogen cavitation, resulting in 100% breakage. (C) NS-1 cells were disrupted by one round of nitrogen cavitation, resulting in 95% breakage. Mitochondria were introduced by electrokinetic injection at $-200\,\mathrm{V\,cm^{-1}}$, and separated at $-200\,\mathrm{V\,cm^{-1}}$. Distributions were corrected based on the electrophoretic mobility of fluorescein. Values shown are averages of three replicate runs + 1 standard deviation and are normalized such that the bins in each histogram containing the greatest number of events are of equal height. A few events with very negative electrophoretic mobilities were omitted for clarity of display.

the data obtained on different days. In summary, run-to-run and day-to-day variations do not seem to impede comparison among the electrophoretic mobility distributions for the various disruption processes as long as the CE analysis of a standard is included in the series of runs.

It was anticipated that the mitochondria in our experiment would have an electrophoretic mobility distribution characteristic of the organelle source (i.e. NS-1 mouse hybridoma cell line) and that the electrophoretic mobility values would be mainly a function of the corresponding organelle surface charge. This expectation is in agreement with previous research in which it was found that the electrophoretic mobility of isolated mitochondrial outer membranes [5] and that of swollen mitochondria [21] did not differ from those of the originating organelle. It is not likely that the electrophoretic mobility is dependent on size since the individual event intensity, corresponding to the cardiolipin content and

Table 1
Corrected electrophoretic mobilities of NS-1 mitochondrial samples[a]

Disruption method	Percent disruption	Number of events	Average electrophoretic mobility[b]	Median electrophoretic mobility[b]
2 × cavitation	100	249 ± 48	−1.09 ± 0.04	−1.06 ± 0.03
1 × cavitation	95	212 ± 144	−1.40 ± 0.24	−1.07 ± 0.07
Mechanical homogenizer	75	378 ± 101	−1.15 ± 0.13	−1.02 ± 0.01

[a] Values shown are averages of three replicate runs, electrophoretic mobilities are adjusted based on the migration time of fluorescein. Other conditions are described in Fig. 1.
[b] Electrophoretic mobility values/10^{-4}($cm^2\ V^{-1}\ s^{-1}$).

thus organelle size [41], does not correlate with migration time.

On the other hand, it was found that the mitochondrial isolation procedure used affected both the general appearance of the electropherograms and the electrophoretic mobility distributions of mitochondria from NS-1 cultured cells ((i) and (ii) in Fig. 1A–C, respectively). In the electropherograms in Fig. 1(i), two migration windows can be seen for mitochondria derived from cells disrupted by a single round of cavitation (Fig. 1C); however, the earlier window is absent from the electropherograms of the other two samples, which either underwent two rounds of cavitation or were disrupted with a mechanical homogenizer. These differences might not be apparent in a comparison based on conventional statistics (Table 1) showing average and median values of electrophoretic mobility for the mitochondrial samples described in Fig. 1.

We offer two possible interpretations for the observed differences in the distributions from mitochondria isolated using different procedures: In the first interpretation (i), cytoskeletal proteins, such as tubulin and plectin, remain attached to the mitochondria after one round of cavitation (Fig. 1C of (i). These two proteins have been found to be closely associated with mitochondria [42] and when liver mitochondria were treated with colchicine, a cytoskeletal inhibitor, their average electrophoretic mobility was significantly altered [43], suggesting that the cytoskeletal components strongly affect the mitochondrial electrophoretic mobility. According to this interpretation, an additional round of cavitation or the shear forces produced by mechanical homogenization could remove some of this cytoskeleton, thus producing mitochondria with more homogeneous electrophoretic mobilities (Fig. 1B and 1A of (i), respectively). In effect, homogenization or multiple rounds of cavitation may “clean” the mitochondria.

For the second interpretation (ii), the mitochondria released by cavitating the NS-1 cells a single time (Fig. 1C of (i) retain a higher degree of structural integrity than those released by doubly cavitating or homogenizing the cells. This would imply that the maxima at approximately $-2.2 \times 10^{-4}\ cm^2\ V^{-1}\ s^{-1}$ in the three electrophoretic mobility histograms correspond to fragmented mitochondria, while the mitochondria with higher electrophoretic mobilities are less damaged. The fragmented and intact mitochondria would have different surface compositions due to varying distributions of proteins, carbohydrates and lipids throughout the organelle. If this explanation is correct, we can conclude that mechanical homogenization results in more mitochondrial breakage than does nitrogen cavitation which does not subject the cells to shear forces. Nitrogen cavitation has been demonstrated to be a gentle method of cell disruption that produces intact organelles based on enzymatic studies [44,45]. Further discussion of the validity of both of these explanations is presented below.

3.2. Fluorescence intensities of mitochondria from NS-1 cells

The intensity distribution of individual events supports explanation (i) over explanation (ii). As mentioned earlier, the intensity of an event is related to the amount of NAO stoichiometrically bound to the cardiolipin found in the corresponding organelle; thus intensity is related to the organelle size [41]. Therefore, fluorescence intensities of the narrow events in the electropherogram shown in Fig. 1(i), represented as histograms in Fig. 2, indicate that the amount

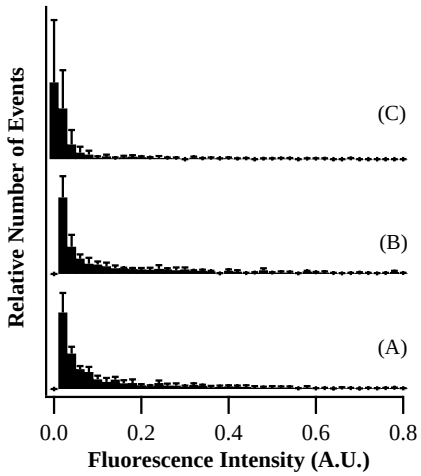

Fig. 2. Fluorescence intensity histograms of mitochondria from NS-1 cells. Fluorescence intensity histograms were normalized such that the fullest bin in each data set contained equal numbers of events. Assignments (A)–(C) are shown in Fig. 1. Values shown are averages of three replicate runs + 1 standard deviation. A few events with very high fluorescence intensities were omitted for clarity of display. The first bin in (C) results from a lower background, thus lower threshold, on the day these data were collected. However, the sensitivity of the instrument was unchanged.

of NAO does not differ between the preparations subject to one or two rounds of cavitation or to mechanical homogenization. The first bin of distribution C in Fig. 2 has events in the first bin, but this is the result of a background change (i.e. threshold change) in the electropherograms that were used to obtain the raw data. Moreover, a change in one single bin cannot account for disruption or aggregation that would cause a dramatic change in the distribution. More specifically, if mitochondria were to fragment or aggregate, then the *entire* distribution would shift towards lower or higher fluorescence intensity values, respectively. Within the error associated with each bin, there are not significant differences between these distributions (Fig. 2), suggesting disruption or aggregation is not occurring after a second round of cavitation or when using mechanical homogenization. Indeed, since the LIF detector used for this work shows an R.S.D. of 20% for the fluorescently labeled microspheres used as standards in flow cytometry, variations of <20% in event intensity distributions corresponding to aggregation or fragmentation would not be detectable. This also suggests that, at most, only a small fraction of mitochondria may be affected by the cell disruption process. Thus, the effects of cell disruption cannot explain the drastic changes in the electrophoretic mobility distributions observed in (Fig. 1(ii)). Altogether, this argument is further evidence that mechanical disruption or repeated nitrogen cavitation is more likely a "cleaning" than a disruption process.

3.3. Effects of freezing and thawing of rat liver mitochondria

Since rat liver mitochondria purified by density gradient centrifugation are considered to be the gold standard in mitochondrial research, we decided to use one of these organelle preparations to further investigate whether CE-LIF analysis can adequately monitor the status of mitochondria in a preparation. In particular, we were interested in determining if freezing in liquid nitrogen, a convenient way of decreasing animal tissue consumption and facilitating the exchange of materials between research groups at institutions located far apart, has an effect on the electrophoretic mobility distributions of mitochondria. In Fig. 3, we compare two electrophoretic mobility distributions: one was produced by mitochondria taken from fresh rat liver, shipped on ice by overnight courier from one research laboratory to another (Part B), and the other by mitochondria from the same batch after it was frozen and thawed out (Part A). The electrophoretic mobility distribution of the fresh mitochondria lacks the second maxima seen in the sample that had been frozen and thawed out. Further statistics (Table 2) indicate that freezing/thawing made the average and median electrophoretic mobility of mitochondria more negative with respect to that of fresh mitochondria. For instance, the median changed from $(-1.88 \pm 0.07) \times 10^{-4}$ to $(-2.75 \pm 0.21) \times 10^{-4}\,\mathrm{cm^2\,V^{-1}\,s^{-1}}$.

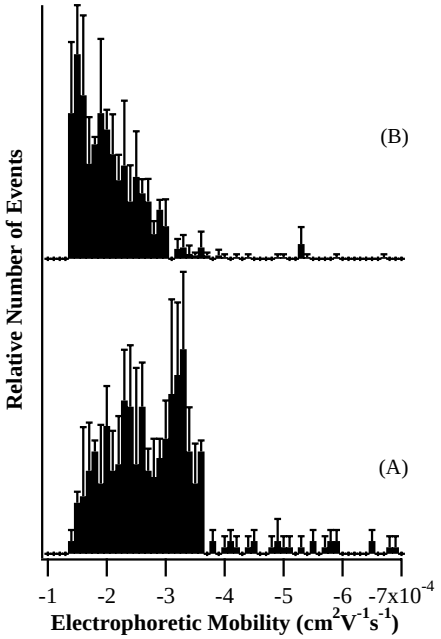

Fig. 3. Electrophoretic mobility distributions from: (A) frozen/thawed out and (B) fresh rat liver mitochondria. A few events with very negative electrophoretic mobilities were omitted for clarity of display. Other conditions are the same as in Fig. 1.

There are two possible explanations for the appearance of the second maxima in the frozen/thawed out sample. First, the DMSO that was used as a cryoprotectant to minimize the formation of damaging ice crystals could have modified the surface of some of the mitochondria, increasing their electrophoretic mobilities. Another possibility is that, even in the presence of the cryoprotectant, the freezing and thawing processes may have damaged some of the mitochondria, exposing biomolecules with more negative surface charges, either as a result of fragmentation due to ice crystal formation, or by inducing changes in the membrane structure, such as permeability changes [42]. However, electron microscopy imaging (data not presented) did not show any apparent mitochondrial damage after freezing and thawing, ruling out fragmentation as a viable explanation for the observed changes in the electrophoretic mobility distribution. An extensive study investigating several freezing/thawing condi-

Table 2
Comparison of electrophoretic mobilities of rat liver mitochondria

Treatment[a]	Number of events	Average electrophoretic mobility[b]	Median electrophoretic mobility[b]
Fresh	357 ± 34	−2.41 ± 0.31	−1.88 ± 0.07
Frozen/thawed	142 ± 30	−3.70 ± 0.25	−2.75 ± 0.21
Mitoplasts	181 ± 46	−7.82 ± 0.98	−2.82 ± 0.21

[a] Values shown are averages of three replicate runs, electrophoretic mobilities are adjusted based on the migration time of fluorescein. Other conditions are described in Figs. 3 and 4.

[b] Electrophoretic mobility values/10^{-4} ($\mathrm{cm^2\,V^{-1}\,s^{-1}}$) and adjusted based on the migration time of fluorescein.

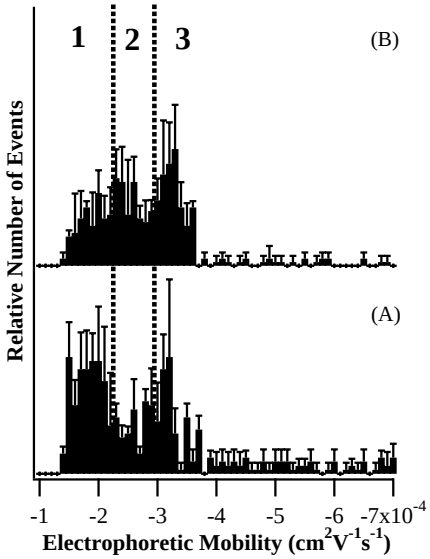

Fig. 4. (A) Electrophoretic mobility distributions of rat liver mitoplasts and (B) rat liver mitochondria. Mitochondria were preserved frozen and then thawed out before mitoplasts were prepared. A few events with very negative electrophoretic mobilities were omitted for clarity of display. Other conditions as in Fig. 1.

tions may help elucidate the causes for the observed transformation in the mitochondrial electrophoretic mobilities.

3.4. Mitoplast formation

The formation of mitoplasts by using a concentrated digitonin solution to remove the outer mitochondrial membranes was also expected to produce significant alterations to mitochondrial topology, charge distribution, and electrophoretic mobility. This has been confirmed by comparison of the electrophoretic mobility distributions of rat liver mitochondria and mitoplasts made from the same sample (Fig. 4).

The distributions shown in Fig. 4 were divided into three regions corresponding to the three apparent groupings of events and are tabulated in Table 3. The percent number of events in regions 1 and 2 of the mitochondrial electrophoretic mobility distributions does not differ (Fig. 4B), whereas the percent number of events in region 1 of the mitoplast electrophoretic mobility distribution is greater than in region 2 (Fig. 4A). Comparison between the mitoplasts and mitochondria shows no difference, within 1 standard deviation, in the percent number of events in region 1. However, the percent number of events within region 2 of the mitochondrion and mitoplasts differed within 1 standard deviation. The more marked difference is found within region 3 where there were more than 2.5 times as many events in the mitoplast distribution than in the mitochondrial distribution. The higher electrophoretic mobility variation observed for the mitoplast preparation (Fig. 4A) may be caused by the partial removal of the outer mitochondrial membrane or the exposure of a more heterogeneous surface (i.e. inner mitochondrial membrane) with different electrophoretic mobility characteristics. These data show that electrophoretic mobility distributions are sensitive to surface composition, and more specifically, that mitoplasts tend to have a more negative electrophoretic mobility than untreated mitochondria

Table 3
Fraction of events in the regions presented in Fig. 4

Sample/percentage of events	Region 1	Region 2	Region 3
Electrophoretic mobility range[a]	1.4–2.2	2.3–2.9	>3.0
Mitochondria (%)	43.6 ± 12.3	48.0 ± 3.6	8.4 ± 2.1
Mitoplasts (%)	46.1 ± 6.3	32.2 ± 6.1	21.7 ± 1.8

[a] Electrophoretic mobility values/-10^{-4} ($cm^2\ V^{-1}\ s^{-1}$). Other experimental conditions are described in Fig. 4.

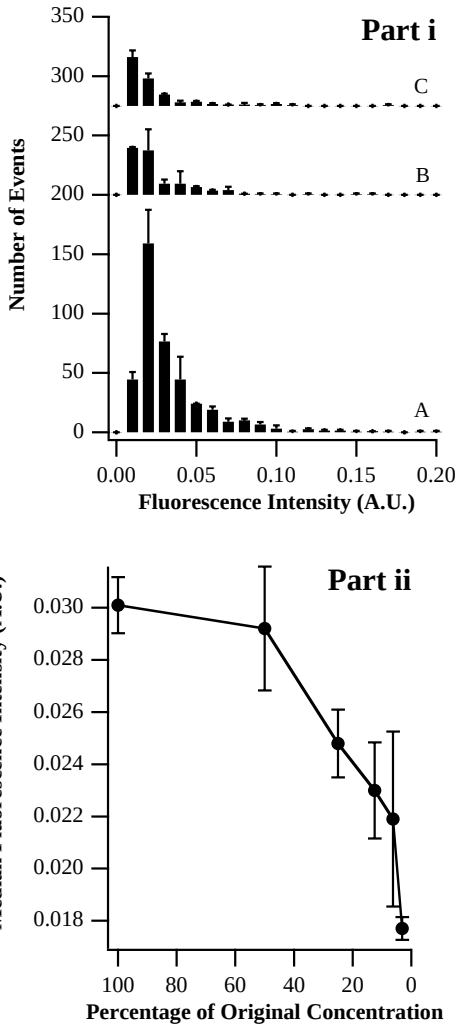

Fig. 5. Changes in intensity distribution with dilution. (i) The fluorescence intensity distributions for three dilutions of frozen rat liver mitochondria. (A) Original suspension; (B) eight-fold dilution of original suspension; (C) 16-fold dilution of original suspension. Values shown are averages of two replicate runs + one-half range. A few events with very high fluorescence intensities were omitted for clarity of display. (ii) The median fluorescence intensity of all fluorescence events is plotted against sample concentration. The conditions for obtaining the initial electropherograms were the same as in Fig. 1.

(Table 2). This is borne out from the data in this table where the the mitoplasts have a higher average electrophoretic mobility $(-7.82 \pm 0.98) \times 10^{-4}\,cm^2\,V^{-1}\,s^{-1}$ than the average of frozen mitochondria that were used to prepare the mitoplasts $(-3.70 \pm 0.25) \times 10^{-4}\,cm^2\,V^{-1}\,s^{-1}$.

3.5. Aggregation of frozen/thawed out rat liver mitochondria

As expected, the corresponding electropherograms (not shown) for the electrophoretic mobility distributions of frozen and thawed out mitochondria (Fig. 3A) do not indicate that event height (i.e. organelle size) is correlated with electrophoretic mobility (cf. electropherogram for NS-1 mitochondria, Fig. 1(i)). Also, no fragmentation or aggregation was revealed by electron microscopy (data not shown).

On the other hand, the texture of the mitochondrial preparation was pasty in appearance, suggesting that organelle aggregation may have occurred. As described earlier in this section, if aggregation is present, the event intensity distributions will be shifted towards higher values. In Fig. 5(i), three fluorescence intensity distributions are shown for samples of varying mitochondrial density numbers. The maxima of the distributions shift increasingly towards lower fluorescence intensities with increasing dilution, indicating that aggregates form at higher concentrations and dissociate as the sample is diluted. The same trend is observed when plotting the median fluorescence intensity versus dilution (Fig. 5(ii)). The events detected in more concentrated samples have a higher median fluorescence intensity than those from more diluted samples. Thus, even when electrophoretic mobility is not expected to be a function of size, assessment of mitochondrial properties by CE-LIF needs to consider the possible presence of aggregates if the sample has not been adequately diluted.

4. Conclusions

CE-LIF analysis makes it possible to detect individual mitochondria as discrete events. By evaluating the migration times of the sampled mitochondria, we can construct electrophoretic mobility distributions. Since it is believed that electrophoretic mobility is a direct probe of mitochondrial surface characteristics, it is possible to use the mobility distribution as a parameter that distinguishes among samples of mitochondria produced using different cell disruption techniques, as well as between mitochondria before and after undergoing freezing/thawing or chemical modification into mitoplasts. It was determined that freezing fresh mitochondria changes their electrophoretic mobility distribution, either due to surface modifications resulting from the cryoprotectant used, or to changes induced by the freezing process. Similarly, the production of mitoplasts by removing the mitochondrial outer membrane causes the electrophoretic mobility distribution to change. In both cases, sample preparation is clearly introducing alterations in the mitochondrial surface charge distribution. Likewise, the method used to disrupt NS-1 cells influences the electrophoretic mobility distribution of the isolated mitochondria. In the case of purified mitochondria, even though there is no significant change in electrophoretic mobility, the fluorescence intensities clearly indicate the presence of aggregates in the more concentrated samples.

Overall, this work demonstrates the ability of CE-LIF to measure changes in the electrophoretic mobility of mitochondria that result from the preparation procedure or handling. Electrophoretic mobility distributions can also be used to gain more insight into mitochondrial surface attributes. For instance, collecting mitochondria with differing electrophoretic mobilities after their detection for further characterization by electron microscopy, atomic force microscopy, or mass spectrometry in order to correlate their surface characteristics with electrophoretic mobility would seem to be a worthwhile direction for future research. In addition, electrophoretic mobility distributions can be used as a guide when designing preparative strategies for purifying mitochondria or other organelles in bulk.

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