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Simultaneous Laser-Induced Fluorescence and Scattering Detection of Individual Particles Separated by Capillary Electrophoresis

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This technical note describes a detector capable of simultaneously monitoring scattering and fluorescence signals of individual particles separated by capillary electrophoresis. Due to its nonselective nature, scattering alone is not sufficient to identify analyte particles. However, when the analyte particles are fluorescent, the detector described here is able to identify simultaneously occurring scattering and fluorescent signals, even when contaminating particles (i.e., nonfluorescent) are present. Both fluorescent polystyrene particles and 10-nonyl acridine orange (NAO)-labeled mitochondria were used as models. Fluorescence versus scattering (FVS) plots made it possible to identify two types of particles and a contaminant in a mixture of polystyrene particles. We also analyzed NAO-labeled mitochondria before and after cryogenic storage; the mitochondria FVS plots changed with storage, which suggests that the detector reported here is suitable for monitoring subtle changes in mitochondrial morphology that would not be revealed by monitoring only fluorescence or scattering signals.

Capillary electrophoresis (CE) has been employed in the study of metal nanoparticles, liposomes, subcellular organelles, and other biologically important particles.[1,2] Common CE detectors employ UV−vis absorption,[3] surface-enhanced Raman[4] and refraction or dynamic adsorption.[3] In addition, laser-induced fluorescence (LIF) detectors have been used for CE analysis of individual fluorescent submicrometer particles.[5] While LIF detectors provide information about the content of fluorescent molecules in the analyte particles, scattering may provide additional information in the CE analysis of individual particles. For instance, light scattering (LS) detection would provide information on scattering-related properties (e.g., size and, to some extent, internal structure).[6] Unfortunately, scattering detectors are nonspecific and contaminating particles may overwhelm the scattering signal of the analyte particles.

In this technical note, we describe a light scattering laser-induced fluorescence (LS-LIF) detector for individual submicrometer particle CE analysis. With the use of this detector configuration, the fluorescence signals allow us to identify the corresponding scattering signals of the particles of interest (e.g., latex microspheres or mitochondria) in the presence of other, nonfluorescent particulates. The LS-LIF detector further allows for identification of particle types even when the electrophoretic mobility of the particles is similar and for detection of changes in mitochondria that have been kept under cryogenic storage. While this report exemplifies the feasibility of measuring scattering signals of analyte particles, further developments in scattering detectors for CE (e.g., dual-angle scattering[7]) may facilitate the direct sizing of organelles, which presently relies on the accumulation of fluorescent markers.

REAGENTS AND METHODS

Reagents. *N*-(2-Hydroxyethyl)-piperazine-*N*-(ethanesulfonic acid) (HEPES), *N*′,*N*′,*N*′,*N*′-tetramethylethylenediamine (TEMED), sodium dodecylsulfate (SDS), 4-(1,1,3,3-tetramethylbutyl) phenyl-polyethylene glycol (Triton X-100), ammonium persulfate, sulfuric acid, fluorescein, 3-aminopropanol, acryloyl chloride, acrylonitrile, dichloromethane, acetone, methanol, KOH, and TLC plates were purchased from Sigma (St. Louis, MO). Allyltriethoxysilane was purchased from Gelest (Morrisville, PA). Fused-silica capillaries were purchased from PolyMicro (Phoenix, AZ). Alexa-488-labeled microspheres with diameters of 200, 500, and 1000 nm were purchased from PolySciences (Warrington, PA). 10-*N*-Nonyl acridine orange (NAO) was purchased from Invitrogen (Eugene, OR). Carboxy-cellulose membranes were purchased from Fisher (Florence, KY).

Buffer Preparation. The CE buffer for the analysis of latex microspheres contained 10 mM HEPES titrated with 1 M KOH to pH 7.4 (HEPES buffer). This buffer was modified for the analysis of mitochondria by adding sucrose to obtain a final concentration of 250 mM (sucrose−HEPES buffer). Buffer for the isolation of mitochondria contained 210 mM D-mannitol, 70 mM sucrose, 5 mM HEPES, and 5 mM EDTA, adjusted to pH 7.4 with potassium hydroxide (isolation buffer). Buffers were filtered through 400 nm carboxy-cellulose membranes before each experiment. Very low particulate buffers, used in experiments aimed at extensive scattering background reduction, were prepared by an additional filtering step through 20 nm pore alumina membranes (Whatman Inc., Clifton, NJ).

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1-Acryloly-3-hydroxyaminopropanol (AAP) Synthesis. AAP has been synthesized using a modified procedure.[8] Briefly, a 10% solution of acryloyl chloride in dichloromethane was slowly added to 2-fold excess of 10% 3-aminopropanol in dichloromethane at −35 °C. The AAP solution in dichloromethane was separated from a viscous layer and purified on a silica gel column with acetone as the eluent. AAP was stabilized by 0.004% (w/w) of *p*-hydroxyanisol, ∼10-fold excess of D_2O was added (D_2O was used instead of H_2O to simplify further monitoring of quality), and the acetone was evaporated at 0 °C. The product was analyzed by NMR and LC−MS and stored as ∼10% solution in D_2O at −20 °C.

Capillary Modification. To decrease interactions between the analyte particles and the capillary walls, the walls were modified by AAP. The sol−gel procedure was used for introduction of allyl anchors, and copolymerization of AAP and the anchors was performed as described.[8] A 5 m long piece of fused-silica capillary (50 μm i.d.) was flushed with 0.1 M NaOH for 3 h at 35 psi, followed by water for 10 min, 2% HCl for 1 h, and methanol for 1 h at the same pressure. Next, the capillary was dried with Ar (40 psi) at 140 °C for 1 day. Then a 5% solution of allyltriethoxysilane in toluene was flushed through the capillary for 10 min at 35 psi. The capillary ends were capped, and the capillary was incubated in an oven at 140 °C for 2 days, followed by a toluene flush. Electroosmotic flow (EOF) was measured as previously described[9] and was found to have decreased from 1.28×10^{-4} cm^2 V^{-1} s^{-1} in the bare capillary to 0.70×10^{-4} cm^2 V^{-1} s^{-1} in the treated one, indicating a decrease in the amount of free siloxane groups. Two 1 m long capillary pieces were flushed with 3% AAP, 0.04% ammonium persulfate, and 0.1% TEMED in water for 5 min at 35 psi. This treatment was followed by 1 h of incubation without pressure. Next, the capillary contents were flushed out with water at 35 psi. The capillary was cut into ∼30−35 cm long segments. After measuring the final EOF (ex. 2.87×10^{-6} cm^2 V^{-1} s^{-1}), the capillaries were flushed with water and Ar at 35 psi for 15 min each, capped, and stored at room temperature.

Mitochondria Sample Preparation, Characterization, and Labeling. The mitochondria samples were prepared from liver of freshly sacrificed rat (kindly provided by Dr. LaDora Thompson). The liver was cut into ∼1 mm^3 pieces, suspended in isolation buffer, and homogenized mechanically (clearance 0.0035−0.0055″ × 90 strokes; clearance 0.001−0.001″ × 90 strokes) at 0 °C. The cell debris was removed by 2 × 10 m centrifugation at 2kG, and mitochondria were sedimented at 12 kG during 20 m, resuspended in isolation buffer, and immediately used for labeling or frozen in liquid nitrogen until needed (typically 1−2 months).

The identity of the mitochondria in the samples used (fresh or frozen) was confirmed by TEM (Figure 4C−F). The samples for TEM were prepared by fixing the mitochondria using gluteraldehyde, followed by embedding them in a resin.

Fluorescent labeling was performed by resuspension of ∼1 mm^3 of the mitochondrial pellet in 1 mL of sucrose−HEPES buffer, followed by addition of 10 μL of 100 μM NAO in the same buffer and incubation for 20 min at 4 °C. The labeled mitochondria were immediately used in CE experiments.

Capillary Electrophoresis. A custom-built CE-LIF instrument, used as the basis for this work, has been previously described.[5]

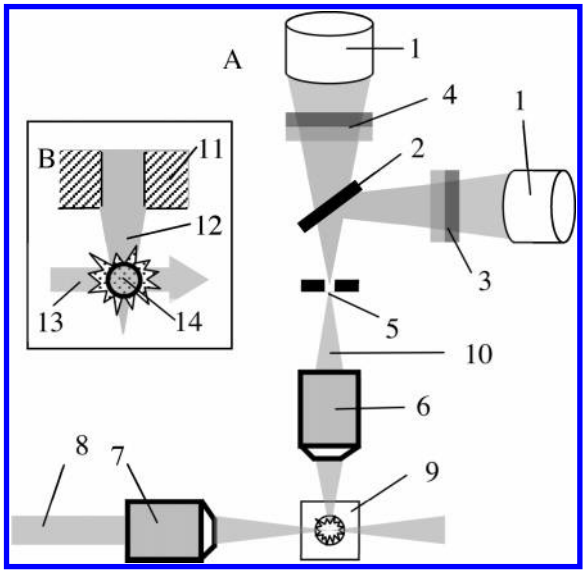

Figure 1. Scheme of the LS-LIF detector. A: The detector for simultaneous analysis of scattering and fluorescence signals, 1—PMTs, 2—beam splitter, 3—scattering filter set (narrow band-pass for 488 nm and neutral density filters), 4—fluorescence filter set (long-pass above 510 and 535 nm band-pass), 5—pinhole, 6—signal collection objective, 7—laser-focusing objective, 8—laser beam, 9—sheath-flow cuvette, 10—collected light. B: The side view of the detection volume defined by the overlapping region of the capillary outflow and the laser beam, 11—capillary walls, 12—capillary outflow, 13—laser beam, 14—volume from which light is collected.

Briefly, the instrument uses a sheath flow cuvette to house the end of the CE capillary and employs postcolumn laser excitation and collection of the optical signal. A 488 nm line from an argon-ion laser (Melles Griot, Irvine, CA) was used for excitation. Both fluorescence and scattering signals were collected 90° from the laser beam with a 60× objective with NA 0.7 (Mitutoyo, Japan). A 1 mm diameter pinhole was used to spatially select the fluorescence and scattering light from the detection volume. The selected light was then separated into two channels by a glass beam splitter positioned at 45°. This splitter was made from a microscope glass slide, which has an expected reflection efficiency of 6%. The less intense reflected light was used for scattering detection. In this detector, the light passed through a neutral density filter (OD = 2), narrow band-pass filter (488 ± 1.5 nm, NB3, Omega Optical, Bratteboro, VT), and was detected by a photomultiplier tube (PMT) (R1477, Hamamatsu Corp., Bridgewater, NJ). The light transmitted by the beam splitter (∼94%) was directed toward the fluorescence detector. In this detector, the light passed through a 505 nm long-pass filter and a 530 nm band-pass filter (Omega Optical, Bratteboro, VT) and was detected by a second PMT (Figure 1A). The PMT outputs were electronically filtered (RC = 0.01 s), digitized using a PCI-MIO-16E-50 I/O card at 100 Hz data collection frequency, and stored as a binary file. No significant cross-talking was observed for signals from the analyzed particles.

This detector uses a sheath flow cuvette to house the end of the CE capillary and employs postcolumn laser excitation and collection of fluorescence and scattering signals, thereby avoiding scattering from capillary walls (Figure 1B). The sheath flow was driven by application of 300 Pa pressure, which was applied by elevating a reservoir containing the same buffer used for the CE separation. This pressure results in a 1.25 mm/s sheath flow linear

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velocity, which is sufficient to wash out particles as they migrate out from the capillary. We chose to use a relatively long distance of ~200 μm between the capillary edge and the laser beam because it resulted in more consistent trajectories of particles through the detector volume.

Before installing a capillary into the sheath flow cuvette of the LS-LIF detector, the capillary end was polished by pushing it against an abrasive rotating disk. To remove the outer polyimide coating without damaging the internal AAP coating, the capillary end was inserted into hot (95 °C) sulfuric acid, while water was constantly flowing through the capillary. Next, the capillary's internal surface was flushed with running buffer for 1 h at 10 psi. The instrument was aligned with 10^{-9} M fluorescein solution in HEPES buffer for a maximal signal-to-noise (S/N) ratio resulting in a limit of detection of ~1 zmole.

Sample injection was performed electrokinetically by placing the capillary end into the particle suspension and applying a −300 V/cm electric field for 10 s. Separations were performed using the same electric field. Detection of mitochondria and particles with diameters of 200 nm was performed at 1000 V PMT bias, whereas the PMT bias was reduced to 800 V for particles with 500 and 1000 nm diameters.

Data Analysis. Raw data were analyzed using in-house written IgorPro automated procedures (available upon request). The raw binary data files contain three separate waves (arrays): one for the scattering signal, one for the fluorescence signal, and one for the electrical current. The electrical current wave was used to monitor the capillary condition and eliminate data points that were not collected during the separation. All subsequent operations were performed on the scattering and fluorescence waves. These waves were processed with the WildPoint procedure,[10] and the resulting waves, describing the background drifting and broad electropherogram features, were subtracted from the original data. The resulting waves were transformed into waves of maximum intensity points for sharp peaks, which exceed a threshold (i.e., 3 times the standard deviation of the background), called peakwaves. The data containing the maximum intensities were stored in the fluorescence and scattering peakwaves and represented as histograms of peak intensities. Usually, a histogram representation was used to define a new threshold, which eliminated peaks that did not belong to the distribution of fluorescent analyte particles. In addition, for each peak intensity value in a given peakwave, a corresponding migration time value was stored in a matching array called timewave. The values in the timewave arrays were used to identify those detected events that were simultaneously detected in the fluorescence and the scattering detector (i.e., within 20 ms of each other). Both the scattering and the fluorescence maximum intensities of the simultaneously detected events were stored in new waves referred to as "common" peakwaves and common timewave. These "common" peakwaves were used to plot fluorescence versus scattering peak intensities (i.e., FVS plots). Patterns in the FVS plots were analyzed, and subgroups were defined graphically. When a comparison between these subgroups was needed, the scattering and fluorescence peak intensities and the migration times or electrophoretic mobilities of these subgroups were represented as histograms or as FVS plots.

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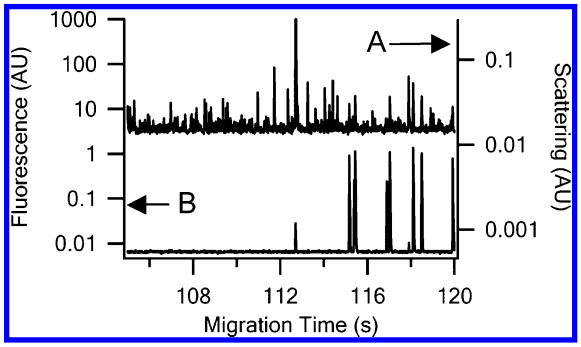

Figure 2. Beginning of the migration time window in an electropherogram of polystyrene microspheres. The sample contains a mixture of 500 and 1000 nm diameter fluorescent microspheres. The separation was performed at −300 V/cm in HEPES buffer. PMT bias at 800 V. A 17.5 mW, 488 nm argon-ion laser was focused down to ~20 μm diameter beam. Traces A and B are the scattering and fluorescence signals, respectively. Average peak widths are 53 ms for scattering and 40 ms for fluorescence, with standard deviations of 18 and 10 ms, respectively. AU stands for arbitrary units.

RESULTS AND DISCUSSION

Simultaneous Fluorescence and Scattering Detection of Latex Microspheres. The detection of individual particles is essential to determine how properties and contents vary within particle ensembles. In the CE analysis of individual particles detection is commonly performed using LIF, a detection scheme that offers high sensitivity and selectivity. Light scattering could also be a sensitive detection scheme for the CE analysis of individual particles, but due to its poor selectivity, scattering detection has not used for the CE analysis of particles. In this technical note, we describe a simple detection scheme that makes it feasible to measure the scattering signals of analyte particles.

Using the detector shown in Figure 1, we simultaneously detected scattering and fluorescence signals. Figure 2 is an expansion of an electropherogram around the time at which polystyrene microspheres reach the detector (i.e., ~112 s). (The whole electropherogram is available in the Supporting Information, Figure S2.) It is clear from Figure 2 that both fluorescence (bottom trace) and scattering (upper trace) signals are continuously monitored and that these signals describe different behaviors. The scattering signal alone (Figure 2, upper trace) shows that, even before the polystyrene microspheres begin to appear (Figure 2, lower trace), there is a high number of detected scattering events. Thus, scattering detection alone does not provide selectivity in the detection of the polystyrene microspheres. Other scattering species appear to originate from the sample, the CE separation buffer, and the sheath flow buffer. These other sources of scattering species were identified by measuring the scattering response of the detector when the separation electric field was on or off and by injecting control samples (i.e., without microspheres) (data not shown). Although elimination of these unwanted scattering species would be beneficial, we found that even for the most carefully degassed and filtered buffers, after 24 h of flushing the lines and detector, many scattering events were still detected (Supporting Information, Figure S3). Thus, it was necessary to rely on the fluorescence signal to identify the scattering response of the analyte particles.

To identify the scattering response of analyte particles (e.g., polystyrene microparticles) in the presence of other scattering events, the LIF signal of fluorescent polystyrene microparticles

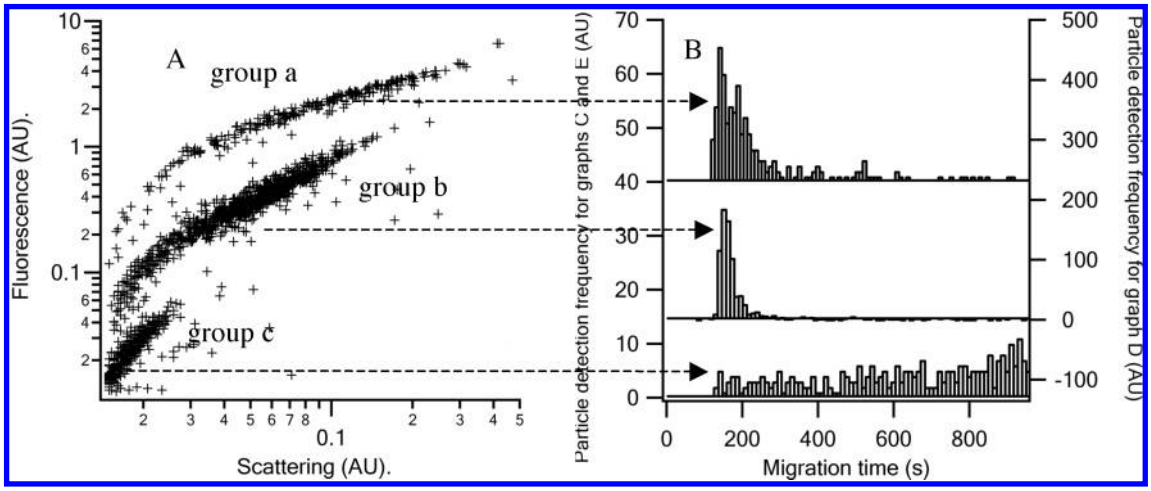

Figure 3. FVS plots and migration time distributions of particle mixtures. Part A shows the FVS plot of a mixture, containing 500 and 1000 nm diameter microspheres. The plot corresponds to the electropherogram shown in Figure 2S (Supporting Information) and partially shown in Figure 2. A total of 1500 events (17% of scattering events) have matching migration times in both detectors and form three well-defined groups with 229 (group a), 875 (group b), and 386 (group c) events. In addition, there are 8998 and 196 events that appear only in the scattering or fluorescence detectors, respectively. These peaks are not represented in this plot, and they are attributed to scattering by contaminant particles in the system and background noise, respectively. PMTs were biased at 800 V. Part B shows the migration time histograms for groups identified in part A. The left *y*-axis is for the histograms of the "group a", offset 40 AU, and the "group c". The right *y*-axis is for the histogram for "group b". Bin widths are 10 s.

was employed (Figure 2, lower trace). The appearance of fluorescent events after ~112 s is in agreement with previous reports on the CE-LIF analysis of individual polystyrene microparticles.[11] With the use of the IgorPro routines described in the Reagents and Methods, the scattering and fluorescent peaks with matching migration times (within 20 ms) were selected. For instance, this approach allowed for the identification of ~1500 scattering peaks of analyte particles detected among ~10 500 scattering peaks caused by other species. (Raw data can be found in the Supporting Information, Figure 2S.)

Figure 3A shows an FVS plot for the analyte particles detected in an electropherogram resulting from the analysis of a mixture of polystyrene microspheres (cf., Figure 2S in the Supporting Information). Three distinct groups, assigned to two particle sizes (i.e., 500 and 1000 nm diameter) and a group of lower intensity events (possibly pieces of fractured analyte particles or aggregates of surfactants, used by the manufacturer to stabilize the polystyrene particles), are seen in this plot.

For each group in the FVS plot, the elongated shape is caused the variations in the particles' trajectories as they travel through the detector (Figure 1A); particles crossing the center or the periphery of the laser beam will be illuminated differently. The efficiency of the optics used to direct photons from the detection volume to the PMTs is also trajectory-dependent. Thus, while the differences in the trajectories of particles result in PMT signals that change consistently they also result in groups that stretch into lines in the FVS plots. (cf., Figure 3A and Figures S1 and S4, Supporting Information).

More homogeneous illumination is obtained by defocusing the laser beam. When this optical alignment is used, FVS plots show elliptical, nearly circular areas for homogeneous particles (see Figure S4, Supporting Information). However, this configuration causes a decrease in the instrument's average sensitivity, we preferred to continue our studies using a tightly focused laser beam (cf., Figure 1).

Each group of particles produces a characteristic group of events on a FVS plot (Figure 3A and Figure 1S, Supporting Information). Since a fluorescence signal is proportional to the particle's volume, and the scattering signal generally increases with the particle's diameter, larger particles define groups with high scattering and intensity values in an FVS plot. For example, the groups a and b in Figure 3A correspond to the 1000 and 500 nm diameter microspheres, respectively. FVS plots for other particles sizes, such as 200 nm diameter, are available in the Supporting Information (Figure S1).

If only scattering or only fluorescence signals were being detected, it would be impossible to distinguish the three groups shown in Figure 3A, as is appreciated by projecting the values of each group onto a single axis. Similarly, it would be impossible to determine the migration times for the species in each group. Fortunately, the IgorPro routines described in the Reagents and Methods make it possible to obtain the migration times corresponding to the events falling into each one of the groups shown in an FVS plot. Figure 3B shows the migration times for the events in each of the groups of Figure 3A. Thus, the migration times for the "group a" (1000 nm diameter particles) and "group b" (500 nm particles) can be extracted from the FVS plots, making information about the electrophoretic behavior of these particles available.

The migration times for the species in "group c" (Figure 3A) reveal the very distinct nature of these species. They start migrating out from capillary at about the same time as those particles in groups a and b, which indicates that they originate in the mixture of microspheres that was injected into the separation capillary. However, they do not form a well-defined migration time band, as observed for the groups a and b (Figure 3B); instead they continue to migrate out of the capillary until the end of the experiment. This behavior, and their lower signal intensities, suggest that these species are either fragments of analyte particles or surfactant aggregates that have higher affinity toward the coated surface of the capillary.

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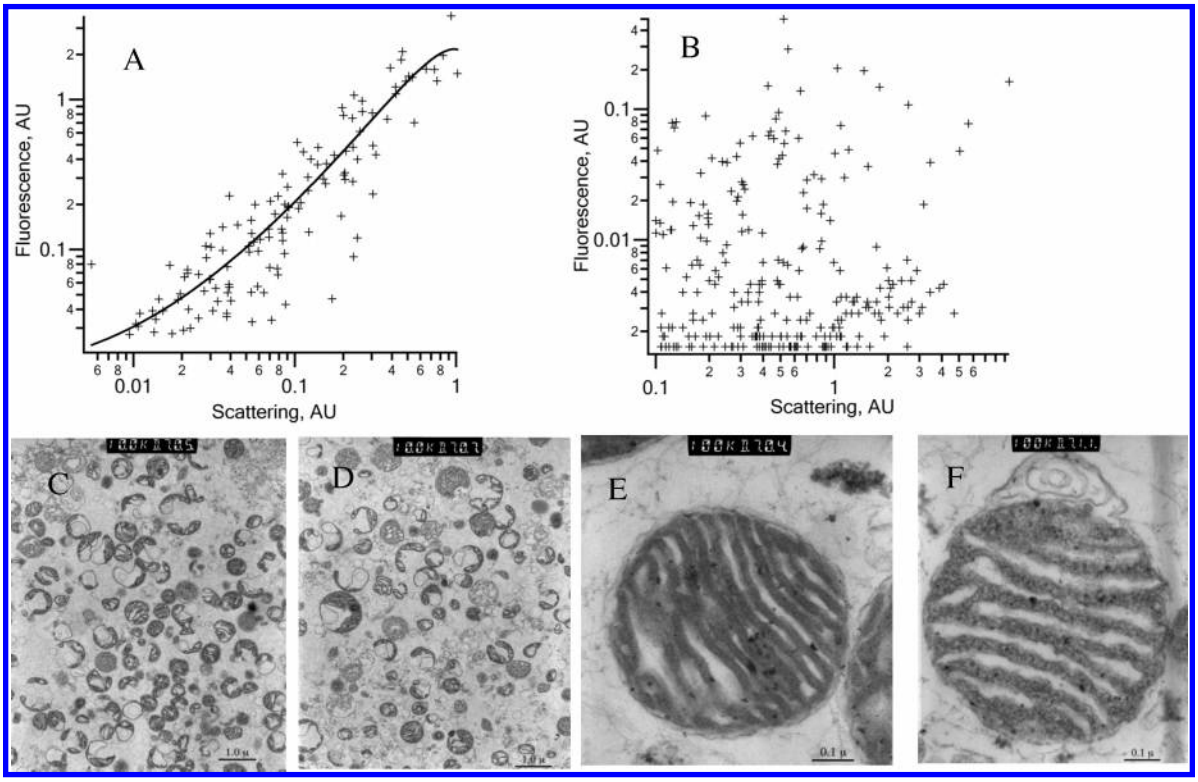

Figure 4. Fresh and frozen mitochondria. Freshly prepared (A) and frozen (B) rat liver mitochondria were stained with NAO. Detection conditions are as in Figure 2, but the PMTs were biased at 1000 V. TEM reveals structures of freshly prepared mitochondria (C and E) and mitochondria from frozen stock (D and F).

Fluorescence and Scattering Detection of Mitochondria. Mitochondria were fluorescently labeled with a mitochondrion-selective probe, NAO, that binds to cardiolipin in the inner mitochondrial membrane.[12] Figure 4A shows an FVS plot of freshly prepared mitochondria and demonstrates a nearly linear pattern. This pattern shows more dispersion than the one for polystyrene microspheres in "group a", Figure 3A (standard deviations of 4-term polynomial fits are 0.16 and 0.25, with x average 0.10 and 0.15 and y average 2.14 and 0.037, respectively). The increased dispersion of the pattern on FVS plot for the freshly prepared mitochondria (compared to the polystyrene particles) was to be expected from the heterogeneous sizes, morphologies, internal structures, and molecular composition of mitochondria. The first three parameters would be associated with the heterogeneity in the scattering peak intensity, while variations in chemical composition (e.g., cardiolipin content in individual mitochondria) are associated with variations in fluorescence intensity.

Surprisingly, the FVS plot of mitochondria stored in liquid nitrogen for 1.5 months (Figure 4B) shows a drastic change compared to the FVS plot associated with freshly isolated mitochondria (Figure 4A). At first glance, the electron microscopy images of isolated fresh and frozen mitochondria appear indistinguishable (Figure 4, parts C and D, respectively). However, close inspection of higher magnification electron microscopy images shows differences in the cristae of fresh (Figure 4E) and frozen (Figure 4F) mitochondria. More systematic studies in which the mitochondrial internal structure is purposely altered (e.g., by inducing swelling in media with different osmolarities or rapid ice crystal formation in liquid nitrogen) may provide further insight on the relevance of this parameter to the FVS plots of these organelles.

In summary, this technical note describes a simple scattering detector for CE analysis of individual particles even when these particles are in the presence of nonanalyte scattering species. This detector relies on LIF detection to identify the analyte particles. Both the fluorescence and scattering signals are represented as FVS plots, which are extremely useful for identifying the components in mixtures and for monitoring the status of mitochondrial preparations. Further developments of the LS-LIF detector described here, particularly by monitoring scattering at different angles, may provide intrinsic corrections for variations in the index of refraction of mitochondria.[7,13] These corrections are important for understanding the dependence of scattering signals on both the size and index of refraction of mitochondria.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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