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Single-cell analysis using capillary electrophoresis: Influence of surface support properties on cell injection into the capillary

Capillary electrophoresis (CE) is an important tool of chemical cytometry. Whole-cell analysis using CE starts with cell injection into the capillary by either siphoning or electroosmosis. However, strong adherence of the cell to the support surface can prevent efficient cell injection and lead to irreproducible analysis. Here we evaluated several surfaces as potential cell supports for HT29 cells (human colon adenocarcinoma). These cells strongly adhered to the surface of untreated glass or polystyrene. Hydrophobic coating with dimethyldichlorosilane (DMS) or Sigmacote® did not significantly reduce cell adhesion. In contrast, cell adhesion was reduced significantly when the surface was modified with hydrophilic polymers (hydrogels) such as poly(2-hydrohyethyl methacrylate) (PHEMA) and polyvinyl alcohol (PVA). In addition to their pronounced antiadhesive properties, PHEMA and PVA coatings were the most biocompatible (had highest survival of cells in contact with surface). Hydrogel-coated polystyrene plates were tested as a commercial alternative to hydrogel-coated glass slides. The cell adhesive properties of such plates were similar to those of PHEMA and PVA. However, the biocompatibility of the plates was lower than that of the other surfaces tested. Moreover, in contrast to PHEMA- and PVA-coated glass slides, the plates were sensitive to UV light and therefore should not be used when fluorescent image microscopy with UV excitation precedes CE. The analyses of the data obtained showed that PHEMA- and PVA-coated glass slides were the most suitable cell supports for cell injection into the capillary.

Keywords: Single cell analysis / Cell injection / Surface properties EL 3816

1 Introduction

Understanding of many biological processes, for example carcinogenesis and embryogenesis, would be stimulated by the ability to analyze the chemical contents of individual cells. Classical cytometry techniques such as fluorescent image cytometry and flow cytometry allow the simultaneous assay of a limited number of species, determined by the number of spectral channels available. Multicomponent chemical analysis of individual cells can be accomplished, instead, by using microscale separation tools, such as CE [1] combined with highly sensitive detection techniques, such as laser-induced fluorescence (LIF) [2]. To distinguish this approach from classical cytometries we introduced the term “chemical cytometry” [3]. Chemical cytometry uses the tools of instrumental analytical chemistry for qualitative and quantitative analysis of the chemical contents in single cells.

The contents of individual cells can be sampled for capillary electrophoresis in several ways depending on the type of cell studied and problems to be solved [4]. Relatively large cells, for example oocytes or neurons, can be homogenized in a microvial and the homogenate can then be prepurified and assayed in an ordinary manner [1]. For large cells, subcellular sampling is also possible [5]. With small cells, including most mammalian somatic cells, a whole cell can be injected into the capillary [6]. In whole-cell mode, the analysis consists of four major steps: (i) cell injection (ii) cell lysis, (iii) separation of cellular contents, and (iv) reconditioning of the capillary. The quality of analysis requires optimization of all four steps, including cell injection.

For cell injection, the capillary is immersed into the cell suspension so that its orifice is in close proximity to the cell chosen for analysis. The cell is then dragged into the capillary by fluid flow caused by either electroosmosis [4] or siphoning [7]. Therefore, the cell is always injected together with the plug of cell medium. The length of the cell medium plug generally has to be short and reproduci-

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Abbreviations: DMS, dimethyldichlorosilane; **LacNac-TMR**, *N*-acetyllactosamine-tetramethylrhodamine; **PHEMA**, poly(2-hydrohyethyl methacrylate); **PVA**, polyvinyl alcohol; **TMR**, tetramethylrhodamine; **ULAP**, ultra low attachment plates

 0173-0835/00/0404-0767 $17.50+.50/0

ble to minimize deviation in migration time as well as possible disturbance of cell lysis and separation by the excess cell medium. To keep the plug length short the flow rate should be low and cells should just slightly adhere to the support surface.

Cell adhesion to the solid substrate is a fundamental property of most cell types. The interaction of cells with extracellular matrix is important in the control of gene expression [8, 9] and cell function under physiological [10] and pathological conditions [11]. Cell surface proteins play a crucial role in such adhesions [8]. Adhesion can be reduced by changing the physical-chemical properties of the surface, such as electrical charge, hydrophobicity, and chemical structure. A number of chemicals, mainly of two groups, hydrophobic silanes [12] and hydrophilic polymers (hydrogels) [13, 14], have been reported to reduce cell adhesion to the surface. Here we evaluated several substances as potential modifiers of glass and polystyrene surfaces to be used as cell support materials for cell injection into the capillary. Optical properties of the materials were also studied since combining fluorescent image microscopy with capillary electrophoresis is a potentially powerful tool in cell biology [3].

2 Materials and methods

2.1 Materials

The following suppliers were used: Fisher Scientific (Suwanee, GA) for glass microscope slides and ultra low attachment plates (ULAP), Sigma (St. Louis, MO, USA) for Sigmacote®, dimethyldichlorosilane (DMS), and polyvinyl alcohol (PVA), M_r 30 000–70 000, and Aldrich (Oakville, ON, Canada) for poly(2-hydrohyethyl methacrylate) (PHEMA). Tetramethylrhodamine (TMR)-labeled sugars were generously donated by Prof. M. Palcic. The remaining chemicals were commercial reagents of analytical grade and were utilized without additional purification. Deionized distilled water was used in this study after additional filtration with 0.22 μm pore size disposable filters (Millipore, Bedord, MA).

2.2 Capillary electrophoresis

The general design of the electrophoresis system with LIF detection used in this study has been described in detail elsewhere [15]. Separations were carried out in an electric field of 400 V/cm in a fused silica capillary (polymicro, Phoenix, AZ, USA) of 45 cm length, 20 μm inner diameter, and 140 μm outer diameter. The same buffer (10 mM phosphate, 10 mM phenylboronic acid, 10 mM disodium tetraborate, and 10 mM SDS at pH 9.0) was used as separation buffer and as sheath flow fluid.

2.3 Cell culture

An HT29 human cell line (colon cancer) was used in this study. The cells were grown to 80% confluence in Dulbecco's modified Eagle medium, supplemented with 10% fetal calf serum and 40 μg/mL gentamicin at 37°C in 5% CO_2 atmosphere. Then, the cells were harvested, washed and resuspended in PBS. For single-cell injection experiments, the cell suspension was diluted with PBS to a final density of 10^4 cells/mL. For single-cell separation experiments the cells were incubated for 18 h with 25 μM *N*-acetyllactosamine-tetramethylrhodamine (LacNAc-TMR). After incubation the cells were washed 8 times with PBS to remove the residual LacNAc-TMR and its metabolic products from the cell medium. The cells were resuspended in PBS and diluted with PBS to a final density of 10^4 cells/mL.

2.4 Cell injection into the capillary

Fifty microliters of cell suspension were placed on a cell support surface and left for 5 min to settle. Cell injection was carried out under a Model IMT-2 inverted microscope (Olympus America, Melville, NY, USA). A hydraulic micromanipulator (Model MX630R; Newport Bio-Instrument, Nepean, ON, Canada) was used to mount a capillary holder and to lower the capillary until the distance between the capillary and the cell support surface was 30 μm. Moving the microscope stage, the cell chosen for injection was superimposed with the inner bore of the capillary. Then, a 1 s pulse of negative pressure was applied to the opposite end of the capillary. Cell injection was monitored visually under a microscope. We assembled an inexpensive device to control negative pressure used for cell injection. A 1 L filtration flask served as a vacuum ballast reservoir. The flask was connected to a vacuum pump through its side opening and a manual valve. The reservoir was also connected to three vacuum tubes through the rubber stopper. One tube was open to the atmosphere through a manual valve and was used to set pressure inside the reservoir at atmospheric level. The second tube connected the reservoir to a vacuum gauge to monitor pressure inside the reservoir. The third tube was connected to the capillary through a solenoid valve. The solenoid valve was controlled electronically and allowed us to apply a 1 ± 0.01 s pulse of negative pressure to the capillary. Negative pressure in the ballast reservoir was set by slowly pumping out the air from the reservoir until pressure reached a desirable value. Injection volumes for our experimental conditions (20 μm, ID and 45 cm length of the capillary, 1 s injection time, 1 atm maximal differential pressure) did not exceed 1 nL. Therefore, many injections could be made without significant change of pressure in the 1 L ballast reservoir. The mini-

mum negative pressure required for cell injection was measured in the following manner. The initial pressure was set to a level that was insufficient for cell injection. We decreased the pressure in increments of 0.1 kPa after every unsuccessful injection until ten successful injections were completed in sequence. This pressure was considered the minimum pressure required for injection.

2.5 Glass slide coating and biodeactivation

Before coating, the glass slides were treated with 0.1 M NaOH for 15 min, rinsed vigorously with water, and air-dried. For coating with DMS or Sigmacote, the slides were immersed into 5% DMS in toluene or in Sigmacote solution for 1 min, thoroughly washed with ethanol, and air-dried. For coating with PVA, 7 μL solution of PVA in water was evenly distributed over the slide surface, incubated at 140°C overnight and washed thoroughly with ethanol and water, and air-dried. For coating with poly(2-hydrohyethyl methacrylate) (PHEMA), 7 μL of 7.5% PHEMA solution in 2-methoxyethanol was evenly distributed over the slide surface, air-dried, washed with ethanol and water, and air-dried again. To asses hydrophilicity/hydrophobicity of coated surfaces, the contact angles between the surfaces and water were measured with a contact angle goniometer (Rame-Hart, Mountain Lakes, NJ, USA). The standard procedure for deactivating biohazardous materials is overnight treatment with 10% bleach. If a coating is stable to such a treatment then the hazardous cells can be washed and deactivated and the cell support can be reused. We evaluated the resistance of coated surfaces to the overnight treatment with 10% bleach in two ways. First, the loss of the surface antiadhesion during the bleach treatment was assessed. The minimum pressure required for cell injection was measured before the treatment with bleach, P_1, and after the treatment, P_2. The resistance, R, of the coating to the treatment was calculated using the following expression:

$$R = \left(1 - \frac{P_2 - P_1}{P_0 - P_1}\right) \times 100\% \quad (1)$$

where P_0 is the pressure required for cell injection from the uncoated surface. Second, we evaluated coated surface resistance to bleach by assessing the loss of surface hydrophobicity for coated glass slides and surface hydrophilicity for ULAP. Contact angles between the surface and water were measured both before the treatment with bleach (α_1) and after the treatment (α_2). The resistance of the coating to the treatment was calculated using the following expression:

$$R = \left(1 - \frac{\alpha_2 - \alpha_1}{\alpha_0 - \alpha_1}\right) \times 100\% \quad (2)$$

where α_0 is the contact angle between water and uncoated surface.

2.6 Surface biocompatibility

Cell viability was measured to assess surface biocompatibility. Fifty microliters of a cell suspension containing 5×10^3 cells in PBS was placed on the surface and left at room temperature for 2.5 h. Then, 10 μL of 0.4% trypan blue solution was mixed with this cell suspension. Trypan blue stains only dead cells. A 10 μL aliquot of the suspension was transferred to a hemocytometer and the fraction of viable cells, V_1, was determined. Biocompatibility, B, was calculated using the following expression:

$$B = \frac{V_1}{V_0} \times 100\% \quad (3)$$

where V_0 is the fraction of viable cells before the incubation on the surface.

2.7 Spectral properties

Light absorbance of glass slides and polystyrene plates was measured using a Beckman (Palo Alto, CA, USA) DU 650 spectrophotometer. Fluorescence of support materials was studied using the same fluorescent microscope that was used for cell injection. The microscope was equipped with a dichroic mirror set FX06 (Omega Optical, Brattleboro, VT, USA) permitting UV irradiation (365 ± 12.5 nm) and blue fluorescence observation (450 ± 32.5 nm). The fluorescence intensity was measured with a photomultiplier tube installed on one of the optical ports of the microscope.

3 Results and discussion

Two types of cell supports suitable for cell injection application are commercially available, microscope slides and tissue culture plates. The main material used for manufacturing the microscope slides is glass. Cell culture plates are usually made of polystyrene. Here we evaluated glass slides and polystyrene plates as cell supports for cell injection into the capillary. The data on different surface characteristics are collected in Table 1.

3.1 Cell adhesion

3.1.1 Untreated surfaces

We found that cells adhered strongly to untreated glass or polystyrene surfaces. Cell injection from untreated glass and polystyrene surfaces was impossible even when maximum negative pressure of 88 kPa from a vacuum

Table 1. The surface parameters

Surface material	Injection pressure[a] (kPa)	Resistance to bleach (%)		Hydrophobicity[d] (α^o)	Biocompatibility[e] (%)	Fluorescence intensity[f] (a.u.)
		Based on pressure[b]	Based on angle[c]			
Glass	> 88	ND[g]	100	9 ± 3	64	0.04
Polystyrene	> 88	ND	100	92 ± 1	77	0.93
Sigmacote	> 88	ND	100	91 ± 1	63	0.05
DMS	> 88	ND	100	91 ± 1	78	0.04
PHEMA	0.7	> 99	100	47 ± 1	89	0.04
PVA	0.7	0	0	63 ± 3	88	0.04
ULAP	1.7	0	0	52 ± 1	54	1.00

a) Minimal pressure required for cell injection under standard conditions (see Section 2.4)
b) The residual antiadhesive ability (%) after overnight treatment with 10% bleach determined by measuring the minimal injection pressure (see Section 2.5). Since pressure, P_0, required for injection from uncoated glass and polystyrene was determined only as a lower limit, then according to Eq. (1) only the lower limit of reistance could be evaluated.
c) The residual coating after overnight treatment with 10% bleach determined by measuring the contact angle (see Section 2.5)
d) Contact angle between the surface and water (see Section 2.5). Mean ± standard deviation for 6 experiments
e) Percentage of live cells after 2.5 h incubation on the surface (see Section 2.6)
f) Fluorescence was excited by 365 nm and registered at 450 nm (see Section 2.7)
g) Not determinable since minimum negative pressure required for cell injection was higher than maximum pressure generated by the vacuum pump

pump was applied to the distal end of the capillary. Moreover, under such negative pressure the solubility of air dissolved in the buffer decreases and air bubbles form in the capillary. Air bubbles are very undesirable in CE since they can disturb both separation and detection. Cell adhesion can be reduced if the surface is modified with an antiadhesive coating. Two groups of chemicals, hydrophobic silanes [12], and hydrophilic polymers (hydrogels) [13, 14], have been most often used in biological studies to reduce cell adhesion to the surface. Here, we studied the feasibility of glass and polystyrene surfaces modified with representatives of these two groups to be used as cell supports for cell injection into the capillary.

3.1.2 Hydrophobic coating

Silanization is the chemical modification applicable to the glass surface. It is based on a covalent binding of silane groups of a modifier to the silanol groups on a glass surface. Depending on the structure of a silane molecule, silanization can provide different physical-chemical parameters to the glass surface. We coated glass slides with two silanes, DMS and Sigmacote. Silanization, being a covalent surface modification, gives rise to a coating that is resistant to chemical attack. We found that both DMS- and Sigmacote-modified surfaces could be bio-deactivated with 10% bleach overnight without significant change in its properties (see Table 1). Our measurements of contact angle showed that the DMS and Sigmacote surfaces were highly hydrophobic ($\alpha > 90^o$; see Table 1). Such surfaces forced the drop of aqueous cell suspension to acquire a sphere-like shape. This shape prevented cells from quickly settling on the surface and also introduced optical aberrations that made viewing the cells more difficult. Unfortunately, it was not possible to inject cells from the DMA- and Sigmacote-modified surfaces at a pressure drop of 88 kPa, the maximum pressure difference created by our pump. Thus, hydrophobic surfaces did not significantly reduce cell adhesion, which may be due to the presence of transmembrane proteins with exposed hydrophobic moieties that can interact with the surface and facilitate cell adhesion.

3.1.3 Hydrophilic coating

Hydrogels are hydrophilic polymers. Coating with hydrogels is known to prevent strong protein and cell adhesion to the surface [13, 14]. The surface can be modified by creating a physical layer of a hydrogel or by covalent binding of a hydrogel to the surface material. Here we used two polymers, PHEMA and PVA, for physical modification of the surface. PHEMA can be applied to both

glass and polystyrene. PVA is applicable only to glass surfaces. To allow PVA to form a highly cross-linked layer of polymer, it must be incubated at 140°C; polystyrene is distorted above 65–80°C. However, there are commercially available polystyrene plates (Corning Costar, Acton, MA, USA) with a covalently bound hydrogel (the structure of the hydrogel is not disclosed) that are sold as ULAPs.

PHEMA, PVA, and ULAP had considerably less cell adhesion than untreated surfaces. The pressures required for cell injection from these surfaces were lower than 2 kPa (see Table 1). PVA coating and ULAP lost their antiadhesion upon treatment with 10% bleach. PHEMA coating survived 16 h incubation in 10% bleach without significantly deterioration in its antiadhesion ability (see Table 1). All three hydrogel-modified surfaces were more hydrophobic than uncoated glass and less hydrophobic than silanized surfaces or uncoated polystyrene (see Table 1). The drop of cell suspension on these surfaces was round enough to reduce the rate of liquid evaporation and yet flat enough to avoid optical aberration and to allow rapid settling of the cells to the surface. The latter ensured that there were no cells floating in the suspension that could adhere to the outer wall of the capillary and introduce artifacts to CE separation results.

3.2 Biocompatibility

Anchoring to the surface is an important property of most human cell types. Inability to adhere to the surface is known to disturb normal cell proliferation [16]. Therefore, in most cases the cells have to be grown on surfaces with a high adhesion index and should not be allowed to stay on antiadhesive surfaces for prolonged periods of time. Here we assessed surface biocompatibility by measuring the percentage of cells surviving 2.5 h incubation on antiadhesive surfaces. PHEMA- and PVA-coated surfaces were the most biocompatible (see Table 1). ULAP, in contrast, had the lowest biocompatibility among the surfaces tested. Therefore, if ULAP is used, the cells should only be placed on this surface immediately before injection for each single-cell experiment. There was one more reason, common for all antiadhesive surfaces, not to prolong cells contacting the surface: when the cells had been on an antiadhesive surface for a long time they tended to form an aggregate (Fig. 1). Such an aggregate made it difficult to inject individual cells.

3.3 Spectral properties

Fluorescent image microscopy is potentially an efficient tool in cell selection for CE analysis [3]. Combining fluorescent image microscopy with CE of single cells requires that cell support material be light-transparent, nonfluorescent, and photostable. All surfaces studied were transparent for visible light but absorbed in the UV (Fig. 2). ULAP

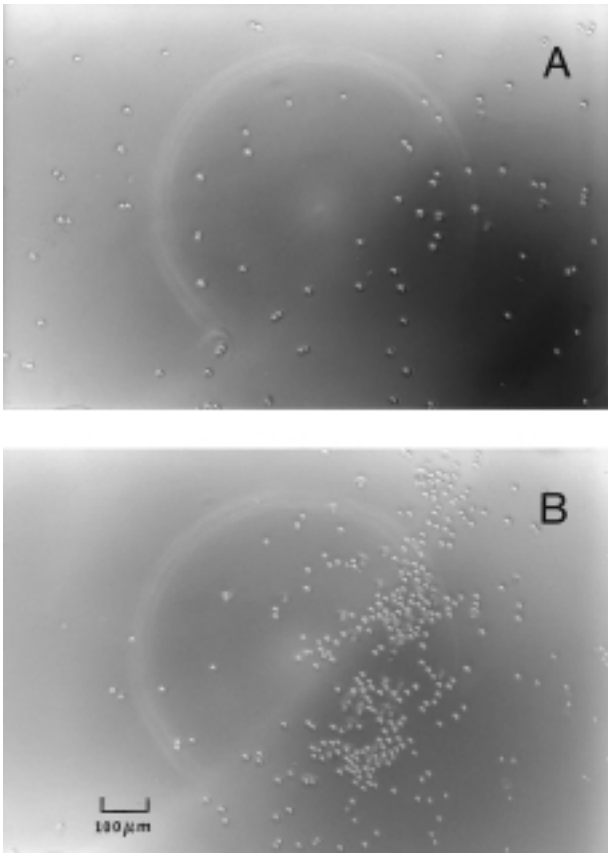

Figure 1. Formation of cell aggregate on glass surface coated with antiadhesive layer of PVA. (A) Cells 5 min after being placed on the slide; (B) cells 1 h after being placed on the slide.

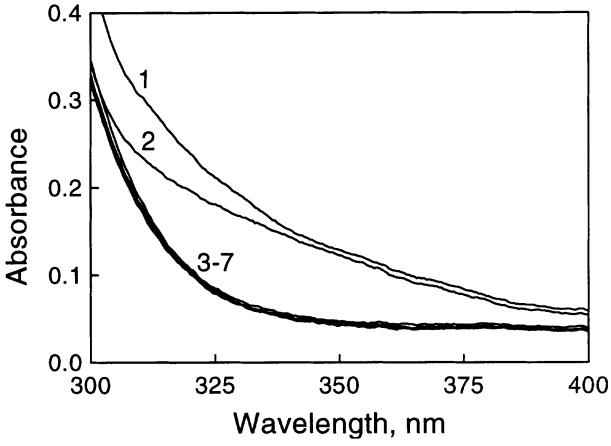

Figure 2. Absorption spectra of cell supports in near UV region. 1, Polystyrene of ULAP; 2, polystyrene of ordinary cell culture plate; 3, untreated glass slide; 4, DMS-modified glass slide; 5, PVA-coated glass slide; 6, PHEMA-coated glass slide; 7, Sigmacote-coated glass slide.

polystyrene absorbed the most. Uncoated polystyrene had intermediate absorbance. Uncoated and coated glass slides had the least absorbances. Fluorescence intensity of polystyrene plates under UV irradiation was roughly 25 times higher than that of the glass slides (see Table 1). Polystyrene plates not only intensely fluoresced when excited with 365 nm line of an Hg lamp but also photobleached. Figure 3 shows fluorescence decay under continuous UV irradiation due to photobleaching. This photodestruction was a relatively complex process since the kinetic curve was far from being a single exponential (unimolecular) process or hyperbolic (radical-radical) process. Therefore, it was difficult to correct for instability of polystyrene fluorescence.

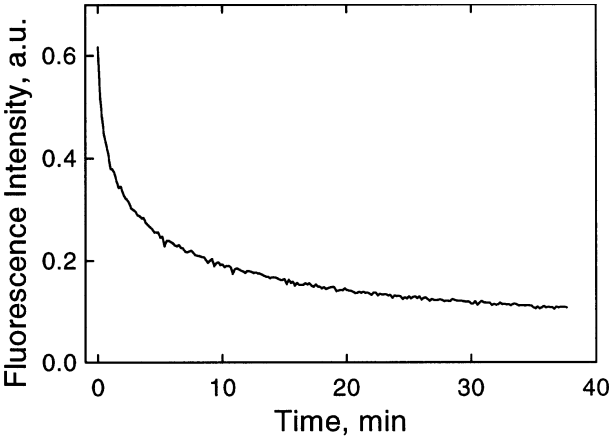

Figure 3. Fluorescence of polystyrene registered at 450 ± 32 nm under constant irradiation by UV light (365 ± 12.5 nm) of the mercury lamp.

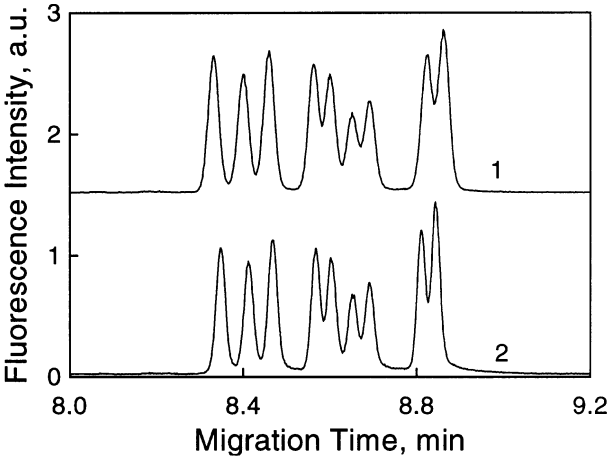

Figure 4. Electropherograms generated by a sample injected from (1) the uncoated slide and (2) PVA-coated slide. The sample was an equimolar mixture of TMR-labeled sugars [15].

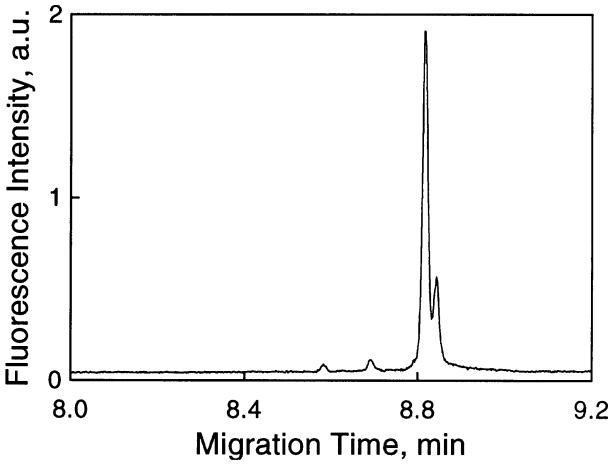

Figure 5. Separation of the metabolic products of LacNAc from a single cell injected from a PVA-coated slide.

3.4 Separation

The quality of a CE separation can deteriorate if a surface modifier is not bound to the surface strongly and is instead injected into the capillary together with the cell. To check such a possibility we injected a liquid sample from both modified and unmodified surfaces by placing the capillary in intimate proximity to the surface. The distance between the capillary tip and the surface was equal to 10 μm. We found no detectable influence of the modifier on the quality of CE separation (see, for example Fig. 4). Cell injections were carried out at a larger distance between the capillary tip and the surface (30 μm) than in these experiments with liquid samples. The larger distance ensured that single-cell separations were not affected by surface modification, providing the peaks of the same quality as those of the standards (Fig. 5).

4 Concluding remarks

In this study we evaluated a number of support surfaces for cell injection into the capillary for analysis by CE. We concluded that hydrogel-coated surfaces were the most suitable for cell injection into the capillary. PHEMA- and PVA-coated glass slides had the best biocompatibility. PHEMA slides could be biodeactivated with 10% bleach and reused many times. Both PHEMA- and PVA-coated slides were not fluorescent under UV excitation and thus could be used for fluorescent image analysis with UV excitation.

The authors thank Dr. Karel Smetana, Jr. (Charles University, Czech Republic), Dr. George Truskey (Duke University, USA), and Mr. Hossein Ahmadzadeh (University of Alberta, Canada) for their valuable advice on preparation of antiadhesive surfaces. This work was funded by

the Natural Sciences and Engineering Research Council of Canada.

Received May 25, 1999

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