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Preparation and electrophoretic separation of Bodipy-Fl-labeled glycosphingolipids

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ABSTRACT

Several glycosphingolipids were labeled with the fluorphore Bodipy-Fl and analyzed using capillary electrophoresis with laser-induced fluorescence detection. GM1-, LacCer-, and Cer-Bodipy-Fl were prepared through acylation using the N-hydroxysuccinimide ester of Bodipy-Fl. Several other glycosphingolipids including GT1a-, GD1a-, GM2-, GM3-, GD3-, and GlcCer-Bodipy-Fl were enzymatically synthesized. Micellar electrokinetic capillary chromatography with a TRIS/CHES/SDS/α-cyclodextrin buffer produced better separation than an established borate/deoxycholate/methyl-β-cyclodextrin buffer. The nine Bodipy-Fl-labeled glycosphingolipid standards were separated in under 5 min, theoretical plate counts were between 640,000 and 740,000, and the limit of detection was approximately 3 pM or 240 ymol analyte injected onto the capillary.

1. Introduction

Glycolipids constitute over 80% of the conjugated saccharides in the mammalian brain, making them one of the most abundant molecules on neuronal cell surfaces [1]. Glycosphingolipids consist of a polar sugar head group and a ceramide hydrophobic lipid tail made up of a sphingosine and a fatty acid. Sialic-acid containing glycosphingolipids are termed gangliosides and play a crucial role in cellular signaling [2–5], cell differentiation [6–8], oncogenesis [9], auto-immune disorders [10], infectious disease [11–15], and lysosomal storage disorders such as Tay-Sachs and Sandhoff Diseases [16]. The structure of the ganglioside GM1 is shown in Fig. 1A.

Thin layer chromatography [17,18] and high performance liquid chromatography [19,20] are the traditional approaches for the separation of glycosphingolipids. Glycosphingolipids are amphiphillic, which complicates their separation. TLC is rapid, simple, and requires little sample preparation [21], but glycosphingolipids with the same sugar moiety can migrate to different positions on the TLC plate due to differences in carbon chain length within the ceramide tail [22]. HPLC offers several advantages over TLC including improved resolution, reproducibility, and the ability to easily interface with mass spectrometry [23]. Mass spectrometry is frequently used as the detection method to produce detailed structural information about these complex glycolipids. However, gangliosides can be difficult to analyze by mass spectrometry due to the fragile nature of metastable sialic acid-containing ions [24]. In addition, it is difficult to obtain proper separation conditions that are compatible with mass spectrometry [23,25]. Limits of detection for these hyphenated mass spectrometry techniques often range between 0.1 and 1 pmol [24,26–29], which is approximately 8–10 orders of magnitude poorer than what is required for monitoring metabolism in single cells.

Our lab coined the term "metabolic cytometry" to describe the process of probing metabolism within single cells by capillary electrophoresis with laser induced fluorescence detection [30,31]. In a typical experiment, the fatty acid of a glycosphingolipid is replaced with a fluorescent label to create a fluorescent substrate [32]. Cells are incubated with this fluorescent substrate, and any metabolic products due to the addition (anabolism) or removal (catabolism) of sugars will also be detected (Fig. 1B). After incubation, cells are injected onto the capillary, lysed, and the identities of the metabolic products are determined by co-migration with standards [32,33]. We have used this approach to investigate metabolism in mammalian cellular homogenates [34] and single cells [35], and lipid uptake by the malaria-causing parasite *Plasmodium falciparum* [36].

The separation of glycosphingolipids by capillary electrophoresis is complicated because it requires the separation of anionic gangliosides and several neutral metabolic products including

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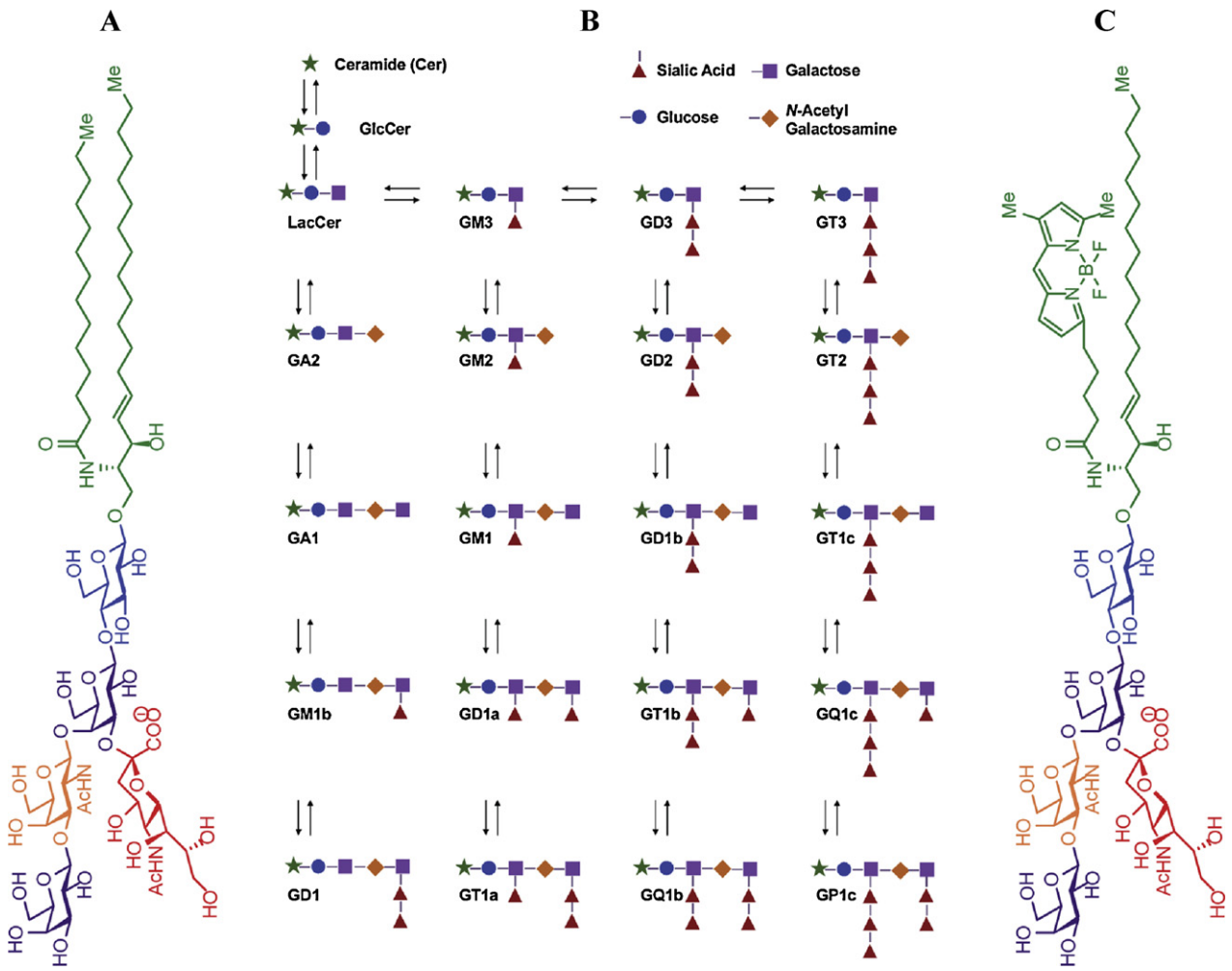

Fig. 1. Glycosphingolipid structure and metabolism. (A) Structure of the ganglioside GM1. (B) Metabolic pathway of glycosphingolipids. (C) Bodipy-Fl-labeled GM1.

lactosyl ceramide (LacCer), glucosyl ceramide (GlcCer), and ceramide (Cer). Most of the previous work focused on capillary zone electrophoresis based on a borate, phosphate, and α-cyclodextrin buffer [37–40]. Because glycosphingolipids are amphiphillic, they are known to form micelles in solution [41]. α-Cyclodextrin adds improved separation through an equilibrium partitioning process [25,38]. This composition is excellent at separating ganglioside isomers, but neutral glycosphingolipids co-migrate [38].

We have previously used a micellar electrokinetic capillary chromatography (MEKC) approach to separate charged and neutral glycosphingolipids based on a borate/deoxycholate/methyl-β-cyclodextrin buffer [42,43]. This buffer allowed for rapid separation (<5 min) of tetramethylrhodamine labeled mono-sialylated and neutral glycosphingolipids with theoretical plate counts between 300,000 and 550,000 [32,34,35]. However, here we show that this buffer composition is insufficient to separate higher ordered gangliosides (*i.e.* GD and GT series). Here we report on the preparation and separation of several Bodipy-Fl (boron dipyrromethene difluoride, λ_{ex} = 505 nm) labeled glycosphingolipids (Fig. 1C). Bodipy-Fl is a green-emitting dye (λ_{ex} = 505 nm) that has been used to study glycosphingolipid transport within cells [44,45]. Here we show that MEKC based on a TRIS/CHES/dodecylsulfate/α-cyclodextrin buffer separates polysialylated, monosialylated, and neutral glycosphingolipids in a rapid, highly efficient, and ultrasensitive manner.

2. Experimental

2.1. Preparation of Bodipy-Fl-labeled glycosphingolipids

2.1.1. Chemicals and Cer-, LacCer-, and GM1-Bodipy-Fl synthesis

BODIPY®-FL-C5 succinimidyl ester (Bodipy-NHS ester) was purchased from Invitrogen (San Diego, CA). D-Sphingosine and C18-lyso-lactosylceramide (LacCer) were purchased from Avanti Polar Lipids (Alabaster, AL). C18-lyso-GM1 was prepared as previously reported [32]. N,N-Diisopropylethylamine (DIEA) and β-galactosidaseses (from *Escherichia coli* and from bovine testes) were purchased from Sigma (St. Louis, MO). All small molecule salts and solvents were also purchased from Sigma–Aldrich (St. Louis, MO). Sialyltransferase (MalE fusion protein from *Campylobacter jejuni*) were expressed and purified as previously reported [46,47]. Cytidine 5′-monophospho-N-acetyl-β-D-neuraminic acid (CMP-Neu5Ac) was purchased from IEP GmbH (Wiesbaden, Germany). Alkaline phosphatase was purchased from Roche (Basel, Switzerland). C18 Sep-Pak cartridge was purchased from Waters Corporation (Milford, MA) and activated by washing with MeOH (20 mL) followed by water (20 mL) before use. MALDI-TOF MS spectra were recorded on Bruker (Billerica, MA) Microflex instruments using a α-Cyano-4-hydroxycinnamic acid solution [20 mg dissolved in 1 mL of 50/50 (v/v) acetonitrile/water with 0.1% TFA] as matrix.

Cer-Bodipy: To a mixture of D-sphingosine (1.5 mg, 5.0 μmol) and Bodipy-NHS ester (1.5 mg, 3.6 μmol) in DMF (1.0 mL) were added DIEA (2.0 μL, 12 μmol). After stirring at room temperature for 24 h, the reaction mixture was evaporated with toluene and then purified by column chromatography on silica gel (chloroform/MeOH = 50/1) to give the product (2.0 mg, 92%).

LacCer-Bodipy: To a mixture of C18-lyso-LacCer (2.0 mg, 3.2 μmol) and Bodipy-NHS ester (2.4 mg, 5.8 μmol) in DMF (1.0 mL) was added DIEA (5.6 μL, 32 μmol). After stirring at room temperature for 2 days, the reaction mixture was evaporated with toluene and then purified by column chromatography on silica gel (chloroform/MeOH/water = 8/1/0.1 to 4/1/0.1). The purified product was loaded on double connected C18 Sep-Pak cartridges with 40% aq. MeOH (20 mL) and the cartridges were flushed with 40% aq. MeOH (20 mL), 60% aq. MeOH (60 mL), 80% aq. MeOH (80 mL) and MeOH (50 mL). The product was eluted with 80% aq. MeOH giving 2.3 mg (77%).

GM1-Bodipy: To a mixture of C18-lyso-GM1 (2.3 mg, 1.8 μmol) and Bodipy-NHS ester (1.8 mg, 4.3 μmol) in DMF (1.0 mL) was added DIEA (3.1 μL, 18 μmol). After stirring at room temperature for 2 days, the reaction mixture was evaporated with toluene and then purified by column chromatography on silica gel (chloroform/MeOH/water = 2/1/0.1 to 1/1/0.1). The purified product was loaded on double connected C18 Sep-Pak cartridges with 40% aq. MeOH (20 mL) and the cartridges were flushed with 40% aq. MeOH (20 mL), 60% aq. MeOH (60 mL) and 80% aq. MeOH (80 mL). The product was eluted from 60% to 80% aq. MeOH giving 2.4 mg (84%).

2.1.2. Enzymatic preparation of other Bodipy-Fl-labeled standards

After each enzymatic reaction, the resulting reaction mixture was loaded on a C18 Sep-Pak cartridge, previously activated with MeOH (10 mL). The cartridges were washed with sample loading solution (100 mL) and the Bodipy-compounds were eluted with MeOH (10 mL). Finally, the eluent was evaporated to yield the desired product.

GlcCer-Bodipy: The reaction was performed at room temperature in a mixture (30 μL, pH 6.8) containing LacCer-Bodipy (0.010 mg, 11 nmol), β-galactosidase from *E. coli* (1 U), sodium phosphate (100 mM), NaCl (50 mM) and $MgCl_2$ (6.6 mM). After 18 h of incubation, the resulting mixture was loaded onto Sep-Pak column with 40% aq. MeOH (1 mL) and purified.

GM3-Bodipy and GD3-Bodipy: The reaction was started at room temperature in a mixture (200 μL, pH 7.5) containing LacCer-Bodipy (0.045 mg, 50 nmol), sialyltransferase (0.27 mg), CMP-Neu5Ac (500 nmol), alkaline phosphatase (125 U), MOPS (50 mM), $MgCl_2$ (60 mM). After 15 and 24 h incubation, enzymes and donor solution [27 μL; sialyltransferase (0.18 mg), alkaline phosphatase (125 U) and CMP-Neu5Ac (250 nmol)] was added to the reaction mixture. After totally 2 days of incubation, the resulting mixture was loaded onto Sep-Pak column with 30% aq. MeOH (1 mL) and purified.

GM2-Bodipy: The reaction was performed at 37 °C in a mixture (20 μL, pH 4.5) containing GM1-Bodipy (0.01 mg, 11 nmol), β-galactosidase from bovine testes (26 mU), sodium acetate (100 mM). After 18 h of incubation, the resulting mixture was loaded onto Sep-Pak column with 30% aq. MeOH (1 mL) and purified.

GD1a-Bodipy and GT1a-Bodipy: The reaction was started at room temperature in a mixture (200 μL, pH 7.5) containing GM1-Bodipy (0.040 mg, 25 nmol), sialyltransferase (0.27 mg), CMP-Neu5Ac (300 nmol), alkaline phosphatase (125 U), MOPS (50 mM), $MgCl_2$ (60 mM). After 15 and 24 h incubation, enzymes and donor solution [25 μL; sialyltransferase (0.18 mg), alkaline phosphatase (125 U) and CMP-Neu5Ac (200 nmol)] was added to the reaction mixture. After totally 2 days of incubation, the resulting mixture was loaded onto Sep-Pak column with 30% aq. MeOH (1 mL) and purified.

2.2. Capillary electrophoresis with laser-induced fluorescence detection

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO). All solutions were prepared in 18.2 MΩ distilled deionized water (Barnstead Nanopure System, Thermo Scientific, Waltham, MA) and filtered twice at 0.2 μm. MEKC was used to perform the electrophoretic separations. One buffer consisted of 10 mM sodium tetraborate, 35 mM sodium deoxycholate, and 5 mM methyl-β-cyclodextrin with an operating voltage of 18 kV [36,48]. Another buffer consisted of 100 mM tris(hydroxymethyl)aminomethane (TRIS), 100 mM 2-(cyclohexylamino)ethanesulfonic acid (CHES), 20 mM sodium dodecyl sulfate (SDS), and 5 mM α-cyclodextrin with an operating voltage of 24 kV.

A Spellman (Hauppauge, NY) CZE-1000R power supply was used to supply voltage for the separation on a 34 cm, 30 μm ID/150 μm OD fused silica capillary (Polymicro Technologies, Phoenix, AZ). Sample introduction was performed by electrokinetic injection (1 kV for 1 s), housed in a locally constructed injection block [33]. Unless otherwise stated, all optical components were purchased from Thorlabs (Newton, NJ) and CVI Melles Griot (Alburquerque, NM). A post-capillary sheath flow cuvette was used for fluorescence detection, as described elsewhere [49,50]. A 473 nm diode pumped solid-state laser (Lasermate Group, Inc., Pomona, CA) at 10 mW was used for excitation of the Bodipy-Fl-labeled glycosphingolipids. The emission was collected with a 0.7 NA microscope objective lens, bandpass filtered (505 ± 10 nm), imaged onto a fiber optic, and detected using an avalanche photodiode (SPCM-AQR-13-FC, PerkinElmer, Vaudreuil, Quebec). All data was collected using locally constructed hardware and software based on the LabVIEW (National Instruments, Austin, TX) programming environment. Data was digitized using a PCI-6035E card (National Instruments) and the fluorescence emission was monitored at 50 Hz.

Data analysis was performed in locally written software using the LabVIEW and MATLAB (Mathworks, Natick, MA) programming environments. All electropherograms were filtered with a 5-point median filter to remove spikes and smoothed with a 0.1 s full width at half height Gaussian function. Peak widths were estimated using a least-squares fit of each peak to a Gaussian function. Separation efficiencies (N) were calculated according to Eq. (1)

$$N = \left(\frac{t_m}{\sigma_t}\right)^2 \quad (1)$$

where t_m is the migration time of the analyte and σ_t is the standard deviation of the Gaussian peak [51]. Apparent mobilities (μ_a) were calculated as

$$\mu_a = \frac{L_{eff} L}{t_m V_{run}} \quad (2)$$

where L_{eff} is the effective capillary length (to the detector), L is length of the capillary, and V_{run} is the running voltage. Concentrations of the Bodipy-Fl-labeled lipids were estimated by comparing the fluorescence intensity to that of a known quantity of free Bodipy-Fl dye.

3. Results and discussion

3.1. Preparation of Bodipy-Fl-labeled glycosphingolipid standards

Mass spectrometry was used to characterize the Bodipy-Fl-labeled glycosphingolipid preparations, Table 1. The MALDI mass spectra are shown in Fig. 2. The mass spectrum of the GT1a/GD1a mix showed a peak corresponding to GM1, but an MEKC separation

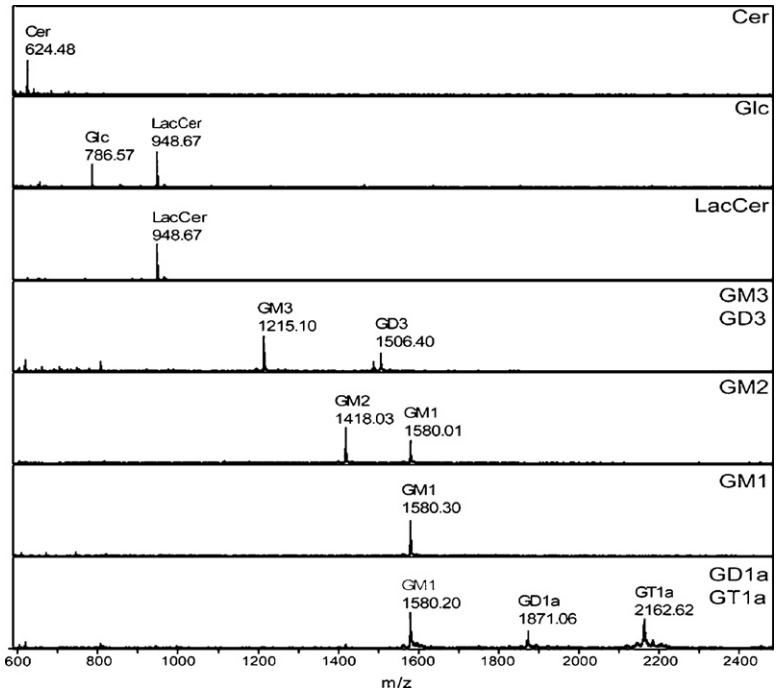

Fig. 2. MALDI mass spectra for the prepared Bodipy-Fl-labeled glycosphingolipid standards.

using 10/35/5 (*vide infra*) showed only two peaks corresponding to a 1.0:1.4 GT1a to GD1a ratio. This result is consistent with the fragile nature of ganglioside metastable ions and highlights the disadvantage of mass spectrometric detection of glycosphingolipids. Comparing peak heights using MEKC, enzymatic conversion of GM1-Bodipy-Fl to GM2-Bodipy-Fl gave a 3:1 ratio of GM1 to GM2, a 4:1 GM3 to GD3 ratio, and a 2:1 ratio of LacCer-Bodipy-Fl to GlcCer-Bodipy-Fl.

3.2. Glycosphingolipid separation in borate/deoxycholate/methyl-β-cyclodextrin

A mixture containing 1–3 nM Bodipy-Fl labeled GT1a, GD1a, GD3, GM1, GM2, GM3, LacCer, GlcCer, and Cer was separated in a10 mM sodium tetraborate, 35 mM sodium deoxycholate, and 5 mM methyl-β-cyclodextrin (10/35/5) buffer at 18 kV. The electropherogram is presented in Fig. 3A and B, showing poor resolution of the polysialic acid-containing glycosphingolipids.

GM1 migrated as a shoulder on the GD1a peak. In addition, GD3 and GM3 comigrated, even though GD3 contains two sialic acids that are deprotonated in this buffer (pH 9.2). The gangliosides migrated before the neutral glycosphingolipids, but did not follow any predictable order based on size, charge, or hydrophobicity. Gangliosides are known to form complexes with borate in aqueous solution [25] so the observed separation pattern is likely due to a balance between the interaction of the analyte with the outer ionic surface of deoxycholate micelles and the inner structure of neutral methyl-β-cyclodextrin molecules.

For the peaks that were resolved, separation efficiencies ranged between 440,000 and 550,000 theoretical plates except for GlcCer, which had over 1.4 million plates. The measured current across the capillary was 18 μA for the 530 V/cm separation and apparent electrophoretic mobilities of the glycosphingolipids varied between 3.0 and 3.3×10^{-8} m^2 V^{-1} s^{-1}. The limit of detection (3-σ, measured with free Bodipy-Fl dye) was 5.8 ± 1.0 pM or 315 molecules injected onto the capillary.

Table 1
MALDI ion information for Bodipy-Fl-labeled glycosphingolipids.

Bodipy-Fl analyte	Theoretical ion	Theoretical *m*/*z*	Actual *m*/*z*
Cer	$C_{34}H_{54}BF_2N_3NaO_3^+$	624.41	624.48
GlcCer	$C_{40}H_{64}BF_2N_3NaO_8^+$	786.46	786.57
LacCer	$C_{46}H_{74}BF_2N_3NaO_{13}^+$	948.52	948.67
GM3	$C_{57}H_{90}BF_2N_4O_{21}^-$	1215.62	1215.1
GD3	$C_{68}H_{107}BF_2N_5O_{29}^-$	1506.71	1506.4
GM2	$C_{65}H_{103}BF_2N_5O_{26}^-$	1418.7	1418.03
GM1	$C_{71}H_{113}BF_2N_5O_{31}^-$	1580.75	1580.3
GD1a	$C_{82}H_{130}BF_2N_6O_{39}^-$	1871.84	1871.06
GT1a	$C_{93}H_{147}BF_2N_7O_{47}^-$	2162.94	2162.62

3.3. Glycosphingolipid separation in TRIS/CHES/SDS/α-cyclodextrin

The composition of the 10/35/5 buffer had previously been optimized for the separation of amphiphillic lipid species [42]. For enhanced separation of polysialic acid glycosphingolipids, a previously successful TRIS/CHES/SDS buffering system [52] was modified. α-Cyclodextrin was chosen instead of methyl-β-cyclodextrin as an additive because α-cyclodextrin is the only cyclodextrin that has been shown to separate di- and trisialogangliosides [25,38].

The concentration of TRIS and CHES were held constant at 100 mM while the concentration of SDS and α-cyclodextrin varied. It has been shown that ganglioside separations can be enhanced in a buffer with high ionic strength [37]. The composition of the buffer that achieved the best separation was 100 mM TRIS, 100 mM CHES, 20 mM SDS, and 5 mM α-cyclodextrin (henceforth referred to as TCS-α). Lower SDS and α-cyclodextrin concentrations resulted in poor resolution. Increasing the α-cyclodextrin concentration above 15 mM caused severe excessive peak tailing, consistent with literature [38]. The voltage was increased from 18 kV to 24 kV, improving peak efficiencies without excessive Joule heating.

A separation of the same nine Bodipy-Fl-labeled glycosphingolipids in TCS-α at 24 kV is shown in Fig. 3C and D. Although

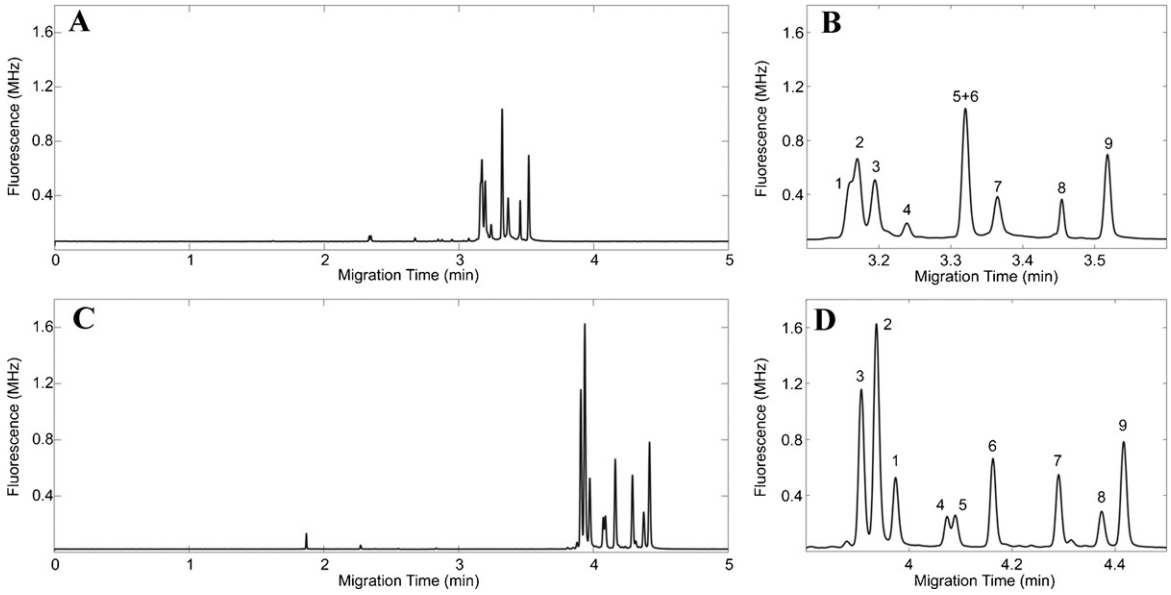

Fig. 3. Separation of Bodipy-Fl-labeled glycosphingolipid standards (1–3 nM). (A) Separation in 10 mM sodium tetraborate, 35 mM sodium deoxycholate, and 5 mM methyl-β-cyclodextrin buffer at 18 kV. (B) Expansion of the relevant portion of (A). (C) Separation in 100 mM TRIS, 100 mM CHES, 20 mM SDS, and 5 mM α-cyclodextrin buffer at 24 kV. (D) Expansion of the relevant portion of C. The numbered labels correspond to GM1 (1), GD1a (2), GT1a (3), GM2 (4), GD3 (5), GM3 (6), LacCer (7), GlcCer (8), and Cer (9).

baseline separation of GM2 and GD3 was not achieved, all species were resolved. Separation efficiencies ranged from 640,000 to 740,000 theoretical plates. Compared to the 10/35/5, the apparent electrophoretic mobilities of the glycosphingolipids were lower in TCS-α ($1.8–2.0 \times 10^{-8}$ m^2 V^{-1} s^{-1}), slightly increasing the time of the separation.

Besides improving the separation of glycosphingolipids, TCS-α also offered three other advantages over the 10/35/5 buffer. First, the current across the capillary was lower in TCS-α compared to 10/35/5, 12 μA for the 700 V/cm separation. Second, the migration order of the analytes was predictable, following a decreasing number of sugars attached to the lipid tail (Fig. 1B). GM2 and GD3 both have four sugars, which could explain why these two analytes had similar migration times. Finally, comparing Fig. 3A and C shows that TCS-α offers an improved limit of detection. The TCS-α buffer generated lower fluorescence background, improving the limit of detection by half to 3.0 ± 0.6 pM or 150 molecules injected onto the capillary.

4. Conclusions

Here we have demonstrated the synthesis, electrophoretic separation, and ultrasensitive detection of several Bodipy-Fl-labeled glycosphingolipids. A MEKC separation based on a TRIS/CHES/SDS/α-cyclodextrin buffer provided superior resolution and a lower limit of detection compared to a borate/deoxycholate/methyl-β-cyclodextrin buffer. Since the separation of glycosphingolipids in TCS-α depended on the number of sugars the analyte contained, it is possible that the TCS-α buffer may not separate structural isomers such as the GD1a/GD1b or GT1a/GT1b couple. Fluorescent b-series gangliosides are challenging to synthesize, but when prepared, their separation will be evaluated in TCS-α. These results, taken together with previous work using tetramethylrhodamine [32], should allow for the construction of an ultrasensitive two color laser-induced fluorescence assay for probing multiple complex glycosphingolipid metabolic pathways simultaneously in single cells.

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