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Post-capillary reaction detection in capillary electrophoresis based on the streptavidin–biotin interaction Optimization and application to single cell analysis

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Abstract

A class-selective post-capillary reaction detection method for capillary electrophoresis is described in which a streptavidin–fluorescein isothiocyanate (streptavidin–FITC) conjugate is used to detect biotin moieties. The selective binding of biotin moieties to the streptavidin–FITC conjugate causes an enhancement of fluorescence proportional to the concentration of biotin present. After capillary electrophoresis the separated analytes react with streptavidin–FITC in a coaxial reactor and are then detected either by a benchtop spectrofluorometer (2.5 μM detection limit) or by an epi-fluorescence microscope ($1 \cdot 10^{-7}$ M detection limit). The method is used to examine biotinylated species in a crude mammalian cell lysate which was found to contain 83 ± 3 fmol in 3600 cell volumes. In addition, it is used to examine the uptake of biotin by individual sea urchin oocytes. The results indicate that, in the oocytes, biocytin is the prevalent form of biotin and its concentration varies widely between cells (mean$=2 \pm 2$ μM).

Keywords: Derivatization, electrophoresis; Detection, electrophoresis; *Arbacia punctulata*; Biotin

1. Introduction

In the past few years, several methods of post-capillary reaction detection have been developed for use in capillary electrophoresis (CE). The most common methods involve derivatization of amino acids or amines with fluorescent reagents such as *o*-phthaldialdehyde (OPA) [1–6], fluorescamine [1,7], or naphthalene-2,3-dicarboxaldehyde (NDA) [8]. These methods have resulted in high-resolution separations with as low as attomole detection limits for some diluted, standard proteins. The disadvantage of these reagents is that, while they improve the sensitivity of the detection compared to direct UV absorbance, they are not selective for a specific analyte, i.e., they will label any species containing a reactive group, such as primary amines or amino acids. It has been shown that post-capillary coupling of fluorescence-based homogeneous binding assays to CE cannot only improve detection limits over absorbance and native fluorescence measurements,

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but provides a highly selective detection system [9–11]. In this article, a coaxial capillary reactor was used to introduce a streptavidin–fluorescein isothiocyanate conjugate (streptavidin–FITC) into the sample stream in order to couple a post-capillary binding assay for biotin to capillary zone electrophoresis (CZE).

In this system, a biotin-selective binding assay (BA) was coupled to CE. The advantage of a coupled CE–BA system is that, while CE can separate structurally similar compounds based on charge-to-mass ratio, the binding assay has the ability to discriminate among different families of compounds. After separation by CE, the effluent stream of the separation capillary is mixed with streptavidin–FITC. The vitamin biotin is strongly bound by the protein streptavidin ($M_r \approx 60\,000$) with a dissociation constant of $\approx 10^{-14}$ M [12]. Streptavidin is a tetrameric protein capable of binding one biotin or biotinylated molecule per subunit, for a total of four bound molecules per molecule of streptavidin. Covalent labeling of streptavidin with fluorescein yields a conjugate that can detect biotin compounds via fluorescence enhancement upon binding [13]. This increase has been attributed to microenvironmental pH changes and to changes in dynamic quenching of the fluorophore [14]. This results in an increase in fluorescence of up to 3.5-fold for biotin and 6.5-fold for biocytin. The amount of fluorescence enhancement is also concentration-dependent for both compounds [13].

Using a coaxial reactor, a post-capillary reaction detection system for biotin and its derivative biocytin was examined using this reagent. The selectivity of the fluorescence binding assay means that the separation conditions must only be optimized for species containing biotin; indeed, the binding assay will not respond to those species which do not contain biotin so that co-elution with other species (i.e., proteins, carbohydrates, etc.) is not a problem [15]. The described detection system was used to detect biotin/biocytin at levels below those possible with direct UV absorbance, and is demonstrated to be useful for detection in complex samples. Specifically, the method is used to detect biotin in a crude lysate of mammalian fibroblasts and to examine biotin uptake by individual sea urchin oocytes.

2. Experimental

2.1. Apparatus

A diagram of the electrophoresis system using detection by a benchtop spectrofluorometer is depicted in Fig. 1a. In both set-ups the anodic and ground reservoirs were isolated inside plastic boxes to prevent accidental touching of the electrodes and to prevent discharge onto the metal components of the apparatus. The direction of electroosmotic flow (EOF) is thus towards the ground electrode. All potentials given should be considered to have negative polarity. Post-capillary reaction took place by means of a coaxial reactor as shown in Fig. 1b–d. In the first step, a 25 cm×77 μm I.D.×365 μm O.D. fused-silica capillary (the separation capillary) was etched along its final 1.5-cm length by first removing the polyimide coating then immersing the stripped end in concentrated hydrofluoric acid for 65 min. The etched end of the separation capillary was then inserted into a 125 cm×144 μm I.D. fused-silica capillary. All capillaries were from Polymicro Technologies (Phoenix, AZ, USA) and were used without further modification. The assembled capillaries were then epoxied to a glass microscope slide for stability. A detection window was created by removing a 1-cm length of the polyimide coating 20 cm from the junction of the separation and reaction capillaries. A 20-μl fluorescence flow cell (NSG Precision Cells, Farmingdale, NY, USA) was then placed over the window to guard against breakage while the capillaries were being inserted into the detector. The inlet and outlet reservoirs were placed at the same height, with the reactor positioned 10 cm above them. Post-capillary reaction detection was carried out using a SPEX Fluorolog-2 spectrofluorometer (SPEX Industries, Edison, NJ, USA), equipped with a 450-W xenon arc lamp, in photon-counting mode. The excitation wavelength was set at 495 nm, while the emission was monitored at 518 nm. The excitation and emission slitwidths were set at 1 mm each. Light from the source was focused on the capillary detection window using a 1 mm diameter target slit. Voltage was supplied using a ±30 kV high-voltage power supply (Gamma High Voltage Research, Model RR30-1R; Ormond Beach, FL, USA).

Another detection system was investigated using a

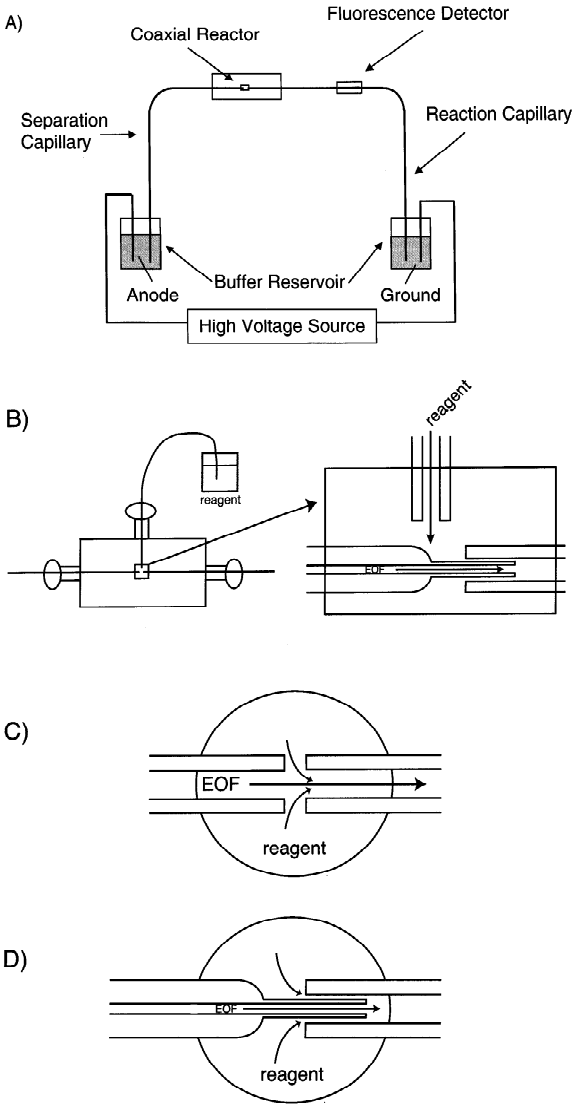

Fig. 1. Schematic of the CE post-capillary reaction system. The overall set-up is seen in (A). (B), (C) and (D) show different types of reactors used in this study. In (B) a coaxial reactor mounted in a stainless steel mixing tee was assembled that gave control of the reagent flow-rate via changing the height of the reagent reservoir compared to the inlet and outlet, but resulted in severe reagent backflow. Shown in (C) a gap reactor was assembled by aligning capillaries under a microscope. This reactor had high reagent flow-rates, but did not allow the sample to cross the gap. The final reactor (D) is a cross between the first two in that it consists of a free-solution coaxial reactor that gave no backflow problems and resulted in good reagent flow and large sample peaks. The reactors in (C) and (D) are epoxied to a glass microscope slide for stability and the union between the capillaries is immersed in the reagent solution. The drawings are not to scale.

Nikon Diaphot 200 inverted epi-fluorescence microscope (Nikon, Melville, NY, USA). When using the epi-fluorescence microscope, the relative heights of the reservoirs and the injection parameters were preserved. The detection window was taped to a glass microscope slide and set on the stage of the microscope. A 40× fluorescence objective was used to focus on the center of the capillary. Once the run had begun, the light path was switched from the ocular to a photomultiplier tube (PMT) (SPEX Industries F-1911) in photon-counting mode mounted on the side-arm of the microscope. The fluorescence source was a 100-W Hg short arc lamp focused on the capillary through the objective using a 470/40 nm bandpass filter (Chroma Technology, Battleboro, VT, USA). Fluorescence was also collected through the objective and was filtered using a 510 nm cut-off dichroic mirror and a 520 nm long-pass filter (Chroma Technology).

2.2. Reagents

Biotin, biocytin and monobasic sodium phosphate were purchased from Sigma (St. Louis, MO, USA). Streptavidin labeled with fluorescein isothiocyanate (FITC) (5.9 fluoresceins per streptavidin molecule) was obtained from Vector Labs. (Burlingame, CA, USA) as a 1 mg/ml solution. Assuming the molecular mass of the conjugate to be ca. 60 000, this corresponds to a concentration of 16.7 μ*M*. Deionized distilled water (Milli-Q Water Purification System; Millipore, Bedford, MA, USA) was used to prepare all solutions, which were then filtered through 0.2-μm filters and degassed before use.

The running buffer for all electrophoretic separations was 30 m*M* sodium phosphate, prepared from the dibasic salt and titrated to the desired pH with 1 *M* NaOH. Stock solutions of the analytes were prepared by diluting in this buffer. The streptavidin–FITC solutions were prepared fresh daily by serial dilution in 30 m*M* sodium phosphate. The capillaries were rinsed with 1 *M* NaOH every morning for 10 min, then with several column volumes deionized water, and then with several column volumes of sodium phosphate buffer, and finally allowed to equilibrate in the phosphate buffer for another 15 min before use.

2.3. Optimization of applied voltage

In order to determine the optimum voltage necessary to carry out these experiments, the 30 m*M* sodium phosphate buffer was subjected to an Ohm's law analysis. In this analysis, the buffer and reagent were placed in the capillaries and a voltage applied to the apparatus. After a 1-min equilibration the current was measured. The current was then plotted against the applied voltage.

2.4. pH study

In order to determine the effect of pH on the separation and fluorescence signal of the streptavidin–FITC conjugate, the pH of the streptavidin–FITC solution (1.67 μ*M*) and of the phosphate buffer were varied, as was the pH of the sample (50 μ*M* biotin). The pH of all three solutions was the same. The sample was hydrodynamically injected for 10 s at a height of 11 cm.

2.5. Calibration curve

A calibration curve was constructed in order to determine the relationship between peak area and biotin/biocytin concentration. Standards (10 m*M*) of both biotin and biocytin were prepared in degassed running buffer and filtered through 0.2-μm filters before dilution. Samples were diluted in the running buffer and were hydrodynamically injected for 10 s as before. The concentration of streptavidin–FITC was 0.84 μ*M*. For the microscope studies, it was 16.7 n*M*.

2.6. Cell lysate

A crude lysate of cloned rat embryo fibroblasts (CREF cells) was prepared using a 1.0-ml culture of CREF cells ($1 \cdot 10^6$ cells/ml). The cells were centrifuged for 5 min at 6000 *g*, the supernatant discarded, the cells resuspended in running buffer, and centrifuged again at the same speed. This was repeated for a total of three washes. The final supernatant was discarded and the cells were resuspended in 50 μl of 2.5 m*M* deoxycholic acid (in water) and incubated for 20 min at 4°C. The cell lysate was then centrifuged for 15 min at 12 000 *g* to remove cellular debris. The supernatant was hydrodynamically injected into the capillaries for 40 s and the fluorescence monitored. The sample was then spiked with 1/10th volume of 100 μ*M* biotin and the sample run again under the same separation conditions.

2.7. Biotin uptake by sea urchin oocytes

Oocytes from the sea urchin *Arbacia punctulata* were collected by injecting the coelomic cavity of a sea urchin with 2 ml of 0.5 *M* KCl, causing the urchin to expel its eggs. The oocytes were collected in artificial sea water (425 m*M* NaCl, 9 m*M* KCl, 9.3 m*M* $CaCl_2$, 25.5 m*M* $MgSO_4$, 23 m*M* $MgCl_2$, 2 m*M* $NaHCO_3$, pH 8.0) and rinsed several times to remove any debris or fecal matter. To study biotin uptake, 100 μl of this suspension was made to 1 μ*M* biotin and the oocytes incubated in the biotin solution for 1 h. They were then briefly centrifuged (30 s, 2000 *g*) and resuspended in 1 ml of 30 m*M* sodium phosphate buffer. This process was repeated three times. After washing, a 10-μl drop of oocytes in buffer was placed on a microscope slide and viewed under a second light microscope at 20× magnification (Bausch & Lomb Optical, Rochester, NY, USA). The injection end of the capillary set-up was moved in the oocyte suspension using a Narishige MN-188 micromanipulator (Narishige, Tokyo, Japan) so that the capillary opening was close to a lone oocyte. A small suction was applied to the capillary outlet via a syringe equipped with a high-performance liquid chromatography (HPLC) injection needle fitted with a small piece of PTFE tubing whose I.D. (0.010 in.; 1 in.=2.54 cm) is approximately the same size as the O.D. of the reaction capillary. Inserting the end of the reaction capillary into the PTFE tubing formed an effective seal, so that pulling back on the syringe plunger provided enough suction to inject the oocyte (see Fig. 4). The inlet and then the outlet were manually replaced in their respective reservoirs and the 15 kV potential applied. After a few seconds the potential was increased to 25 kV for 5 s to rupture the oocyte. The run then continued as before using 16.7 n*M* streptavidin–FITC.

3. Results and discussion

Biotin lacks a strong chromophore, making it difficult to detect at low levels using direct UV absorbance methods. Detection must also take place at 220 nm, a wavelength at which many compounds absorb. Pre-column derivatization techniques have been used to impart fluorescence to the molecule, but derivatization of the biotin moiety occurs through the carboxylate groups, leading to neutralization of the molecule and making the technique more difficult for use in CZE [16,17]. As a post-column reagent, avidin has been used to selectively detect biotin through competitive binding assays [18,19]. These assays depend upon the competition between biotin/biocytin and a dye for binding sites on avidin. Because biotin and biocytin have a higher affinity for avidin than does the dye, the dye is displaced, leading to a change in signal. Avidin– or streptavidin–FITC as a post-column reagent in HPLC to make fluorophore-linked protein binding assays for biotin has also been accomplished [15,20,21]. These methods gave detection limits on the order of 100 pg for biotin and biocytin, an improvement of 1–2 orders of magnitude compared to UV detection. Because of the enhanced stability of the streptavidin conjugate, the assay using streptavidin–FITC as the reagent was used to study a post-capillary reaction detection system for biotin and biocytin. It should be noted that this reagent has been used before to study the kinetics of the reaction between biotin and streptavidin after separation by CZE using laser-induced fluorescence [11]. It is the intention of this paper to optimize the separation and reaction conditions and then apply the results to cellular samples.

Three types of reactors were examined for use in this system. Two other types of reactors were constructed before the coaxial set-up used in the main body of this work. In the first, a coaxial reactor of the same type as described in the Experimental section was built, except that the union was mounted in a stainless steel mixing tee (Fig. 1b). The reagent capillary was also mounted in the tee, and reagent flow controlled hydrodynamically by raising and lowering the reagent reservoir. Several engineering problems precluded the use of this set-up, including an inability, because of the height of the spectrofluorometer, to find a height for all reservoirs sufficient for the proper operation of the capillaries. In short, no matter at what height the reservoirs were placed there was a significant backflow problem which could not be overcome by the EOF. Another problem was that tightening of the ferrules of the tee resulted in torsion of the capillaries, leading to breakage of the fragile tip of the separation capillary at the coaxial union. Unfortunately, since there was no way to see inside the tee, the only way of knowing if this occurred was by seeing zero reagent flow to the detector. The last problem was clogging of the reagent capillary at the point of entry to the tee. Since these tee-mounted systems have been successfully used with organic reagents [3–5], the problem apparently lies in the use of the proteinaceous streptavidin–FITC solution as the reagent.

After this a gap reactor was then assembled (Fig. 1c). One 144 μm I.D. capillary and one 77 μm I.D. capillary were aligned under a microscope at a distance of approximately 77 μm and epoxied in place on a glass microscope slide. The gap was then filled with the streptavidin–FITC solution and the set-up placed in the detector. In theory, the electric field should be sufficient to allow the eluent of the separation capillary to cross the gap. Because of the disparities in the I.D.s of the two capillaries, streptavidin–FITC should be drawn into the reaction capillary along with the effluent of the separation capillary. In point of fact, a reactor was also constructed using a 144 μm I.D. separation capillary, but neither reactor produced peaks when the sample was applied. Since the fluorescence of the streptavidin–FITC could be seen, it must be that the biotin sample failed to efficiently traverse the gap, a problem in gap junctions with misaligned capillaries [22]. Even attempting to aid the flow of the biotin across the gap via diffusion by changing the ionic strength of the running buffer as opposed to the reagent buffer did not fix the problem. It was also a very tedious and time-consuming process to properly align the two capillaries under the microscope. Often, it was difficult to tell if they were properly aligned without visualizing the flow in the reactor under a microscope using colored dyes.

For this reason, a hybrid reactor using the advantages of both the coaxial and the gap reactors was constructed (Fig. 1d). These two different types of reactors were combined by removing the tee in the

first set-up and immersing the coaxial reactor in the streptavidin–FITC solution. The capillaries were fixed to a microscope slide after inserting the etched end of the separation capillary into the reaction capillary. This hybrid reactor worked very well, despite having the drawback of giving no external control over the reagent flow-rate; because of the dimensions of the spectrofluorometer, the height of the reactor compared to the inlet and outlet reservoirs could not be changed. Despite this, the reactor was extremely easy to fabricate and clean and gave very reproducible results. Also, the coaxial union was clearly visible and could be easily repaired if the tip of the separation capillary broke. A schematic of the entire CE system can be seen in Fig. 1a.

After the reactor was assembled, the first step in optimization of the separation of biotin and biocytin was to determine the appropriate voltage to apply to the system. A 30 m*M* sodium phosphate buffer at neutral pH was chosen as the background electrolyte. One limitation to using a phosphate buffer is, however, its limited thermostating capabilities [23]. At higher applied voltages, phosphate buffers have been seen to cause excessive Joule heating, which can lead to temperature-related peak distortion. This cannot be overcome by changing the ionic strength of the buffer, but is rather a characteristic of the buffer itself. In order to determine the best voltage to use in our system, an Ohm's law plot was constructed [24]. At voltages above 15 kV, there is a deviation from linearity that becomes severe above 17.5 kV (data not shown). Several attempts were made to separate the biotin analogues at potentials greater than 17.5 kV, but the heat generated in the capillaries caused them to become prone to breakage. There was also evidence of the phosphate buffer and streptavidin–FITC reagent evaporating, leading to variations in background fluorescence and EOF. Separations carried out at 15 kV showed none of these problems, and were used in the rest of the experiments.

After the optimum voltage was determined, the effect of changing the pH of the buffer on the separation and on the fluorescence signal of the streptavidin–FITC conjugate was examined. In addition, the effect of changing pH on the electroosmotic flow of the system was determined. As seen in Fig. 2a, the electrophoretic mobilities of both biotin and

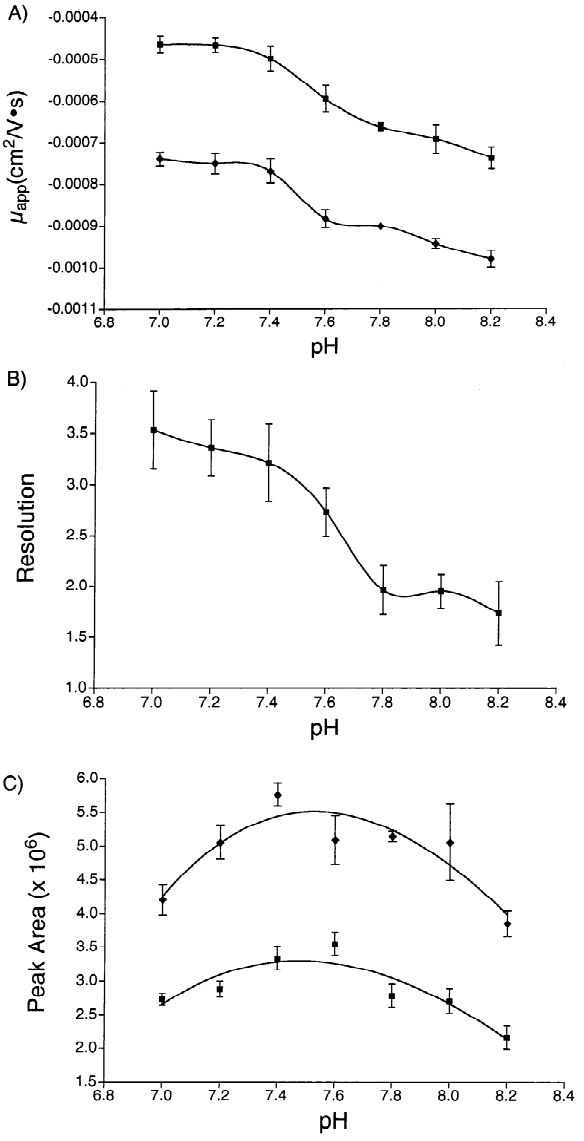

Fig. 2. Effect of buffer pH on electropherograms. The concentration of streptavidin–FITC is 1.67 μ*M* and the concentration of biotin (■) and biocytin (♦) is 50 μ*M*. The buffer is 30 m*M* sodium phosphate and the applied potential is 15 kV. Samples were hydrodynamically injected at 11 cm for 10 s. Data points are average±1 standard deviation (n=4). The effect of pH is shown on (A) the electrophoretic mobilities, (B) the resolution between the biocytin and biotin peaks, and (C) the amount of fluorescence enhancement upon binding of biotin/biocytin with streptavidin–FITC.

biocytin are retarded at lower pH. This effect is more pronounced for biotin, the acid, than for biocytin, the zwitterion. Between pH 7.0 and pH 7.4, the maximum resolution is attained (Fig. 2b), although all peaks are resolved across the pH range studied.

It has previously been shown that the fluorescence of FITC is highly pH dependent, with a maximum signal at slightly basic pH [25,26]. As stated earlier, the assay used in this system depends upon enhancement of the fluorescence of the streptavidin–FITC conjugate upon binding of biotin/biocytin to the streptavidin moiety. One possible explanation for this occurrence is that the fluorophore (FITC) thus becomes more exposed to the solvent upon binding. If this were the case, then changing the pH of the buffer would have an effect on the amount of fluorescence enhancement, due to the effect of pH on FITC. As seen in Fig. 2c, there is a pH-dependent difference in the peak areas for both biotin and biocytin, reaching a maximum for both biotin and biocytin at pH 7.4–7.6. The change in pH also has more of an effect on the peak areas of the biocytin complex than on those of the biotin complex. This is not an effect of changing electrophoretic mobilities causing greater residence time in the detector or more time for the interaction of the analyte with the streptavidin–FITC conjugate, because the mobility of the biocytin peak is not significantly changed between pH 7.0 and 7.4, nor is the rate of EOF altered significantly between these pH values (the calculated difference is less than 4% between pH 7.0 and 7.4). However, the amount of fluorescence enhancement is almost half again as much at 7.4. The effect must therefore be related to the streptavidin–FITC conjugate itself, but whether this effect is due to changing affinities for the two molecules, or to a change in the properties of the fluorescein label is unknown.

The peaks in this system are somewhat wide for a CE separation, due to several factors. The nature of the reactor itself contributes to band-broadening, due to radial dispersion, diffusion, and backpressure [4,27]. Calculation of the flow-rates under the optimized conditions gives an electroosmotic flow-rate of 2.7 nl/s and a reagent flow-rate of 0.7 nl/s (data not shown). This data is similar to that obtained by Rose and Jorgenson, who determined an inverse relationship between reagent flow-rates and peak area, i.e., at low flow-rates there is a large peak area due to increased residence time in the detector and a comparatively small dilution effect of the reagent [4]. The same experiments showed that a small difference between the inner diameter of the reaction capillary and the outer diameter of the separation capillary is desirable because it gives the highest peak area–efficiency product. The calculated outer diameter of the separation capillary is 141 μm, giving a space of 1.5 μm around the separation capillary for reagent influx (data not shown). This, coupled with the small hydrodynamic force on reagent flow, contributes to the very low flow-rates of the reagent, resulting in a large peak area. The trade-off between peak area and resolution is easily resolved in this case, since there will be very few peaks in class-selective detection (high efficiency is not necessary), but the peaks might be rather small (large peak area is desirable). Also, experiments carried out using UV absorbance of biotin and biocytin using the same coaxial reactor set-up (data not shown) show that the peaks are much narrower (approximately half the width obtained in fluorescence measurements), leading to the conclusion that immunodiffusion is partially responsible for the band-broadening.

Once the optimal separation conditions had been determined, a calibration curve for biotin and biocytin was constructed. By varying the concentration of biotin/biocytin injected, the relationship between peak area and concentration could be determined. The calibration curve is linear over a range of 2.5–25 μ*M* biotin and biocytin. The range is limited due to the restrictions on radial diffusion of biotin/biocytin into the reagent layer imposed by the coaxial reactor [4,27]. In both cases the amount of biotin/biocytin present is already much greater than that of the streptavidin–FITC, leading to the conclusion that saturation of binding sites on the conjugate is not the major limiting factor. The lowest concentration that could be discerned above background was 2.5 μ*M* for both compounds. Using Poiseuille's equation, we estimate that, for a 10-s injection the sample volume is 7.2 nl [28]. A typical electropherogram under the given conditions is shown in Fig. 3A.

In order to determine if this particular set-up could be used on a complex sample matrix, a lysate of

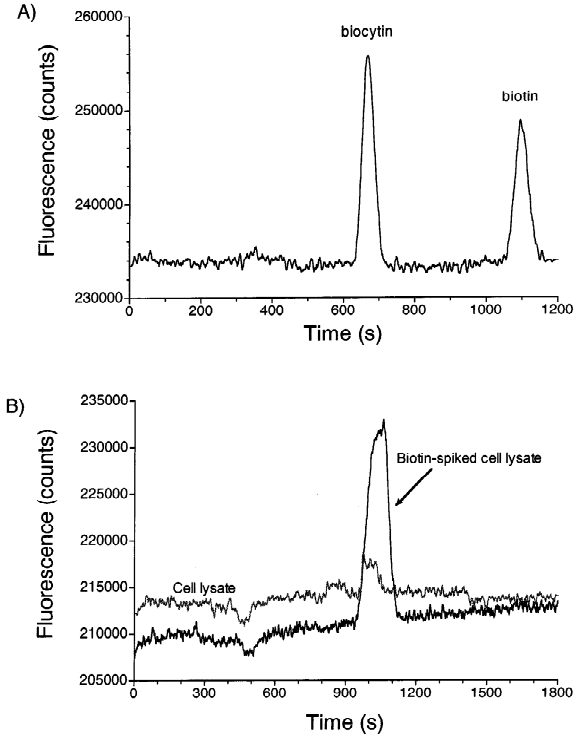

Fig. 3. Electropherograms obtained using the benchtop spectrofluorometer. (A) Typical electropherogram for the system showing 17.5 μ*M* biotin and biocytin. (B) Electropherogram of a crude CREF cell lysate and the lysate spiked with 1/10th volume 100 μ*M* biotin. The biotin peak is distorted due to the concentration difference between the lysate and the running buffer. Conditions for both: 7.2 nl sample volume, 15 kV applied voltage, 0.84 μ*M* streptavidin–FITC, running buffer: 30 m*M* sodium phosphate, pH 7.4.

CREF cells was prepared. Injection parameters had to be changed to account for the increased viscosity of the sample. A 40-s injection was necessary to inject a volume of 14.4 nl, or roughly 3600 cell volumes. As can be seen in Fig. 3B, two peaks are present. The second taller peak was identified as biotin by spiking the sample. The other peak, whose area is roughly half that of the larger peak, could not be identified. Preincubation of the lysate with avidin produced an electropherogram containing no peaks. Spiking with biocytin (data not shown) gave a peak with a different electrophoretic mobility. It is possible that the peak is composed of unresolved biotinylated cellular proteins, since these are likely to have a smaller charge-to-mass ratio than biotin and would, therefore, elute closer to the electroosmotic flow than biotin. Vertebrates are known to have four biotinylated proteins, so their presence here would not be surprising [29]. The second peak also appears to be distorted to a large degree. This is most likely due to the high ionic strength of the cell lysate compared to the running buffer, which causes an increased current and temperature-related peak distortion. The amount of biotin present in the sample is 83±3 fmol ($n=3$), equivalent to 23.1 amol per cell.

After the set-up had been optimized for detection in a benchtop spectrofluorometer, the capillary apparatus was moved to an inverted epi-fluorescence microscope for further testing. The optics of the microscope are better suited for measurement of such small samples than the spectrofluorometer. Using the optimized conditions from the spectrofluorometer, it was found that the microscope could use a lower concentration (16.7 n*M*) of the streptavidin–FITC conjugate than was possible on the larger instrument. This had the effect of giving a more sensitive calibration curve for biotin/biocytin that was linear over three orders of magnitude. The detection limit for the microscope set-up is approximately $1\cdot10^{-7}$ *M* for both species. This is similar to the detection limit achieved by Kelly et al. using laser-induced fluorescence, although the linear range in this case is much larger [11].

The system was then used for the measurement of biotin uptake in single sea urchin oocytes. It was simply too difficult to inject individual mammalian cells using this system because they are so small compared to the inner diameter of the separation capillary. This is also a problem because when they lyse they become diluted to a much larger degree than the oocytes, making detection difficult. As stated in the Experimental section, oocytes were incubated with 1 μ*M* biotin for 1 h, then washed and injected individually onto the capillaries as seen in Fig. 4. One concern is that during the move from the oocyte solution to the buffer reservoir, the cell will become dislodged or otherwise damaged. Sea urchin oocytes are extremely tough, so it was necessary to increase the potential to 25 kV in order to lyse the cells. We performed a series of experiments to observe the fate of the oocyte after the move (data not shown). First, we injected the oocyte as usual, moved the to its reservoir, then placed the inlet back

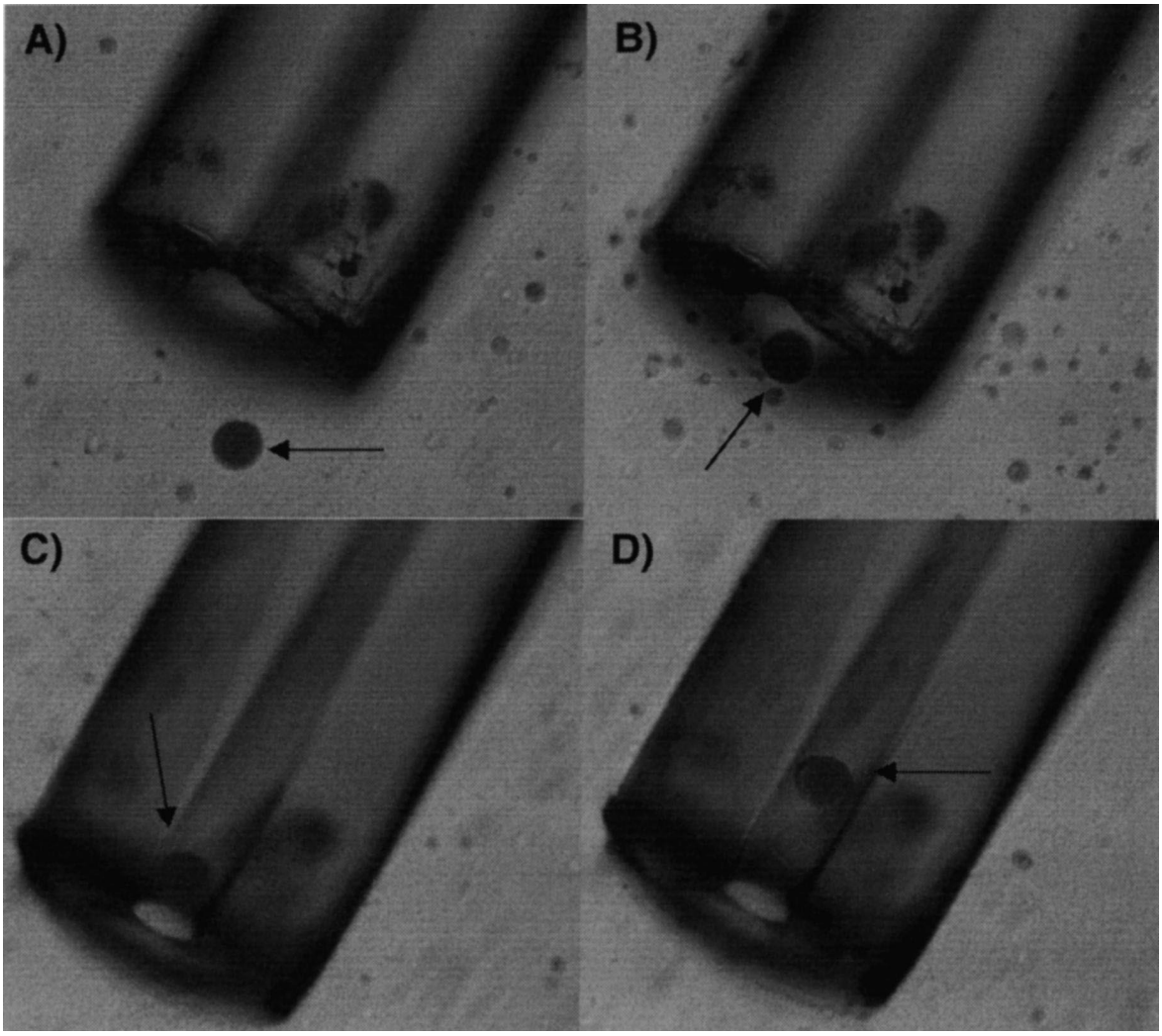

Fig. 4. Injection of individual sea urchin oocyte into the separation capillary. The I.D. of the bare fused-silica capillary is 77 μm. The oocyte is the indicated sphere. In this series of photomicrographs (taken at 20× magnification) the capillary inlet is first positioned near the oocyte (A), then a small suction is applied to the outlet causing the oocyte to move closer to the inlet (B) and become aspirated (C). At time point (D) the oocyte is fully aspirated and the capillary inlet is returned to its reservoir.

under the microscope in order to see if the oocyte remained aspirated. By elevating the outlet reservoir and causing a backflow, the intact oocyte was redeposited on the microscope slide. The same experiment was repeated several times. In each case, the oocyte was recovered intact, indicating that it had remained inside the injection capillary and that it was undamaged by the journey from slide to reservoir. Another set of experiments went on to the next step – applying the potential to the system. When the inlet was moved back to the slide and the contents ejected after moving and briefly electrophoresed the oocyte was seen to be mostly intact when 15 kV was used and highly fragmented when 25 kV was used. Again, this experiment was repeated several times and the results were similar each time, i.e., the oocyte was lysed.

The major problem encountered with the oocytes was that the system had to be flushed with clean buffer and fresh reagent supplied after each oocyte.

This was due to irreproducible electropherograms being obtained after running the second oocyte in the same buffers as the first. To test this, a standard containing both biocytin and biotin was run after every oocyte injection. After the first oocyte was run, the standard showed peak areas, residence times, and background fluorescence within the expected range as obtained from the calibration curve. However, if this was repeated after the second oocyte had been run in these buffers, the standards showed a change in one or more of these characteristics. Whether this was due to fouling of the capillaries by the oocyte or to denaturation of the reagent by the 25 kV pulse is not clear, but to avoid this problem the capillaries were washed with clean running buffer and fresh reagent was added to the reservoir after each oocyte run.

Representative electropherograms of some of the oocytes, as well as a typical electropherogram of biocytin/biotin under the same conditions, can be seen in Fig. 5. The amount of biotin/biocytin in an individual native oocyte (one which has not been incubated with biotin) is undetectable in this system and, therefore, produces no peaks. It is readily apparent that simply microinjecting the oocyte with streptavidin–FITC would not be accurate, as there is a large mass of cellular contents that cause an increase in fluorescence from the conjugate, perhaps biotinylated proteins, protein fragments, or metabolites. The major peak in the electropherogram corresponds to the placement of the biocytin peak, suggesting that in the oocytes, free biotin is not the predominant species. Ideally, the peak should be identified by mass spectrometry. However, since it is known that streptavidin–FITC selectively binds to biotin-containing compounds and only shows an increase in fluorescence from these compounds in complex samples [15], it can be assumed that a fluorescent peak with the same electrophoretic mobility as biocytin is indeed biocytin.

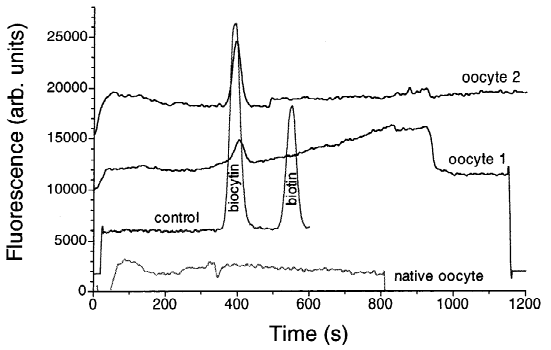

Fig. 5. Electropherograms of individual oocytes. Two individual oocytes that have been incubated in 1 μ*M* biotin for 1 h, are washed and injected onto the capillaries. They are shown compared to a typical electropherogram using 25 μ*M* biocytin and biotin and one electropherogram of a native oocyte. Conditions are: 15 kV applied voltage, 16.7 n*M* streptavidin–FITC, running buffer: 30 m*M* sodium phosphate, pH 7.4.

In animals, biotin exists mainly as a covalent attachment on certain proteins that use it as a cofactor [30]. The proteolytic digestion of these proteins produces ε-biotinyl-L-lysine (biocytin), which is then further digested to free biotin and reused [31]. By integrating the area under the major peak, and comparing this to the calibration curve for biocytin under the same conditions, it was seen that the oocytes varied greatly in terms of free biocytin content (Table 1, mean=2±2 μ*M*, 95% RSD, $n=4$). This could be due to many factors, including variations in biotin uptake and incorporation, proteolytic activity, etc., and supports the theory that individual cells respond differently under the same external stimulus. In order to examine the recovery of biocytin from these cells, an experiment was conducted in which 50 μl of oocytes (approximately 95 000 oocytes) were incubated with biotin, washed, and boiled to break open the cells. This crude lysate was then clarified by centrifugation to remove solid particles that might clog the capillaries. The clarified lysate was then injected onto the capillaries after dilution in running buffer to give an injection

Table 1
Analysis of biocytin content of individual sea urchin oocytes

Oocyte	Peak area	Concentration (μ*M*)
1	199 700	5.13
2	67 330	0.79
3	132 500	2.53
4	50 600	0.48

equivalent to 1 oocyte's contents (1 oocyte=525 pl, injection volume is 14.4 nl, dilution factor is 1:27). The concentration of biocytin was found to be 1.6 ± 0.1 μM in a volume equivalent to that of one oocyte. This value is not significantly different from the average biocytin content measured in the individual oocytes.

Table 1 only shows the results from four oocytes because that was the most that could be run on the same day as they were collected. This was the same day that the sea urchins were received. Because there were no facilities for keeping the urchins alive, the eggs were collected immediately and used the same day. On top of this, as mentioned above, injecting an oocyte is extremely tedious. After they are incubated with biotin, washed, resuspended, they are placed on a microscope slide for aspiration into the capillaries. This requires mounting the inlet in a micromanipulator, moving it around on the slide to find an oocyte, placing the inlet just so next to the oocyte to aspirate, perhaps trying again if more than one is taken up, etc. Once the oocyte is inside, lysed, and run, then the capillaries have to be cleaned out and refilled with the various solutions. After all of this was done, the capillaries had been through some heavy usage and the separation capillary broke off after the eighth oocyte (four incubated with biotin, four not incubated with biotin). In terms of a success rate, looking at Fig. 5 reveals that all unincubated oocytes showed no peaks while all incubated oocytes showed a peak at a retention time equivalent to that of the biocytin peak.

In conclusion, we have shown that post-capillary reaction detection using a spectrofluorometer is possible in CE. The use of streptavidin–FITC as a reagent in CE, while posing some challenges, leads to a sensitive method for the detection of biotin species (i.e., biocytin) in small volumes and in single cells. The ability to study biotin uptake and metabolism on the level of the individual cell is important in a number of cases. First, irregularities in biotin content in terms of both biotin metabolites such as biocytin and in the relative amounts of the four biotin-containing enzymes have been shown in various forms of colon cancer [32,33]. Second, it would allow for the study of the effects of toxins such as ethanol on vitamin uptake [34] and perhaps lead to a greater understanding of the mechanism by which this takes place.

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